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Ciclo XXX

Exploring liver cell fate in co-transplantation studies and animal tumor models

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Abstract

Liver is the largest internal organ of the body. It has an exceptional regenerative capability: where as little as 25% of a liver can regenerate into a whole liver. This is predominantly due to two factors: hepatocytes that are re-entering the cell cycle, and the presence of bipotential stem cells, called hepatic oval cells, which can differentiate into hepatocytes. Liver replacement using the orthotopic technique (OLTx; normal, whole liver) represents the standard care for end-stage liver disease and many liver-based metabolic conditions. Organ shortage and patient instability, however, have emphasized the need for other non-traditional approaches for liver replacement, such as auxiliary liver transplantation, hepatocyte transplantation, xenotransplantation and bio artificial liver assist devices. Cell therapy (i.e hepatocyte transplantation) represents an alternative method to overcome OLTx, it has a substantial therapeutic potential, however multiple barriers still restrict engraftment and proliferation of transplanted hepatocyte. Although hepatocyte transplantation is currently performed in the clinic, it is only a temporary solution due to the delays in organ transplantation. As such, more reliable protocols for hepatocyte transplantation that can bring long-lasting benefits, are clearly needed. Herein, we show that co-transplantation of liver cell combinations (i.e. hepatocytes, liver sinusoidal endothelial cells, and Kupffer cells) after conditioning, can dramatically ameliorate hepatocytes engraftment and long-term functions of the transplant. Our goal is to achieve a consistent engraftment through the induction of hepatocyte proliferation, while favoring the interaction between resident and transplanted cells. To achieve our goal, we must also investigate the best conditions to induce proliferation after transplantation (*in vivo*), not only in the terms of pretreatment, but especially in the sense of cell-cell interactions to understand how to lead the hepatocytes to do what they already do physiologically. All cell types were transplanted under physiological conditions (67% hepatocytes, 22% LSECs, and 11% KCs) to reproduce the niche generating the normal cell-cell interactions and cell signaling. This ability of LSECs, KCs and hepatocytes to reconstitute the liver by co-transplantation will be helpful for basic studies of cell-cell interactions for the development of advanced protocols for liver cell therapy. Moreover, a stable hepatocyte model (*in vitro*), which maintains the original characteristics of liver parenchymal cells, is needed. For this purpose, we describe the isolation and characterization of two rat hepatocyte cell lines as tools for *in vitro* studies. Long-term stable cell lines were obtained from a HCC-bearing rat exposed to the Resistant-Hepatocyte protocol (RH cells) and from a rat subjected to the same model in the absence of

carcinogenic treatment, thus not developing HCCs (RNT cells). The presence of several markers identified the hepatocytic origin of both cell lines and confirmed their purity. Although morphologically similar to normal primary hepatocytes, RNT cells were able to survive and grow in monolayer culture for months, did not form colonies in soft agar and spheroids when grown in 3D and were not tumorigenic in vivo. On the contrary, RH cells displayed tumor-initiating cell markers, formed numerous colonies in soft agar and spheroids when grown in 3D and were highly tumorigenic and metastatic after injection into syngeneic rats and immunocompromised mice. Moreover, RNT gene expression profile was similar to normal liver, while that of RH resembled HCC. In conclusion, the two cell lines here described represent a useful tool to investigate the molecular changes underlying hepatocyte interaction with the other liver cell type and their transformation in HCC development.

Riassunto

Il fegato è il più grande organo interno del corpo. Ha un'eccezionale capacità rigenerativa: dove solo il 25% di un fegato può rigenerarsi in un fegato intero. Ciò è dovuto principalmente a due fattori: gli epatociti che rientrano nel ciclo cellulare e la presenza di cellule staminali bipotenziali, chiamate cellule ovali epatiche, che possono differenziarsi in epatociti. La sostituzione del fegato con la tecnica ortotopica (OLTx, fegato normale e intero) rappresenta la cura standard per la malattia epatica allo stadio terminale e molte condizioni metaboliche a base di fegato. La carenza di organi e l'instabilità del paziente, tuttavia, hanno enfatizzato la necessità di altri approcci non tradizionali per la sostituzione del fegato, come trapianto di fegato ausiliario, trapianto di epatociti, xenotrapianto e dispositivi di assistenza epatica artificiale bio. La terapia cellulare (cioè il trapianto di epatociti) rappresenta un metodo alternativo per superare l'OLTx, ha un potenziale terapeutico sostanziale, tuttavia le molteplici barriere restringono ancora l'attecchimento e la proliferazione degli epatociti trapiantati. Sebbene il trapianto di epatociti sia attualmente eseguito nella clinica, è solo una soluzione temporanea a causa dei ritardi nel trapianto di organi. Pertanto, sono chiaramente necessari protocolli più affidabili per il trapianto di epatociti che possano portare benefici duraturi. Qui, mostriamo che il co-trapianto di combinazioni di cellule epatiche (cioè epatociti, cellule epiteliali sinusoidali del fegato e cellule di Kupffer) dopo il condizionamento, può migliorare notevolmente l'attecchimento degli epatociti e le funzioni a lungo termine del trapianto. Il nostro obiettivo è raggiungere un costante attecchimento attraverso l'induzione della proliferazione degli epatociti, favorendo l'interazione tra cellule residenti e trapiantate. Per raggiungere il nostro obiettivo, dobbiamo anche studiare le migliori condizioni per indurre la proliferazione dopo il trapianto (in vivo), non solo nei termini del pretrattamento, ma soprattutto nel senso delle interazioni cellula-cellula per capire come condurre gli epatociti a fare cosa già fanno fisiologicamente. Tutti i tipi di cellule sono stati trapiantati in condizioni fisiologiche (67% di epatociti, 22% LSEC e 11% di KC) per riprodurre la nicchia generando le normali interazioni cellula cellulare e segnalazione cellulare. Questa capacità di LSEC, KC ed epatociti di ricostituire meglio il fegato mediante il co-trapianto sarà utile per gli studi di base sulle interazioni cellula-cellula per lo sviluppo di protocolli avanzati per la terapia con cellule epatiche. Inoltre, è necessario un modello stabile di epatocita (in vitro), che mantenga le caratteristiche originali degli epatociti. A tale scopo, descriviamo l'isolamento e la caratterizzazione di due linee cellulari di epatociti di ratto come strumenti per studi in vitro.

Linee cellulari stabili a lungo termine sono state ottenute da un ratto portatore di HCC esposto al protocollo Resistant-Hepatocyte (cellule RH) e da un ratto sottoposto allo stesso modello in assenza di trattamento carcinogenico, quindi non sviluppando HCC (cellule RNT). La presenza di diversi marcatori ha identificato l'origine epatocitaria di entrambe le linee cellulari e ne ha confermato la purezza. Sebbene fossero morfologicamente simili ai normali epatociti primari, le cellule RNT erano in grado di sopravvivere e crescere in coltura monostrato per mesi, di non formare colonie o sferoidi in colture 3D in presenza di agar e non erano tumorigeniche in vivo. Al contrario, le cellule RH mostravano marcatori cellulari che iniziavano il tumore, formavano numerose colonie e sferoidi quando venivano depositate in 3D ed erano altamente tumorigeniche e metastatiche dopo l'iniezione in ratti singenici e topi immunocompromessi. Inoltre, il profilo di espressione genica RNT era simile al fegato normale, mentre quello di RH somigliava a HCC. In conclusione, le due linee cellulari qui descritte rappresentano uno strumento utile per studiare i cambiamenti molecolari alla base dell'interazione degli epatociti con l'altro tipo di cellula epatica e la loro trasformazione nello sviluppo di HCC.

1. Introduction

1.1 Liver

The liver is the largest internal organ of the body (weighing up 1.5 kg in adults; Molina and Dimaio, 2012), and it provides many essential metabolic, exocrine and endocrine functions. These include production of bile, metabolism of dietary compounds, detoxification, regulation of glucose levels through glycogen storage, as well as control of blood homeostasis by the secretion of clotting factors and serum proteins such as albumin (Jungermann and Keitzmann, 1996; Zorn, 2008). The human liver consists of two main lobes, which are divided into eight segments (four in each lobe). The segments are then macroscopically divided into hepatic lobules, which may have anatomic variations among different species. Blood enters the liver and circulates within the lobules through liver microvessels, while bile, produced and secreted by hepatocytes, flows in the opposite direction. Bile drains *via* several bile canaliculi that connect with larger ducts to ultimately form the common hepatic duct. The common hepatic duct transports bile to the gallbladder and finally to its destination, the duodenum. The hepatic lobule is the anatomic unit of the liver. Each lobule is made up of thousands of hepatic cells, called hepatocytes, which are separated from one another by large capillary spaces termed "liver sinusoids". The liver is composed of several cell types which are divided into parenchymal and non-parenchymal cells, of which approximately 80% are parenchymal (hepatocytes) and 20% are non-parenchymal liver cells (NPC) (Kmiec, 2001). Specifically, hepatocytes represent the main metabolic cells that execute functions such as glucose homeostasis, bilirubin excretion, protein synthesis and secretion of major plasma proteins. The NPC are composed of Kupffer cells (KC), liver sinusoidal endothelial cells (LSEC), hepatic stellate cells (HSC) and rare cell types such as Pit cells (Blouin et al., 1977). Sinusoids are populated with phagocytic KCs, the resident hepatic macrophages that filter harmful substances, removing large amounts of debris and bacteria. The space between the endothelium and hepatocytes is called the space of Disse, which is home to the stellate cells, also known as fat-storing or Ito cells). The role of stellate cells is to communicate with hepatocytes and modify the extracellular space through the secretion of extracellular matrix (Figure 1)

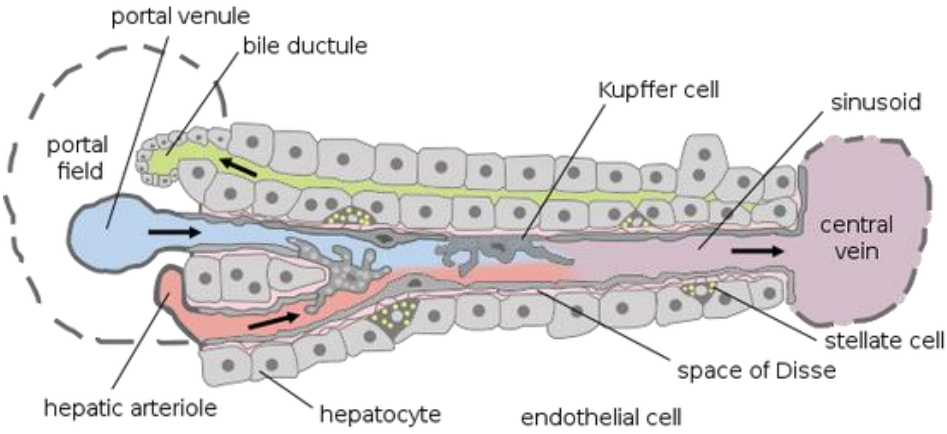


Figure 1. Representation of liver structure. Taken from Frevert et al., 2005.

The liver has an exceptional regenerative capacity, with as little as 25% of a liver capable of regenerating into a whole liver (Michalopoulos, 2007). This is predominantly due to two factors: the hepatocytes re-entering the cell cycle, and the presence of bipotential stem cells called hepatic oval cells that can differentiate into either hepatocytes or cholangiocytes (the later are the cells that line the bile ducts; Bird et al., 2008; Venter et al., 2015).

The livers inability to perform its normal synthetic and metabolic function results in a pathological condition called liver failure. There are several causes of liver failure including cirrhosis, hepatitis, cancer, drug overdose, metabolic or autoimmune disorders, chemical toxins and trauma. Based on the permanence of the injury, two different forms of liver failure are known: acute and chronic failure. To date, liver transplantation remains the only proven treatment for patients with acute liver failure and advanced hepatic encephalopathy. Liver replacement using the orthotopic technique (OLTx; normal, whole liver) is the standard care for end-stage liver disease and many liver-based metabolic conditions (Gotthardt et al., 2007; Qu et al., 2009; Sotil et al., 2009). There are, however, several limitations with this technique, including organ shortage and patient instability. As such other nontraditional approaches for liver replacement have been attempted, such as auxiliary liver transplantation, hepatocyte transplantation, xenotransplantation, extracorporeal perfusion using either xenogeneic approaches or human liver perfusion, and bioartificial liver assist devices (Carpentier et al., 2009; Ekser et al., 2009; Fitzpatrick et al., 2009; Thamara et al., 2009; Waelzlein et al., 2009). A further problem is that the organ when transplanted from donor to recipient undergoes a period of ischemia due to the closing of the vessels. The damage incurred is further amplified and reaches a critical point after replacement, where the opening of the vessels can cause a reperfusion injury. To overcome this problem, the current approach is to resort to a profile of drugs, however, attempts now being made to address alternative approaches. A recent

proteomic study of LSECs and hepatocytes after ischemia and reperfusion injury, highlighted two proteins, sortin nexin 5 (SNX5) and regucalcin (RGN) as potential players in liver protection. The study of these proteins, combined with molecular or intracellular mechanisms leading to their regulation, may contribute to a greater protection of the liver (Mandili et al., 2015).

1.1.2 Anatomy of the liver

The liver is divided into a larger right lobe and a smaller left lobe by the falciform ligament. From a surgical point of view, the liver is divided into right and left lobes of almost equal (60:40) size by a major fissure (Cantlie's line) running from the gallbladder fossa. Each lobe is divided into two sectors: the right one into anterior and posterior by the hepatic vein, while the left lobe is divided into medial (quadrate) and lateral by the left hepatic vein.

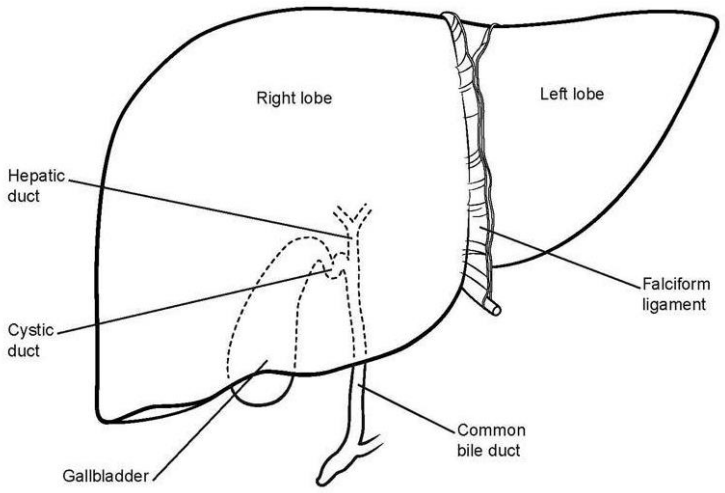
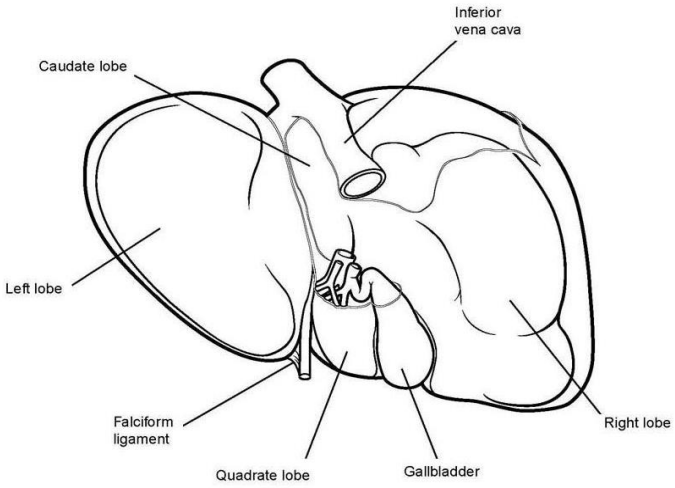


Figure 2. Liver and gallbladder, anterior view. **Vinay Kumar Kapoor, MBBS, MS, FRCS, FAMS Professor of Surgical Gastroenterology, Sanjay Gandhi Post Graduate Institute of Medical Sciences, India**

Sectors are further divided into segments, each with a blood supply and biliary drainage. The anterior sector contains superior (VIII) and inferior (V) segments (Figure 2 and 3). The right lobe in the posterior sector contains the superior (VII) and inferior (VI) segments.



The left lobe (quadrate lobe, segment IV), lies to the right of the midline and it is further divided into a superior sub-segment (A) and an inferior sub-segment (B). The left lobe contains the lateral sector segments II and III. In the lesser sac on the inferior surface of the liver between the inferior vena cava on the right, the ligamentum venous on the left, and the porta hepatic in front, there is the caudate lobe (segment I).

Figure 3. Liver and gallbladder, posterior view.

The Microscopic Anatomy of the surface of the liver is covered by visceral peritoneum (serosa), with a Glisson capsule underneath. At the *porta hepatis*, the Glisson capsule travels along the portal tracts (triads), carrying branches of the hepatic artery, the portal vein, and the bile ducts into the liver substance. Sinusoids are large capillaries lined by endothelial cells between cords of hepatocytes. Sinusoids also contain KCs of the reticulo endothelial system (RES). Bile canaliculi between hepatocytes drain into bile ductules in the portal triad (Figure 4).

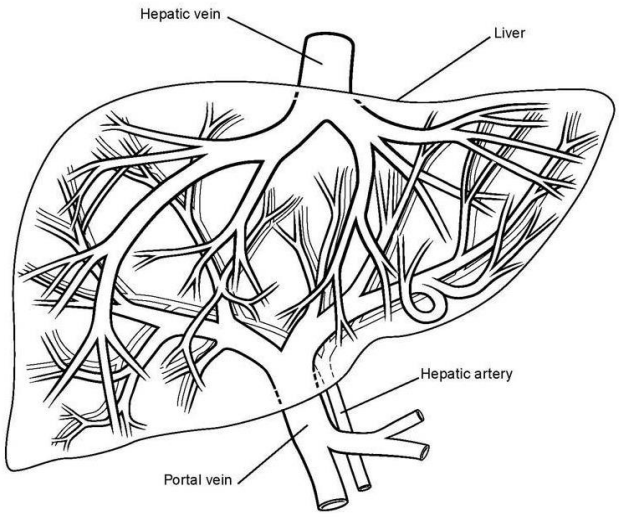


Figure 4. Liver vessel view.

1.2 Parenchymal and non-parenchymal cells

Hepatocytes account for up to 70% of the liver cells. These hepatic parenchymal cells carry out the different functions of the liver, such as protein storage and transformation of carbohydrates, synthesis of cholesterol, bile salts and phospholipids, detoxification,

modification and excretion of exogenous and endogenous substances. In the normal adult liver, hepatocytes are in quiescent state and they turn over very slowly (1-2 times/year). Based on specific conditions, such as a two-third partial-hepatectomy (PH) or acute toxic liver injury, hepatocyte turnover becomes faster and the liver can regenerate within 1-2 weeks (in rodents), increasing up to ~ 1 month in larger animals and humans (Font-Burgada et al., 2015).

1.2.1 Anatomy of hepatocytes

Hepatocytes can be separated from the liver by collagenase digestion, a process that creates a suspension of hepatocytes, which can be immediately used for culture or transplantation. Alternatively, they can be cryopreserved, although they are sensitive to freezing procedures. The use of hepatocyte transplantation as an alternative to the liver transplantation is very useful in case of liver-based metabolic conditions and acute liver failure (Corlu and Loyer, 2015). In fact, hepatocyte transplantation represents a less invasive method than liver transplantation and the cryopreservation of these cells increases the possibility of immediate availability for the treatment of fulminant liver failure. In the case of acute or chronic liver disease, the aim of hepatocyte transplantation is double: to replace tissue function and to allow the engraftment of transplanted cells for liver repopulation. Hepatocytes have a short life in culture so novel cellular models would be beneficial for *in vitro* studies where hepatocytes are required. For example, it could be possible to study toxicity or selectivity of drugs for apoptosis and necrosis of tumor cells, to analyze drugs for blocking proliferation of cancer cells, study the expression of microRNAs involved in disease onset and eventually to develop molecular therapies for the re-adjustment of these microRNAs, as well as investigate new markers.

1.2.2 Functions of hepatocytes

The hepatocytes produce serum albumin, fibrinogen, and the prothrombin group of clotting factors (except for Factor III and IV). These cells can metabolize, detoxify and inactivate exogenous compounds such as drugs (drug metabolism) and endogenous compounds such as steroids. One of the detoxifying functions of hepatocytes is to modify ammonia into urea for excretion.

1.3 Kupffer cells

Kupffer cells (KCs), also known as Kupffer-Browicz cells or stellate macrophages**,** are specialized macrophages located in the liver and constitute part of the reticule endothelial system (RES). The KCs represent approximately 80-90% of the tissue-fixed macrophages in the body and account for 5-15% of all hepatic cells (Froh et al., 2003; Kolios et al., 2006). Identified in 1976 by Karl Wilhem von Kupffer, the specialized function of KCs is their peroxidase activity. In biology, this is termed as the "tolerogenic" phenotype, which is necessary to prevent an immune response to any stimuli (Dixon et al., 2013). In humans and other mammals, the development of Kupffer cells starts in the yolk sac. Primitive macrophages differentiate into fetal macrophages and then enter the bloodstream (Naito et al., 2004). After that, they move to the fetal liver to become mature cells. Due to their localization in the liver sinusoids, KCs are the first barrier against antigens absorbed via the gastrointestinal tract and for this reason; they play a crucial role in identifying bacteria, endotoxins, apoptotic cells and immune complexes as well as removing toxic agents such as ethanol (Bilzer et al., 2006; Gregory et al., 1996).

1.3.1 Anatomy of Kupffer Cell

The KCs present extensions like *microvilli*, *pseudopodia*, *filopodia*, and *lamellipodia* in the plasma membrane, giving them a star-like or worm-like appearance. They are larger than sinusoidal cells and their cytoplasmic volume is generally dense and substantial. Their cytoplasm contains many lysosomes (for degradation of organelles), vacuoles (storage of materials) and phagosomes. They have Golgi bodies in clusters near the nucleus and are involved in the secretion and intracellular transport of vesicles. Rough Endoplasmic Reticulum (RER), known to be responsible for ribosome synthesis, is abundant in KCs. They contain only a single nucleus. Nuclei are mostly oval-shaped and have a finely distributed *euchromatin*. Kupffer cells contain other organelles like free ribosomes, mitochondria and microtubules, simply dispersed in the cytoplasm. They do not contain however glycogen or fat droplets.

1.3.2 Functions of Kupffer Cells

The function of KCs is to remove protein complexes and small particles from the blood. Together with other cells in the sinusoid, KCs are the first line of defense against pathogens entering the liver through the portal vein. This constitutes an important point because

the blood that passes through the portal vein is rich with pathogen-derived products that need to be removed from the circulation to avoid systemic immune activation. Kupffer cells mediate host resistance to infection by releasing pro-inflammatory cytokines, such as interleukin-1 (IL-1), IL-6, IL-12, IL-18 and tumor necrosis factor α (TNFα). Amongst these, IL-12 and IL-18 activate NK cells to produce anti-viral interferon γ (INFγ), while TNFα promotes the neutrophilic granulocyte infiltration to eliminate bacteria and induces apoptosis in hepatocytes under pathological conditions (Schümann and Tiegs, 1999; Tiegs and Lohse, 2010). While activated KCs are the main producer of inflammatory mediators, in the non-inflamed liver, KCs produce and secrete anti-inflammatory signals, such as IL-10, endogenous prostanoids and tissue growth factor β (TGFβ; Ishibashi et al., 2009; Kmiec, 2001; Racanelli and Rehermann, 2006). The release of IL-10 down-regulates the production of IL-6, TNFα and other cytokines (Knoll et al., 1995), contributing to the ability of intrahepatic cells to induce tolerance (Tiegs and Lohse, 2010). Several studies have reported the ability of KCs to induce tolerance *in vivo* after orthotropic liver transplantation (Chen et al., 2008; Liu et al., 2007), as well as the ability of these cells to inhibit DC-induced antigen-specific T cell activation *in vitro* (You et al., 2008). Furthermore, depletion of the KCs compartment after gadolinium injection impairs the generation of systemic immune tolerance following portal injection of all-antigenic leukocytes (Roland et al., 1993). As several liver cell compartments, i.e. hepatocytes, LSECs or hepatic stellate cells may be replaced by transplanted cells (Benten et al., 2005; Follenzi et al., 2008; Fontana et al., 2002; Gupta et al., 1999, 1995), this raises the possibility that KCs can also be replaced. As macrophages, KCs can capture and digest microorganisms and worn-out cells, phagocytize and break down old red blood cells that pass through the liver sinusoids (Nguyen-Lefebvre and Horuzsko, 2016). Added to that, owing to their peroxidase activity, they can also degrade the cell walls of bacterial and other microorganisms. The KCs regulate anti-viral immunity during hepatitis B and C infections, also inhibiting the growth of viral infections in the liver. Together with other macrophages, they contribute to the tissue damage of the infected body part. Kupffer cells also regulate fibrosis (thickening of connective tissue), cirrhosis (scarring of the liver) and hepatocellular carcinoma (abnormal growth of liver cells), which all occur during hepatitis (Karlmark et al., 2009). Kupffer cells have an essential role in maintaining liver homeostasis as well (Werner et al., 2015). A study published by Theurl and colleagues showed that KCs can control ion homeostasis through the expression of hepcidin (Theurl et al., 2008). Hepcidin is a hormone that primarily controls the uptake of iron into the circulatory system of mammals. Remarkably, when hepcidin levels increase (particularly during an inflammation or an immune response), serum iron and the engagement of iron in the

gut decreases. Kupffer cells also exhibit a high degree of adaptability, which depends primarily on their immune environment. Despite technological advancements, understanding the exact mechanisms of KC functions remains rudimentary. Despite their longlife span, KCs seem to have limited possibilities in therapy due to their low or absent proliferating capabilities. At the same time, however, their capacity in producing anti-inflammatory cytokines could assist in promoting the engraftment of other transplanted cells.

1.4 Liver Sinusoidal Endothelial Cells (LSEC)

Endothelial cells (ECs) are a source of physiologically important molecules participating in the formation of platelet and fibrin thrombi (e.g. von Willebrand factor and tissue factor) and contributing to antithrombotic properties of the endothelium (e.g. prostacyclin, thrombomodulin, and heparan sulfate; Van Hinsbergh, 2012; Yau et al., 2015). Endothelial cells also synthesize and secrete plasminogen activator inhibitors (PAIs), molecules regulating the growth of other cells, binding lipoproteins and hormones. Consequently, these cells represent the first barrier between the blood and the extravascular space, but also supply molecules influencing the structural and functional integrity of the circulation (Jaffe, 1987).

1.4.1 Anatomy of LSECs

The LSECs are unique endothelial cells, both morphologically and in their function. The LSECs are small cells, with a diameter of around 6.5 μm when isolated, that are stretched out into a very thin layer lining the hepatic sinusoids (DeLeve, 2013). The LSECs differ from ECs in the blood vessels of other tissues in both structure and function in that they lack a basement membrane and form a fenestrated monolayer organized in sieve plates, which are about 0,1μm in diameter (Aird, 2007; Braet and Wisse, 2002). Among vessels, the liver sinusoid is a specific capillary network system where varieties of metabolic substances are exchanged between hepatic blood flow and hepatic parenchymal cells. The LSECs comprise approximately 50 % of the non-parenchymal hepatic cells.

1.4.2 Functions of LSECs

The LSECs are in the hepatic sinusoids, separating hepatocytes from the circulating blood and play an important role in hepatic microcirculation (Oda et al., 2003). Sinusoidal ECs express a variety of scavenger receptors and eliminate soluble waste molecules from portal venous blood, such as extracellular matrix compounds, acetylated low-density lipoprotein (LDL), denatured albumin and others, via receptor-mediated endocytosis. One aspect of the liver sinusoid is the presence of resident hepatic macrophages, the KCs, on the luminal side of the endothelium. The LSECs and KCs constitute the most powerful scavenger system in the body (Enomoto et al., 2004) removing molecules via endocytosis (LSECs) or phagocytosis (KCs) and contributing to the liver physiology and pathology (Aird, 2007; Carpenter et al., 2005). The LSECs have a crucial role in organogenesis and liver homeostasis and regeneration. In fact, they produce hepatocyte growth factor (HGF) and IL-6 (Cleaver and Melton, 2003; LeCouter et al., 2003; Luna et al., 2004), which act in a paracrine manner to induce hepatocyte proliferation (LeCouter et al., 2003).

Moreover, LSECs synthesize and release Factor VIII (FVIII), a critical co-factor in the intrinsic coagulation pathway (Do et al., 1999; Follenzi et al., 2008). Another relevant role for LSECs is connected to immunity. They express major histocompatibility complex (MHC) class I and II molecules and costimulatory molecules CD40, CD80 and CD86. Although LSECs are not equivalent to DCs in their ability to present exogenous antigens, liver ECs are able to prime T cell responses (Bertolino et al., 2002; Knolle and Limmer, 2001; Racanelli and Rehermann, 2006). Several studies have demonstrated the possibility of isolating murine LSECs and obtaining LSEC-enriched or highly purified LSECs fractions from hepatic NPC (Benten et al., 2005; Follenzi et al., 2008; Kumaran et al., 2005). In turn, these studies also demonstrated that LSECs can be transplanted and will engraft in recipient mice, where the therapeutic potential of these cells has also been demonstrated.

With LSECs expressing factor FVIII of coagulation pathway (Do et al., 1999; Follenzi et al., 2008; Kumaran et al., 2005), it has been demonstrated that LSEC transplantation can correct the bleeding phenotype of Hemophilia A (HA) mice, confirming the use of LSECs in a cell therapeutic approach (Follenzi et al., 2008; Kumaran et al., 2005). As such, the opportunity to isolate and transplant liver ECs together with their ability in modulating immune tolerance to exogenous antigens, has opened the way to new approaches in cell and gene therapy using LSECs as powerful vehicle for the delivery and the expression of therapeutic native genes or transgenes.

1.5 Liver disease

1.5.1 Inherited metabolic liver disorder

Liver-based metabolic defects are usually secondary to a missing enzyme with consequences secondary either to the lack of a normally functioning protein or the upstream accumulation of toxic substances due to impaired metabolism of a protein. These diseases could be further classified into those with no effects on the liver and those leading to liver injury and fibrosis/cirrhosis. Important inherited disorders, causing acute and chronic liver disease, include hemochromatosis (HH), Wilson's disease, and alpha 1-antiprotease (antitrypsin) deficiency. Both Wilson's disease and genetic hemochromatosis involve defects in the transport of heavy metals and their accumulation in hepatocytes. In α 1-antitrypsin deficiency, intrahepatocyte accumulation of defective α 1-antitrypsin occurs. Several studies, concerning cell transplantation, were carried out on these diseases. HH is a genetic disorder in which there is excessive accumulation of iron in the body (iron overload). HH is the most common genetic iron overload disorder among Caucasians (Franchini and Veneri, 2005). It has been demonstrated to result from mutations in several genes involved in the regulation of iron homeostasis such as HFE, TfR2, HJV, HAMP, and Ferroportin. The most common form of HH is associated with the homozygous p.C282Y mutation of the HFE gene, which was first identified by Feder et colleauges (Feder et al., 1996).

Wilson disease is a disorder of copper metabolism that can present with hepatic, neurologic, or psychiatric disturbances, or a combination of these, in individuals ranging from age three years to older than 50 years. Wilson disease is caused by mutations in the ATP7B gene. This gene encodes a protein called copper-transporting ATPase 2, which plays a role in the transport of copper from the liver to other parts of the body.

Mutations in the SERPINA1 gene cause alpha-1 antitrypsin deficiency. This gene encodes a protein called alpha-1 antitrypsin, which protects the body from a powerful enzyme called neutrophil elastase. Neutrophil elastase is released from white blood cells to fight infection, but it can attack normal tissues (especially the lungs) if not tightly controlled by alpha-1 antitrypsin.

1.5.2 Chronic liver disease

Progressive destruction of the liver parenchyma over a period greater than 6 months lead to fibrosis and cirrhosis. According to the Global Burden of Disease 2010 study, liver cirrhosis caused 31 million or 1.2% Disability Adjusted Life Years (DALYs) globally in 2010, and one million deaths or 2% of deaths worldwide in that year (Lozano et al., 2012; Murray et al., 2012). The epidemiology has been evaluated extensively in several developed countries, including Europe and the Americas (Bosetti et al., 2007; Corrao et al., 1997; La Vecchia et al., 1994; Zatoński et al., 2010). Common causes of cirrhosis are HCV infection, alcohol abuse and nonalcoholic steatohepatitis (NASH) (Bataller and Brenner, 2005). In advanced stages, cirrhotic livers contain approximately 6-times more extracellular matrix (ECM) than normal livers. An accumulation of the ECM results from both an increased synthesis and decreased degradation (Arthur et al., 2000). A decreased activity of ECM-removing matrix metalloproteases (MMPs) is mainly due to an overexpression of their specific inhibitors (TIMPs). Treatment of cirrhosis prevents further damage to the liver and holds off the emergence of liver cancer. Liver transplantation is now the only option for.

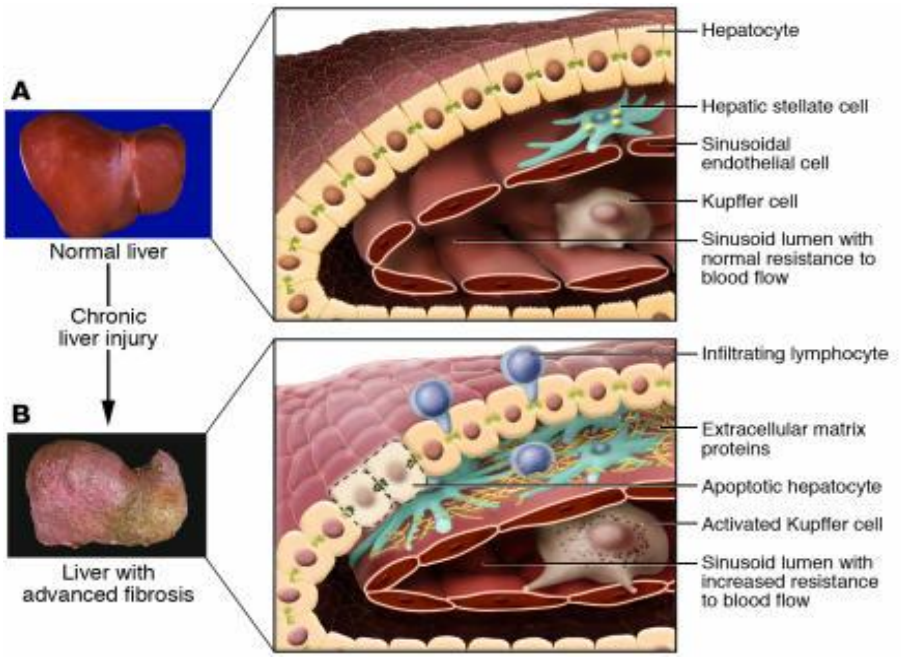


Figure 5. Changes in the hepatic structure (A) associated with advanced hepatic fibrosis (B). Following chronic liver injury, inflammatory lymphocytes infiltrate the hepatic parenchyma. Some hepatocytes undergo apoptosis, and Kupffer cells activate, releasing fibrogenic mediators. HSCs proliferate and undergo a dramatic phenotypical activation, secreting large amounts of extracellular matrix proteins. Sinusoidal endothelial cells lose their fenestrations, and the tonic contraction of HSCs causes increased resistance to blood flow in the hepatic sinusoid. Taken from Bataller and Brenner, 2005.

1.5.3 Hepatocellular carcinoma (HCC)

Hepatocellular carcinoma (HCC) is the most common type of primary liver cancer in adults, and it is the most common cause of death in patients with cirrhosis. In the United States, HCC is recognized as the ninth leading cause of cancer deaths. (CDC, 2010). Chronic viral hepatitis can lead to cirrhosis and/or HCC. It is well accepted that Hepatitis B and C are the most common causes of chronic hepatitis in the world (Takano et al., 1995). Metabolic syndrome, non-alcoholic steatohepatitis (NASH), inherited metabolic liver disorder increase the risk of developing HCC (Ananthakrishnan et al., 2006).

The loss of regulation of the signal, leading to tumor growth, includes many factors such as insulin-like growth factors, hepatocyte growth factor and the Wingless (Wnt) / beta-catenin signaling pathways (Breuhahn et al., 2006; Farazi and DePinho, 2006). These factors are upregulated in HCC. Furthermore, tumor cells can circumvent the mechanisms of apoptosis and facilitate angiogenesis (Farazi and DePinho, 2006). There are also pro-carcinogenic mechanisms that are specific to each type of pathology. These mechanisms follow more than the classical theory of gene mutations in (most affected) cells as a cause of cancer (Kinzler and Vogelstein, 1996). In hepatitis B, viral DNA integration could lead to chromosomal instability and mutations in normal proliferative regulatory factors, including genes such as p53 (Laurent-Puig and Zucman-Rossi, 2006). The accumulation of these alterations leads to HCC.

1.5.4 Acute liver failure (ALF)

Acute liver failure (ALF) is a clinical syndrome, in which there is an acute insult in a patient without a known preexisting liver disease that leads to a rapid loss of liver function. (Trey and Davidson, 1970). ALF is also called fulminant hepatic failure or acute hepatic failure. There is a subdivision of the ALF: hyperacute (0-7 days), acute (8-28 days) and subacute (from 29 days to <26 weeks).

ALF etiology varies among the geographical area, for example in the United States (Stravitz and Kramer, 2009) Europe, and United Kingdom Prognostic utility of the bilirubin lactate and etiology score (Bernal et al., 2009). acetaminophen is the main cause of ALF, in contrast in India and in other Asian countries, viral hepatitis is the main cause (Oketani et al., 2011). Less common causes include ischemia, Wilson's disease, Budd-Chiari syndrome, and pregnancy. Etiology of ALF provides one of the best indicators of prognosis, and it dictates specific management options. In most cases, ALF presents with no previous clinical and

laboratory evidence of disease or significant hepatic injury leading to loss of liver function. Initial laboratory examination must be extensive in order to evaluate both the etiology and severity of ALF. Early testing should include routine chemistries (glucose and hypoglycemia), arterial blood gas measurements, complete blood counts, blood typing, acetaminophen level and screens for other drugs and toxins, viral serologies, tests for Wilson disease, autoantibodies, and a pregnancy test in females. The goal of treatment is to achieve metabolic and hemodynamic stability. Although transplantation is a treatment option for some specific causes of acute liver failure, such treatment is not universally available, and less than 10% of liver transplantations are performed in patients with acute liver failure. The limited availability of liver transplantation has led to the evaluation of other therapies in patients with advanced disease. Hepatocyte transplantation involves intraportal or intraperitoneal infusion of isolated human hepatocytes to augment liver function.

1.6 Orthotopic liver transplantation (OLT)

Orthotopic liver transplantation (OLT) is the standard of care for patients with end-stage liver disease or ALF, and for certain liver-based metabolic defects. (Sze et al., 2009)Currently, it provides a good quality of life and a 5-year survival of 70-80%. The complete transplant procedure is composed of four main stages: the donor hepatectomy, the recipient hepatectomy, the implantation of the graft (4 vascular anastomoses), followed by hemostasis and the bile duct reconstruction. Most of the complications associated with OLT occur in the perioperative period, such as primary graft dysfunction, acute rejection, severe infections, and complications as well as hepatic artery thrombosis or biliary leaks. The adverse effect of the immunosuppressive therapy is one of the most important problems for long-term morbidity and mortality after OLT, they are used to prevent the graft rejection.

The key aspect for long-term management of OLT recipients is the prevention of the metabolic complications, resulting from the use of immunosuppressive drugs, and important, regular screening for malignancy. (Benten et al., 2009) However, successful replacement of lost liver functions by healthy hepatocytes transplantation, in animal models and in people with Crigler-Najjar syndrome due to UGT1 enzyme deficiency, familial hypercholesterolemia due to low-density lipoprotein receptor (LDLR) deficiency, or acute and chronic liver failure indicated that OLT could possibly be avoided. (Fox et al., 1998; Hansel et al., 2014; Sokal, 2014; Strom et al., 1997; Vacanti and Kulig, 2014) This general aspect has been highlight by

similar successes with auxiliary partial orthotopic liver transplantation (APOLT) for enzymatic deficiency states or ALF (Rela et al., 1999). The APOLT clinical experience gives reason to the hypothesis that hepatocyte can be used to correct enzyme defects, metabolic functions, and for failing liver after injury, promoting its regeneration. In addition, successful correction of hemophilia by OLT indicated that cell therapy would be a good way for other classes of diseases. Moreover, cell transplantation is simpler than OLT and APOLT, because cells from a donor liver may be transplanted into multiple recipients, cells can be transplanted via intravascular catheters and not with complex surgery. Cells can also be cryopreserved in a prospective non-emergency setting, and can be transplanted repeatedly, since the native liver is not removed, finally the costs of transplanting cells should be less than that of organ transplantation (Forbes et al., 2015).

1.7 Alternative approach to OLT: cell transplantation

Liver transplantation is presently the only treatment for many medically refractory liver diseases, including end-stage liver disease and many inherited liver diseases. Despite this, approximately 40% of listed patients per year do not receive a liver transplant. Considering these statistics, new therapies are needed in addition to whole-organ liver transplantation.

Table 1. Potential clinical indications for liver cell therapy.

A. Congenital disorders	B. Acquired disorders
• Alpha-1 antitrypsin deficiency*	• Acute liver failure (multiple etiologies)*
• Crigler-Najjar syndrome type 1*	• Fatty liver of pregnancy*
• Familial hypercholesterolemia*	• Acute-on-chronic liver failure (multiple etiologies)*
• Congenital coagulation factor VII deficiency*	
• Hemophilia A	
• Glycogen storage disease type I*	
• Infantile Refsum disease	
• Maple syrup urine disease	
• Neonatal hemochromatosis	
• Progressive familial intrahepatic cholestasis type 2 (PFIC2)	
• Urea cycle defects - ornithine transcarbamylase (OTC) deficiency; arginosuccinate lyase deficiency; carbamoylphosphate synthase type 1 deficiency; citrullinemia*	
• Wilson's disease	
***Indicates conditions treated by cell transplantation in people.**	

The premise for early studies in people was based on transplanting healthy cells to replace deficient functions in acquired or inborn errors of metabolism besides supporting the failing liver, as indicated in Table 1. Innovative cell therapies and animal studies of human liver disease highlight the regenerative capacity of hepatocytes. In the Table 2 were selected examples of animal models used in cell transplantation studies. These studies indicate the possibility of cell therapies as a means of replacing hepatic tissue (Han et al., 2009; Kobayashi et al., 2000).

Table 2. Examples of animal models used in cell transplantation studies.

Mechanisms in cell engraftment and proliferation	**Defects in hepatic detoxification**
Dpp4-deficient F344 rats and Dpp4 knockout mice	Nagase analbuminaemic rat (hypoalbuminaemia)
Transgenic donor mice (HBV, human alpha-1 antitrypsin, betagalactosidase, alb-uPA, etc.)	Gunn rat and MRP-2 knockout mice (Crigler-Najjar syndrome, type-1)
Fumarylacetoacetate hydroxylase (FAH) knockout mice	Histidinemia mice (histidinemia)
Secretory protein deficiency	MSUD knockout mouse (Maple syrup urine disease)
Nagase analbuminaemic rat (hypoalbuminaemia)	Spf-ash mice (OTC deficiency)
Hemophilia A mice	**Diseases of receptor function**
Transport defects	Watanabe heritable hyperlipidemic rabbit (familial hypercholesterolaemia)
TR- rat and Mdr2 knockout mouse (PFIC1)	Apolipoprotein E knockout mouse (hypercholesterolemia)
BSEP knockout mouse (PFIC2)	**Acquired disorders**
Long-Evans Cinnamon rat and Atp7b knockout mouse (Wilson's disease)	Induced acute liver failure (hepatectomy, chemicals, drugs, viruses, physical methods)
	Chronic liver disease (CCl4, thiocetamide, acetylaminofluorene, etc.)

Eiseman in 1965 was the first to report a treatment for liver failure, with an *ex vivo* device using living liver tissue. This was followed by Mata's et al. in 1976, who was the first to perform a hepatocyte transplantation in a rodent (Matas et al., 1976), while Starzl in 1985, performed the first human solid organ liver transplantation (Starzl et al., 1968). Numerous discoveries of the last thirty years have favored the development of innovative therapeutic protocols based on the use of cells in combination or in substitution for organ transplantation. Today we know that, by stimulating the processes of cell regeneration and repair, alternative ways of organ transplantation can be found even if the replacement of an entire organ remains the only life-saving therapy that allows to restore severely compromised functions. The new alternative approaches to transplantation employ specific cell populations that after being subjected to

particular procedures of collection, handling and identification in environments that prevent their contamination, can be stored viable for long periods of time. These cells are then infused into the patient to implement the regenerative processes that will lead to the cure or improvement of the disease. This new treatment perspective is defined by the term regenerative medicine. Cell therapy is potentially able to stop or even reverse the process that causes the disease. Once transplanted, the new cells act through two main mechanisms: i) they are able to replace the damaged cells of the patient by restoring their normal function, and ii) they stimulate the regeneration and repair of the surrounding diseased tissue, helping the patient's cells to reactivate ineffective regenerative processes. This allows producing in a short time a therapeutic effect that remains steadily over time, keeping the patient's organ on site. In addition, cell therapy allows and facilitates the regeneration and repair of diseased tissue by the cells of the same organism, unblocking the processes towards a natural regeneration.

Living donor liver transplantation has been developed to increase the number of donors available for transplantation. This, however, can only be used on a reduced sized liver transplantation. There is an inverse relationship between the sizes of the graft to the degree of non-function. Graft shows delayed and impaired regeneration and frequently leads to liver failure. As such, an immediate regeneration of the mismatched graft is required in a living donor. It has been demonstrated that hepatocyte transplantation may decrease the mortality rate among patients with end-stage liver disease that are waiting for liver transplantation (Pareja et al., 2010). One study suggests that bone marrow mononuclear cell transplantation combined with HGF administration, exhibits a synergistic beneficial effect on improving both functional and histological liver recovery (Jin et al., 2011). Moreover, stem cells including mesenchymal stem cells show promising roles in attenuating ALF. Parekkadan et al., provide the first experimental evidence using mesenchymal stem cells-derived molecules (Parekkadan et al., 2007). Encapsulation of hepatocytes and mesenchymal stem cells improved hepatocyte-specific functions in vitro and in vivo (Shi et al., 2009). Another cell-based therapy for ALF is the bioartificial liver which has been tested in clinical trials (Demetriou et al., 2004; Nyberg and Misra, 1998), as well as other configurations which are under development (Lee et al., 2008; Sussman et al., 2009).

Some research groups have worked to identify the possibility of using iPSCs cells generated in a clinical sense for a rapid and stable liver regeneration (Espejel et al., 2010). Moreover, the iPSC-based cell therapies have been applied with encouraging results, in several animal models (Chun et al., 2010). Also, mesenchymal stem cell (MSCs) therapies may be a novel way for hepatic regeneration and hepatocyte differentiation and thereby support hepatic

function in diseased individuals (Ishikawa et al., 2010). In the Table3 are summarized different types of potential cell therapy depending upon disease scenario.

Table 3. Cell therapy for liver disease

Cell type	Clinical indication	Clinical use	References
Hepatocytes	Metabolic liver disease Paediatric liver failure	Yes	
Mesenchymal stem cell	Liver cirrhosis Liver failure Immune mediated liver disease	Some clinical reports and small number of randomised trials	Moore, J.K et al. 2014 El-Ansary, M., et al 2012
Endothelial progenitor cells	Liver cirrhosis	No	Nakamura, T.et al. 2007
Macrophages	Liver cirrhosis	No	Thomas, J.A. et al. 2011
Embrionic stem cells	Metabolic liver disease Liver failure	No	Hay, D.C., et al. 2008
Induced pluripotent stem cells (iPSCs)	Metabolic liver disease Liver failure	No	Sullivan, G.J., et al. 2010

1.7.1 Co-transplantation

Co-transplantation of hepatocytes, KCs, and LSECs derived from liver may be an alternative approach for orthotopic liver transplantation and hepatocytes only transplantation. In the liver this main cell types together, create a network in which each cell type communicates with others, and each is responsible for the regeneration and proliferation of hepatocytes in case of damage. We report the effect of cell-cell communication in different condition of co-transplantation. Reproducing the physiological regenerative capacity of hepatocytes requires a perfect balance between cells to balance their normal communication. However, it has been seen that the cells of the recipient liver have to get used to the presence of external hepatocytes and the not immediately adequate communication reduces the possibility of the same hepatocytes to cling and above all to activate the proliferation methods. Co-transplanting in addition to hepatocytes, the two main cell types present in NPCs improves communication between cells, reduces inflammation, increases engraftment and begins to promote their proliferation. Some of the molecular mechanisms are known others are being studied and will allow improving the experimental conditions in order to obtain a complete insertion of the transplanted cells, in the network of resident cells, until a complete replacement of the damaged cells without resorting to organ transplantation.

1.8 Sources of hepatocytes

1.8.1 Primary hepatocytes

Isolated hepatocytes have been utilized for extracorporeal and injectable cell transplantation. Being an anchorage-dependent epithelial cell type, primary hepatocytes must attach to an extracellular support matrix to avoid programmed cell death, termed anoikis (Frisch and Francis, 1994), or they require a pre-treatment for anchoring.

For the treatment of some liver diseases, hepatocyte transplantation is an auspicious alternative to liver transplantation. There are numerous sources of primary hepatocytes: they can be isolated from non-transplantable human livers, human liver resections, human fetal livers and healthy donor animals such as the pig.

The quality and metabolic/functional activity of the isolated primary hepatocytes is, however, variable. Bhogal et al. reported that the time between hepatectomy and the beginning of the liver perfusion, and the shortest possible digestion time, are the most important factors in determining the success of the procedure. The success rate was 54% (Bhogal et al., 2011). Patients with biliary cirrhosis provide a high yield of hepatocytes (success rate 71%).

Hepatocytes can also be cryopreserved and stored in hepatocyte banks but they are highly susceptible to the freeze-thaw process and their functionality is significantly reduced compared to freshly isolated hepatocytes (Yagi et al., 2001). For this reason many cryopreservation methods have been reported (Grondin et al., 2009; Katenz et al., 2007; Lloyd et al., 2004; Stéphenne et al., 2005), but functional activities after thawing are still unsatisfactory. Optimal cryopreservation methods still require further research.

1.8.2 Expansion options of primary hepatocyte

Primary hepatocytes continue to be the dominant source of cells for cell therapy. Unluckily, isolated primary hepatocytes have a little proliferation capacity *ex vivo* and the most significant problem is that they rapidly lose their differentiated structures and liver-specific functions. Research for new culture methods for primary hepatocytes therefore continues.

Small scale: in vitro primary hepatocyte culture techniques. Primary hepatocytes when cultured on a single collagen layer produce albumin and urea and demonstrate cytochrome P450 activity, but their functions rapidly decline in the first week of culture. To mimic the matrix, a

second layer of collagen was added on top of the cultured hepatocytes, termed a collagen "sandwich" configuration. This configuration maintains hepatocyte function, polarity and induces apical and lateral membrane formation (Taguchi et al., 1996). To recreate the interactions between parenchymal and non-parenchymal cells, several non-parenchymal cells such as fibroblasts, stellate cells, KCs and endothelial cells have been co-cultured with hepatocytes (Kan et al., 2004). Parenchymal and NPCs self-organize to form simple epithelial structures with an outer layer of biliary epithelial cells, a middle layer of hepatocytes and connective tissue, and an inner layer of endothelial cells (Michalopoulos et al., 2001).

1.9 Generation of hepatocytes from different stem cell sources

1.9.1 Stem cells

Recent advances in cell biology have led to the concept of regenerative medicine, based on the therapeutic potential of stem cells. Stem cells, characterized by their ability to self-renew and differentiate into a wide variety of cell types, have been proposed as an ideal cell source to generate unlimited numbers of hepatocytes. Different types of stem cells are theoretically suitable for liver cell replacement.

Many studies have utilized liver-derived stem cells to generate primary hepatocytes (Lázaro et al., 1998; Zaret and Grompe, 2008). Hepatoblasts are bipotent, being able to become both hepatocytes and bile duct cells. Murine hepatoblast cell lines have been used for their capacity to repopulate the liver upon transplantation in animal models (Zaret and Grompe, 2008). It was reported that human fetal liver cells can be isolated and differentiate into mature hepatocytes in culture (Weber et al., 2010).

Liver regeneration can be achieved by the activation, expansion, and differentiation of the hepatic progenitor-stem cells, such as oval cells and hepatoblasts (Lázaro et al., 1998). However, hepatoblasts comprise only about 0.1% of fetal liver mass, and oval cells comprise 0.3% – 0.7% of adult liver mass. Their total amount is to low and it is difficult to perform the isolation and expansion of these cells for large-scale applications (Haridass et al., 2009; Schmelzer et al., 2006).

Human placental tissue is another promising source of human stem cells. Some work show that human umbilical cord blood stem cells are able to colonize the liver and differentiate

into hepatocytes after acute toxic liver damage in NOD/SCID mice and in immunocompetent rats (Di Campli et al., 2004; Piscaglia et al., 2010).

Other studies have induced embryonic stem cells to differentiate into hepatocyte-like cells. Thus far, many promising studies have shown the therapeutic potential of differentiated derivatives of embryonic stem cells in ameliorating a range of disease in animal models. These derivatives are able to colonize the injured liver and function as mature hepatocytes (Zaret and Grompe, 2008).

Human iPS cells have the potential to provide an unlimited source of hepatocytes. Importantly, iPS cells can bypass the ethical concerns of embryo destruction related to the derivation of embryonic stem cells, as well as issues related to allogenic rejection. They may represent an ideal source of patient-specific and disease-specific adult cells. Hepatocyte-like cells generated from iPS cells secrete human albumin, synthesize urea, and express human cytochrome P450 enzymes (Si-Tayeb et al., 2010).

Researchers have recently reported that iPS cells can revert type-1 diabetes and several liver diseases (Rashid et al., 2010). Transplantation of hepatocytes-like derived from human iPS cells could represent an alternative to liver transplantation in ALF, or for metabolically deficient states.

Several groups have been generating hepatocyte-like cells from other sources, such as monocyte-derived hepatocyte-like cells. Transplantation of hepatocyte-like cells derived from peripheral blood monocyte may represent a possible therapy for people with acute or acute-on-chronic liver disease giving time for whole organ transplantation or in turn promote the regeneration of the liver (Ehnert et al., 2011).

1.10 System for liver repleacement

1.10.1 Bio artificial liver (BAL)

Many other liver sustenance therapies and artificial liver support have been performed over the past 50 years (S. L. Nyberg et al., 1992), including hemodialysis, hepatodialysis, extracorporeal heterologous liver perfusion (Abouna et al., 1970) and homologous, cross circulation, activated charcoal hemoperfusion, simple exchange transfusion, and

plasmapheresis with plasma exchange. There are two positive comments to be made from these early clinical trials. Firstly, the neurological status of hepatic encephalopathy is often improved; however, the long-term survival was not significantly ameliorated in comparison with historical controls (rakela et al., 1985). Secondly, the removal of toxins correlated with recovery from hepatic encephalopathy. Many of these early therapies appeared to have a benefit in case reports and small studies, but none stood the test of a randomized prospective trial (Nyberg et al., 1993a).

A good example of an artificial support therapy is Charcoal hemoperfusion. It appeared to be promising in a small study but it did not stand the test of a randomized prospective trial (O'Grady et al., 1988). Many of the limitations of early liver support therapies include immune responses, reproducibility, functionality, cell dose as well as the duration of therapy.

From bioartificial liver devices, lessons were learned from the membrane used to overcome the problem of cross-circulation where the blood of some subjects was directly exchanged with the blood from others (Nyberg et al., 1999; S L Nyberg et al., 1992).

Similarly, bioartificial liver devices use a membrane to avoid the necessity of immunosuppression to prevent immune responses caused by direct contact of blood or plasma with its hepatocytes. Bioartificial liver was first coined by Matsumura in 1987, where they perfused porcine hepatocytes in an extracorporeal bioreactor (Matsumura et al., 1987). The duration of the treatment with this first bioartificial liver device was limited due to the death of anchorage dependent hepatocytes in suspension culture. The use of hepatocyte spheroids, spherical aggregates of cells have been addressed to overcome the limitations of culture suspension, allowing the cryopreservation of isolated cells prior to extracorporeal use.

1.10.2 Cells sources for BAL (Table 4)

Primary hepatocytes	(Bhogal et al., 2011) (Yagi et al., 2001) (Grondin et al., 2009; Katenz et al., 2007; Lloyd et al., 2004; Stéphenne et al., 2005)
Tumor cell lines	(Ellis et al., 1996; Millis et al., 2002) (Mavri-Damelin et al., 2008; Wang et al., 1998) (Enosawa et al., 2001)

Immortalized hepatocyte cell lines from normal human hepatocytes	(Chen et al., 2010)
Xenotransplantation with primary pig hepatocytes	(Bonavita et al., 2010) (Baquerizo et al., 1999) (Paradis et al., 1999)

1.10.3 Other sistem for liver replacement (Table 5)

Large scale: bioreactor options for cell culture	(Diekmann et al., 2006)
Flat membrane culture systems	(Nahmias et al., 2006)
Hollow fiber system	(Nyberg et al., 1993) (Miki et al., 2011)
Encapsulation technology	(Orive et al., 2003; Weber et al., 2009)
Spherical aggregate culture systems	(Landry et al., 1985) (Sakai et al., 1996) (Nyberg et al., 2005)
In vivo incubators: humanized animal liver	(Meuleman et al., 2005) (Hickey et al., 2011)
Perfusion of decellularized liver matrix and hepatocytes	(Uygun et al., 2010) (Bao et al., 2011)

1.11 Specific cell therapy for various liver disease

1.11.1 Cell therapy for hepatitis

Genetic engineering of endothelial progenitor cells for overexpressing cytokine cardiotrophin-1, enhances the hepatoprotective properties of endothelial progenitor cells and constitutes a therapy for fulminant hepatitis (Fernandez-Ruiz et al., 2011). Autoantigen-specific

regulatory T cells and engineered T cells, respectively, are a potential tool for immunotolerance reconstitution in type-2 autoimmune hepatitis and chronic hepatitis B (Longhi et al., 2011). Vaccination with *ex vivo* activated dendritic cells may be a tool for therapeutic or prophylactic approaches against the Hepatitis B virus (Farag et al., 2010).

1.11.2 Cell therapy for liver cirrhosis

Chronic liver disease is usually accompanied by progressive fibrosis. Phase I trials concerning the injection of autologous bone marrow cells to cirrhotic patients have reported modest improvements (Alison et al., 2007). There was an improvement in Child-Pugh score and albumin levels in subjects with cirrhosis who received portal vein infusion of autologous mesenchymal stem cells (Terai et al., 2006), and a significant increase of liver function post liver resection in subjects with cirrhosis (Ismail et al., 2010).

Transplanted mesenchymal stem cells improved insulin resistance and glucose homeostasis, and ameliorated liver cirrhosis (Jung et al., 2011). Further, autologous peripheral bone marrow cell transplantation is a novel and potentially beneficial treatment for decompensated liver cirrhosis (Han et al., 2008).

1.11.3 Cell therapy for liver cancer

In 2005, autologous CD133+ bone marrow stem cells were transplanted via the portal vein in subjects with liver cancer prior to undergoing portal venous embolization. With the transplantation, these patients following an extensive liver resection had a subsequent degree of clinical improvement (Fürst et al., 2007). In one study, a combination of portal vein embolization and CD133+ bone marrow stem cell administration, showed an increase in the degree of hepatic regeneration, in comparison with embolization alone in subjects with malignant liver lesions (Fürst et al., 2007). In another case, patients with hepatocellular carcinoma receiving prior to surgery, autologous bone marrow stem cells, had a significant increase in liver function post liver resection (Ismail et al., 2010).

1.12 In vitro model

In order to improve knowledge and interpretation at the level of protein expression, gene and specifically molecular mechanisms, in addition to experimentation with animal models, we need cell models that faithfully reproduce what happens in vivo. Our group, with Columbano

and Giordano group, has demonstrated the possibility of obtaining cellular models of normal hepatocytes and tumor hepatocytes, which maintain the characteristics of the original cells but which open the possibility to innumerable in vitro studies that can vary from the effect of drugs on the cells before and after the transformation. Cancer to what happens at the level of expression, communication and interaction between different cell types when put in contact. In addition, this will allow us to understand and improve the transplant conditions more and more in order to be able to replace damaged or diseased cells completely, to understand the mechanisms for reversing tumor processes, until the organ transplant become obsolete.

Michael Conn and James Parker neatly explain the role of the animal model in "The Animal Research War"(2008):

If you are going to study a human disease you can't, for ethical reasons, perform the initial work in humans; you have to develop a model. Some models may be in vitro – literally, in glass tubes – but as you learn more and more, you must eventually test ideas in vivo– in living animals. That means you have to have a way of producing the disease that allows you to study it.

2. Aim of the projects

Aim of the project 1

"Co-trasplantation of mature Liver cells improves hepatocytes engraftment in animal models"

The liver is the largest internal organ of the body. It has an exceptional regenerative capability because as little as 25% of a liver can regenerate into a whole liver. This is predominantly due to two factors: the hepatocytes re-entering the cell cycle and the presence of bipotential stem cells, called hepatic oval cells, which can differentiate into hepatocytes. It remains, however, to be determined how the liver begins to regenerate after cell transplantation, and to find the optimal conditions for engraftment. In recent years, other non-traditional approaches for liver replacement have been attempted with only a partial success. These include either an auxiliary liver transplantation, hepatocyte transplantation, xenotransplantation, extracorporeal perfusion using xenogeneic approaches, or a human liver perfusion and bio-artificial liver assisted devices. Although hepatocyte transplantation is currently performed in the clinic, it is only a temporary solution since it only delays organ transplantation. Thus, more reliable protocols of hepatocyte transplantation are necessary to be able to bring long-lasting benefits. Based on our preliminary data, **we hypothesize that co-transplantation of a combination of liver cells (i.e. hepatocytes, LSECs, and KCs) following liver damage can dramatically ameliorate hepatocyte engraftment and long-term functions of the transplant**. In this proposal, we will determine the best combination of liver cells to be able to replace and replenish liver function. Our goal is to achieve a consistent engraftment through the induction of liver cell proliferation, while favoring an interaction between resident and transplanted cells. For this purpose, we will pursue the following aims:

Aim 1: to determine the best conditions for increasing the engraftment of transplanted cells.

Rationale: Successful hepatocyte engraftment requires liver damage. In order to determine the optimal conditions for engraftment, we will test different transplantation times following pretreatments that reproduce liver injuries/pathologies. Specifically, we aim:

Aim 1.1. To reproduce liver damage and understand the best time for transplantation.

Aim 1.2. To perform experiments that verify liver engraftment.

Aim 1a: To determine the best cell combination for the induction of cell proliferation.

Rationale: In the liver, the cellular network formed by hepatocytes, LSECs and KCs, can activate the regenerative process after injury. In order to obtain engraftment, not only next to the vessels but also in the parenchyma, we will establish the best experimental conditions for inducing proliferation in the parenchyma. We aim:

Aim 1a.1. To reproduce niches capable of making the right condition for cell-cell communication.
Aim 1a.2. To determine the best cell combination able to induce the cell interaction and their activation.

Aim 1b: To characterize the molecular basis of the interaction between hepatocytes, LSECs and KCs *(work in progress).*

Rationale: In the liver niche, cytokines and chemokines produced by resident cells allow their proliferation. We have already established that Ccl2, Cxcl1, Cxcl2 and Timp1 as the most upregulated cytokines, during the first week after transplantation. Thus, we will:

Aim 1b.1. Measure the level of Ccl2, Cxcl1, Cxcl2 and Timp1 *in all the transplant conditions at different time points.*
Aim 1b.2. Determine the functions of the aforementioned cytokines in transplantation, engraftment and proliferation of mature liver cells.

3. Materials and Methods
Aim 1

3.1 Animal

Animal Care and Use Committees of University of Piemonte Orientale approved the studies. Seven to ten-week-old mice were used as donors and recipients. For the first experimental conditions, wild-type C57BL/6J mice and C57BL/6-Tg(CAG-EGFP) 131Osb/LeySopJ mice with ubiquitous green fluorescent protein (GFP) expression (The Jackson Laboratory, Bar Harbor, ME) were used as donors while DPPIV- mice in C57BL/6J background were used as recipients (The Jackson Laboratory). Subsequently, wild type and DPPIV- C57BL/6J donors were used.

3.2 Liver perfusion and cells isolation

The perfusion buffer "Leffert" contains 10 mmol/l HEPES, 3 mmol/l KCl, 130 mmol/l NaCl,1 mmol/l NaH2PO4.H2O, and 10 mmol/l d-glucose, pH 7.4 (Sigma-Aldrich). The portal vein was exposed by laparotomy. The inferior vena cava (IVC) was tied with a wire and another wire between the portal vein and the mesenteric vein was used to maintain the caterer in position. At the initiation of the perfusion, the IVC was cut under the kidney and under the heart to allow an exit for the buffer. The liver was perfused at 5 ml/min via portal vein for 5 minutes with buffer at 37°C containing 1.9 mg/ml EGTA in Leffert buffer (this step removes all the calcium to disrupt cell-cell tight junctions). This was followed for 2 minutes with Leffert buffer lacking EGTA, to remove the excess, and for 13-14 minutes with buffer containing 0.03% (w/v) collagenase and 5 mM $CaCl_2$ (collagenase from Worthington Biochemical Corp.). All the buffers were maintained at 37°C, to avoid both cellular stress and for collagenase activation.The liver was dissociated in perfusion buffer and the cells were passed through an 80-μm pore sized Dacron fabric, after a light scraping. In the preliminary steps of this work, hepatocytes were isolated from C57Bl/6-wt, while KCs and LSECs were isolated from GFP+ mice. We then decided to use only hepatocytes from WT mice, while KCs and LSECs were isolated from DPPIV- mice, in order to evaluate only hepatocyte engraftment and proliferation. Hepatocytes were obtained by differential centrifugation at 50g, firstly for 5 minutes and then twice more at 40g to remove all cells. The NPCs (non-parenchymal cells) were collected by a high-speed centrifugation at 130g for 15 minutes. The LSECs were isolated by immunomagnetic positive selection from NPCs with an anti-CD146 conjugated-antibody, and finally from the negative fraction, KCs were obtained by co-incubation with anti-CD11b and anti-F4/80 conjugated-antibodies. The cell viability was

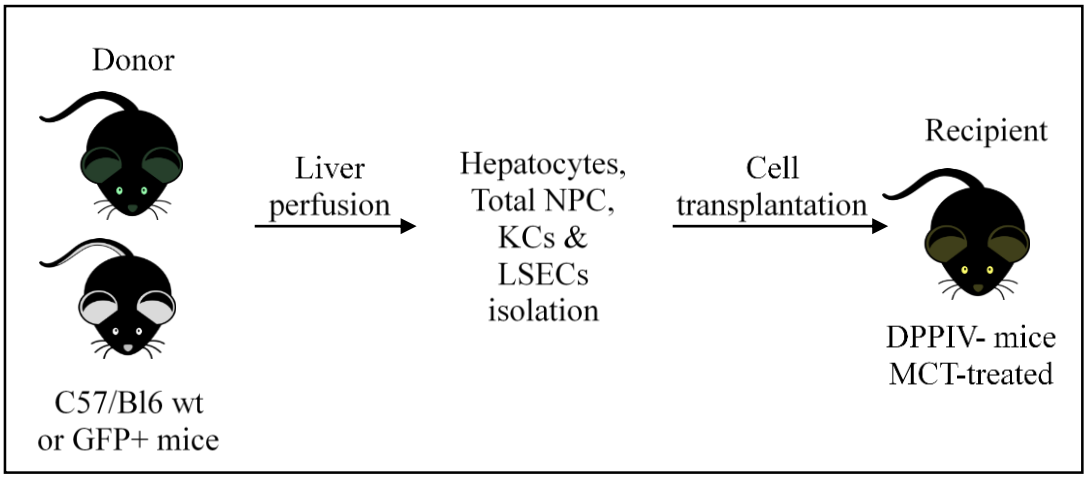
determined by trypan blue dye exclusion, and the purity of the population by cytofluorimetric analysis.

3.3 Cytofluorimetric analysis for LSEC and KC purity

To analyze specific markers, the cells were re-suspended in staining buffer (PBS, 1% FBS, 0.1% NaN_3) followed by an incubation with specific antibodies for 30 minutes on ice. Both CD31 and CD146, F4/80 and CD11b antibodies were used. Finally, the cells were washed twice in Perm/Wash buffer (BD Biosciences) and re-suspended in staining buffer.

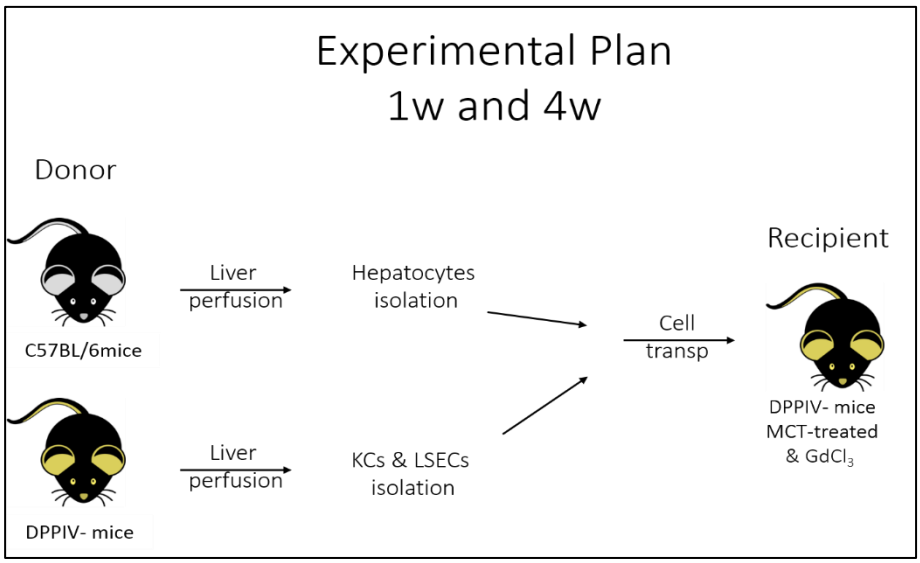
3.4 Pre-treatment for experimental study

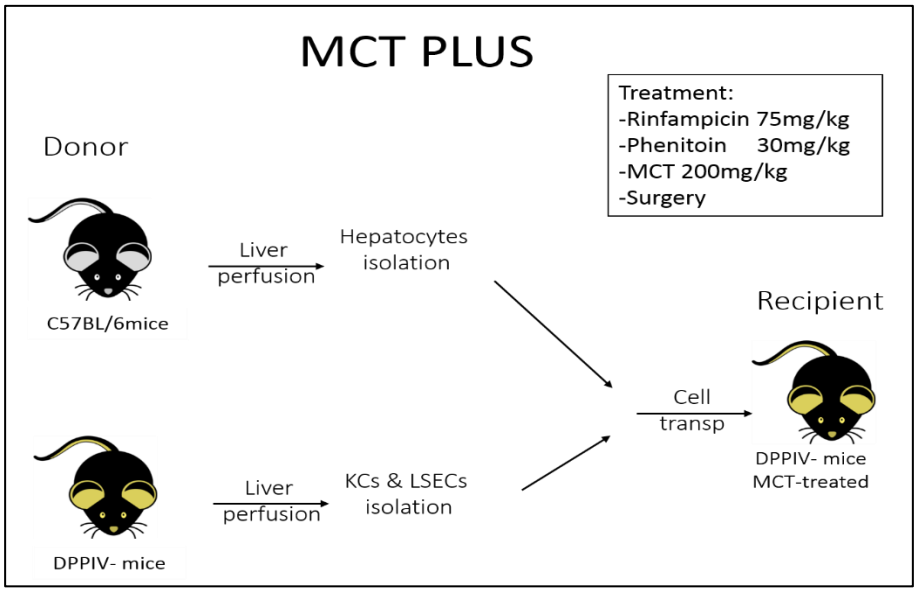
In the primary stage of this work, we used GFP+ mice to isolate GFP+ hepatocytes and C57B/6 wt (DPPIV+) mice for non-parenchymal cells. In the following experiments, we used LSECs, KCs and NPCs, which were all either negative or positive for GFP and DPPIV.



After several preliminary studies with monocrotalin (MCT) pre-treatment, we decided to perform all the transplantations in two ways: with MCT and Gadolinium to evaluate the engraftment, or with MCT plus, to evaluate proliferation. In particular, this required a consecutive three-day treatment with Rinfampicin (75mg/kg) and Phenitoin (30mg/kg), to induce injuries also in the parenchyma, as well as the endothelium. The hepatocytes, LSECs and KCs were used in a physiological condition (respectively 67% - 22% - 11%) without

GFP+ cells (to reduce immune responses), and with only hepatocytes DPPIV+ (because hepatocytes are the most important cells for liver structure and function).





3.5 Transplant

Mice were anesthetized with 5% isoflurane and maintained on 1.5% isoflurane during the surgery. Their hair was shaved and the area disinfected with 70% ethanol. For the transplant group, the portal vein was exposed by laparotomy and injected with 1×10^6 LSECs, or $1x10^6$ KCs GFP+, or $1x10^6$ hepatocytes. For the co-transplantation studies, mice were injected with $1x10^6$ LSECs + $1x10^6$ KC GFP+, or $1x10^6$ hepatocytes + $1x10^6$ LSEC GFP+ in

0.3 ml serum-free DMEM (GIBCO; Invitrogen) using 27-gauge needles. Haemostasis was induced by brief pressure for up to 5 minutes on the injection sites. Following several transplantations, we opted to use different transplantation co-transplantation conditions (with physiological proportions). We used $1.2x10^6$ hepatocytes DPPIV+ alone or in combination with 4×10^5 LSECs or $2x10^5$ KCs both DPPIV- and all three together in serum free HBSS. Finally, the peritoneum of the mice was closed using a 3.0-gauge needle and the skin with clamps. To eliminate pain, mice were injected under the skin with Carpofen-Rimadil.

3.6 DPPIV staining

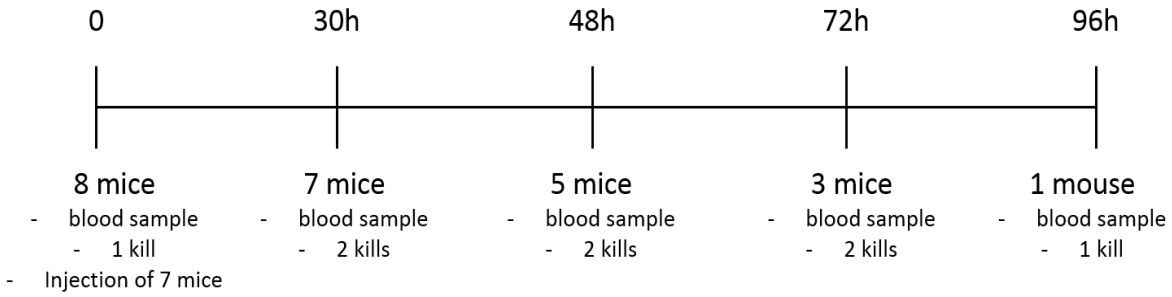
Freshly isolated samples were frozen directly in killik (OCT) and conserved at -80° for DPPIV staining. Sections of 5 µM thickness were cut cryostat, fixed in 1:1 acetone-chloroform for five minutes, air dried at room temperature (RT) and incubated with DPPIV staining solution for 45 minutes. Following the incubation, the sections were rinsed with water or PBS, counter stained with hematoxylin for 15 minutes and finally washed with running water 20 minutes. Images were taken by Nikon microscope or Pannoramic Digital slide scanner and analyzed with Pannoramic Viewer software 1.15.4 (3DHISTECH Ltd).

3.7 Immunofluorescence

One week after transplantation, the mice were sacrificed, and the livers were collected and fixed in PBS-Paraformaldeide (4% PAF). The livers were then equilibrated in 30% sucrose, embedded in killik and conserved at -80°. Fixed samples were cryostat sectioned at a thickness of 5 µM, incubated for 5 minutes in PAF, rinsed in PBS and blocked in buffer containing 5% goat serum, 1% BSA and 0.1% Triton X-100 in PBS. Following blocking, the sections were incubated with rabbit anti-GFP (1:500; Life Technologies) and with rat anti-mouse F4/80 (1:500; Serotec) antibodies. Images were taken with Leica DM5500 microscope and analyzed with LasX Software. All conditions were performed on two mice and for each mouse, two lobes were analyzed. Positive cells were counted in twenty fields/slice and analyzed statistically.

3.8 qPCR for inflammatory response

To establish the optimal time to start transplanting cells after the pre-treatment, an *in vivo* study with MCT and Gadolinium was performed. Mice were pre-treated, and livers were collected at different time points in two different experiments. Total RNA was extracted with Trizol reagent (Invitrogen, Thermo Fisher Scientific) and treated with DNase I (RNase-free DNase; Qiagen, Germantown, MD). The cDNA for each sample was generated from 1 μg total RNA with Omniscript RT Kit (Qiagen). The PCR products were resolved in 2% agarose gels. For quantitative RT-PCR, cDNAs were generated from 2 μg total RNA by RT2 First Strand Kit, followed by RT2 SYBR Green qPCR Master Mix (SABiosciences, Qiagen). The RT2 qPCR Primers for mouse IL1b, IL6, IL10, TGF-b, and GAPDH (SABiosciences) were used.



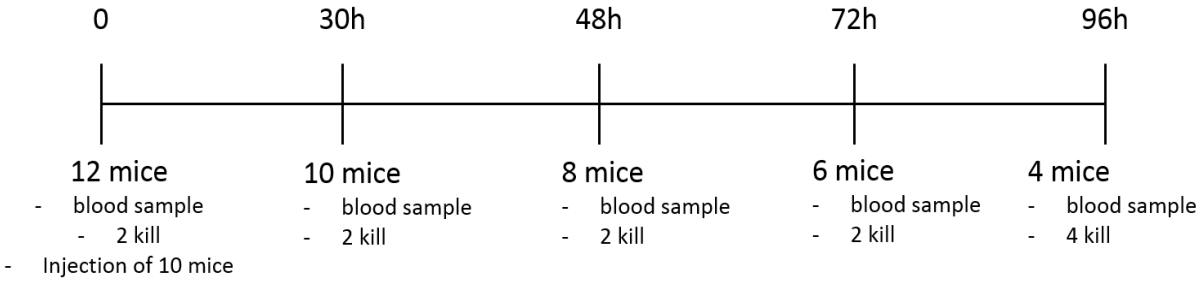
T0: Blood and liver sample from 8 mice (1 mouse sacrificed as a control), 7 mice injected with MCT e Gadolinium.

30h: Blood and liver sample from 7 mice (2 mice sacrificed)

48h: Blood and liver sample from 5 mice (2 mice sacrificed)

72h: Blood and liver sample from 3 mice (2 mice sacrificed)

96h: Blood and liver sample from the last mouse (1 mouse sacrificed)



T0: Blood and liver sample from 12 mice (2 mice sacrificed as controls), 10 mice injected with MCT e Gadolinium.
30h: Blood and liver sample from 10 mice (2 mice sacrificed)
48h: Blood and liver sample from 8 mice (2 mice sacrificed)
72h: Blood and liver sample from 6 mice (2 mice sacrificed)
96h: Blood and liver sample from the last mice (4 mice sacrificed)

3.9 Blood analyses

Blood samples were collected from mice according to the various groups (as indicated previously). Serum was separated and ALT levels were measured with a commercial kit (ALT-GPT LR; Gesan, Campobello di Mazara, Italy), according to the manufacturer's instructions. Differences in animal groups were then compared.

3.10 Statistical analysis

All the experiments were repeated at least three times. All data are expressed as mean values ± standard deviation (SD). The Student t test was performed to confirm the statistical significance ***$p \leq 0{,}001$, ** $p \leq 0{,}01$ and * $p \leq 0{,}05$.

4. Results
Aim 1

4.1 Immuno characterization of isolated cells

Cells were isolated after liver perfusion and collagenase digestion, with hepatocytes obtained by differential centrifugations, while KCs and LSECs were isolated by immunomagnetic selection (as described in materials and methods section 3.3). Purity and viability were evaluated. Both CD146 and CD31 (endothelial markers) were used for LSEC characterization, while F4/80 and CD11b (monocyte and macrophages markers) were used for KCs (fig. 1). We obtained on average a 95% purity for the LSEC population, with a 98% viability, and a 93% purity for the KC population with a 97% viability. These results were the mean of all the isolations.

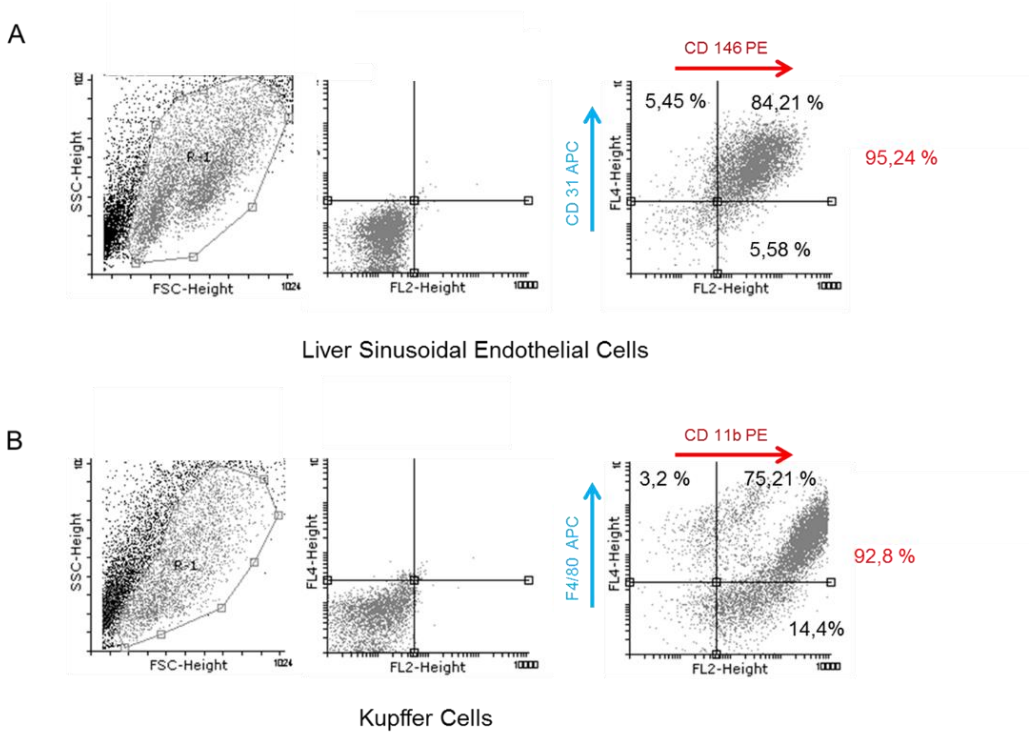


Figure 1. Immuno characterization of LSECs (A) and KCs (B) after immunomagnetic selection.

4.2 Cotransplantation increases the engraftment

To examine if co-transplantation is essential for cell engraftment, we focused on the combination of LSECs, KCs and hepatocytes and their effects on liver repopulation, when compared to hepatocytes, KCs, and LSECs alone. The results show that hepatocytes already have a good engraftment in comparison with LSECs or KCs, when they are individually transplanted (fig 2A and 2B). When transplanted together, LSECs and KCs have a synergistic effect on LSEC engraftment. Moreover, the best results were obtained when hepatocytes were transplanted together with LSECs (fig 2A and 2B).

With GFP staining, only KCs are observed (fig 2B) when co-transplanted with wild type LSECs. In co-transplanted hepatocytes and LSECs, only LSECs were GFP positive. Taken together these results demonstrate that cell engraftment with co-transplanted cells (LSECs+KCs and hepatocytes+LSECs), is greatly increased in comparison to transplantation with a single cell type. Transplantation of LSECs and hepatocytes collectively, however, results in the best engraftment.

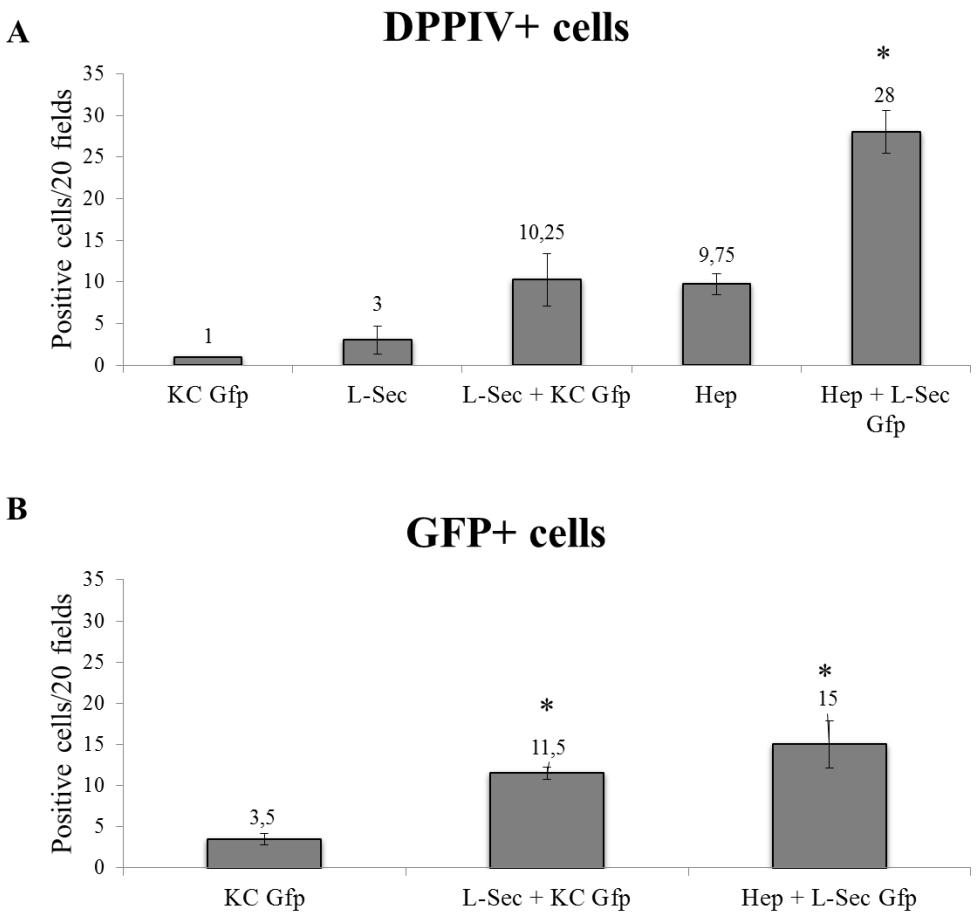


Figure 2. Co-transplantation improves the cell engraftment. Cells transplantations were done with1x10^6 cells per cell type. A) DPPIV staining shows that hepatocytes (Hep) with LSECs and LSECs+KCs increase cell engraftment when compared to KCs, LSECs and hepatocytes alone. B) GFP staining confirms the results obtained with co-transplantation. Normalization was obtained counting the positive cells in 20 different fields under 20X magnification. *p ≤ 0.05

4.3 Cotransplantation with non-parenchymal cells

To understand the optimal cell combination for co-transplantation, several conditions were tested. In this context, NPCs (KCs, LSECst) were co-transplanted. In particular, all the cells transplanted were DPPIV+, but KCs were GFP+ (KCs did not express DPPIV).

Co-transplantation had an increased engraftment when NPCs were present after both 1 week and 1 month (fig 3A and 3B). The NPCs alone or in combination with hepatocytes, increased the engraftment in comparison with all the other conditions (fig 3A). There were, however, no significant differences between 1 week and 1 month, demonstrating that no proliferation was observed.

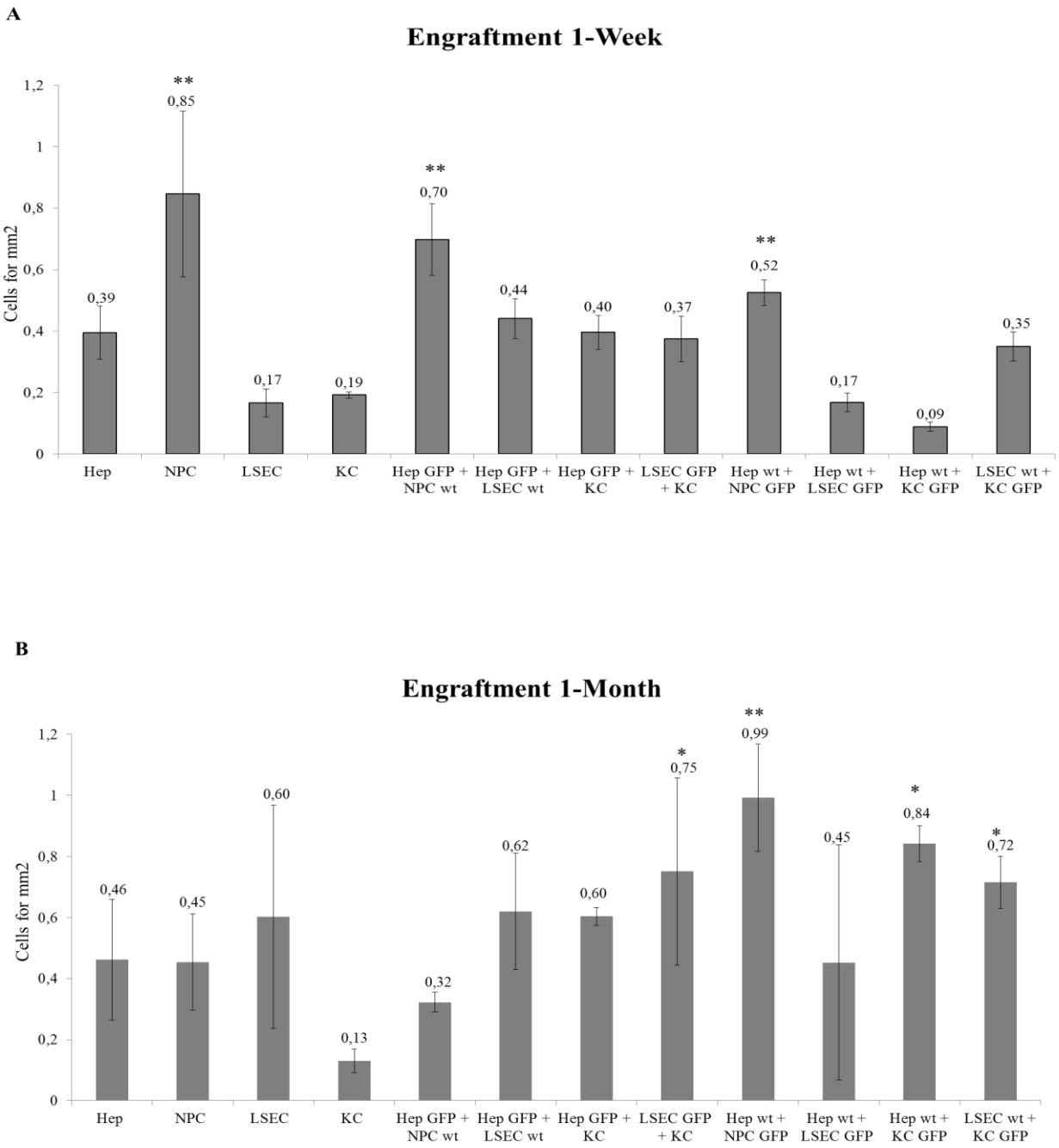


Figure 3. Cell engraftment after 1 week and 1 month. A total of 1x10^6 per cell type, were used. A) After 1 week, NPCs and co-transplanted hepatocytes-NPCs, increased the engraftment compared to other experimental conditions. B) After 1 month, the engraftment did not change in comparison with one-week engraftment. * $p \leq 0.05$, ** $p \leq 0.01$. n=3.

4.4 Hepatocytes, LSECs, and KCs collectively increase the engraftment

Since NPCs play a key role in cell engraftment, and previous data confirmed the involvement of NPCs in the increased cell engraftment, a cell combination of LSECs, KCs and hepatocytes, was used. As observed in figure 4, the engraftment decreased when hepatocyte and KCs were transplanted together, in comparison with hepatocytes alone. These data suggest that KCs increase the inflammatory response against transplanted cells. In addition, GFP positive cells further increase inflammatory response (fig. 5). Moreover, the combination of hepatocytes, LSECs and KCs increases engraftment significantly.

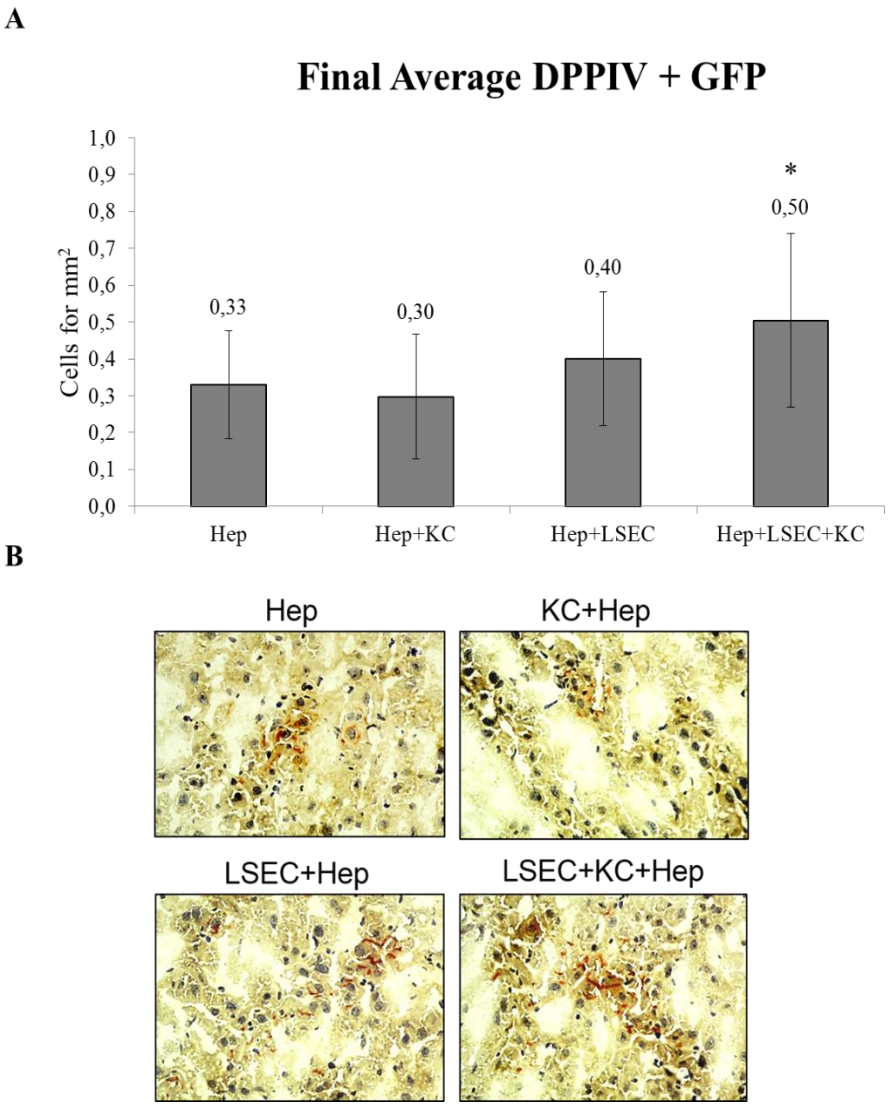


Figure 4. The average of all transplanted mice with DPPIV positive and GFP positive cells. All cells types were transplanted at 1x10^6. A) Count of DPPIV + and GFP + cells. The combination with Hep, LSEC, and KC is statistically significant, with respect to the other conditions. * $p \leq 0.05$ B) Representative images of DPPIV staining. 200x of magnification.

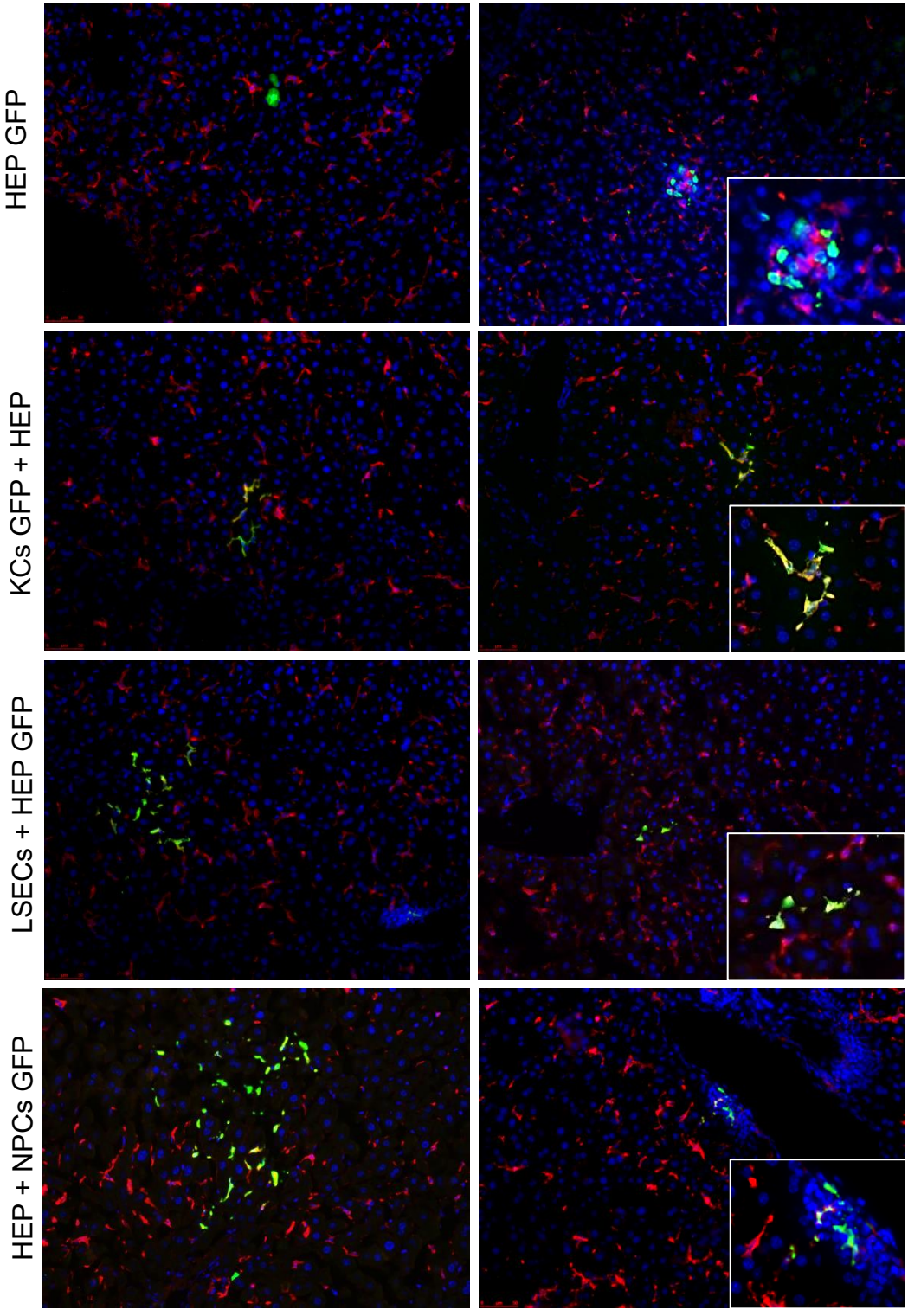


Figure 5. Engraftment and inflammation in presence of GFP positive cells. Merge of the different staining is shown, F4/80 was observed in red, GFP in green, while nuclei were stained with DAPI (blue). 400x of magnification.

4.5 RT qPCR for chemokine expression after pre-treatment:

As observed previously, a non-cooperation between transplanted cells and resident KCs was observed. To inhibit the inflammatory response, we decided to use Gadolinium as an inhibitor of the resident KCs. During the first surgery after gadolinium treatment, all mice died due to bleeding. To understand the best transplantation time after the pre-treatment without surgery, two experiments were performed. Specifically, samples after gadolinium and MCT treatment from different time points were collected, and mice without treatment were used as basal controls. Pro inflammatory (IL-1 β and IL-6) and immunomodulatory (TGF-β and IL-10) cytokines were analyzed (fig 6). As shown by figure 5, all cytokines were up regulated after 30h of gadolinium treatment. Moreover, after 48h IL1β, IL-6, TGF-β and IL-10, were significantly downregulated. Based on this data, we opted to perform the surgeries close to 48h time point after pre-treatment.

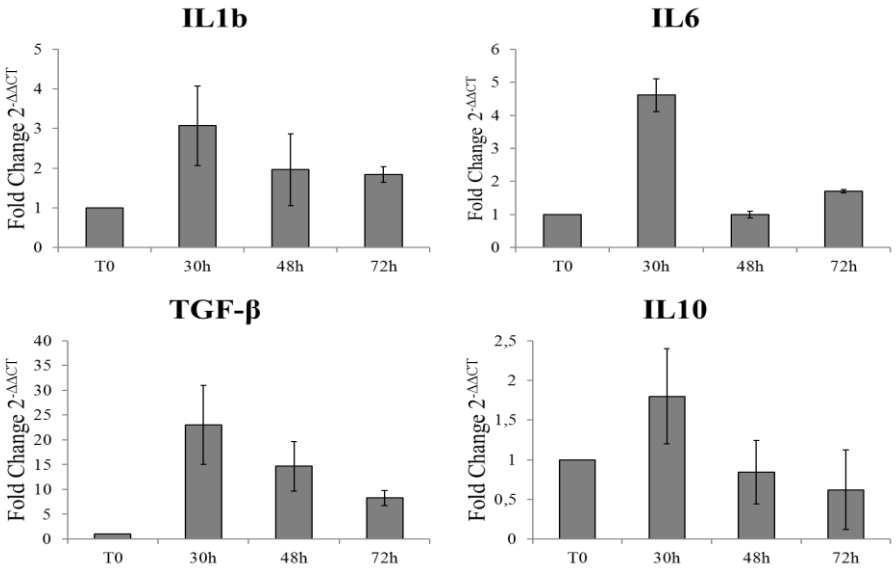


Figure 6. RT qPCR analyses. Graphs show fold changes with respect to T0. T0 are mice prior to treatment with MCT and Gadolinium.

4.6 AST and ALT analyses

The levels of ALT and AST (functional liver marker) in blood sample were measured (fig. 7). Both AST and ALT were increased up to 48h. Taken together, the results of cytokine

expression and ALT and AST analysis, strongly emphasize that 48h is the right time to perform the surgery.

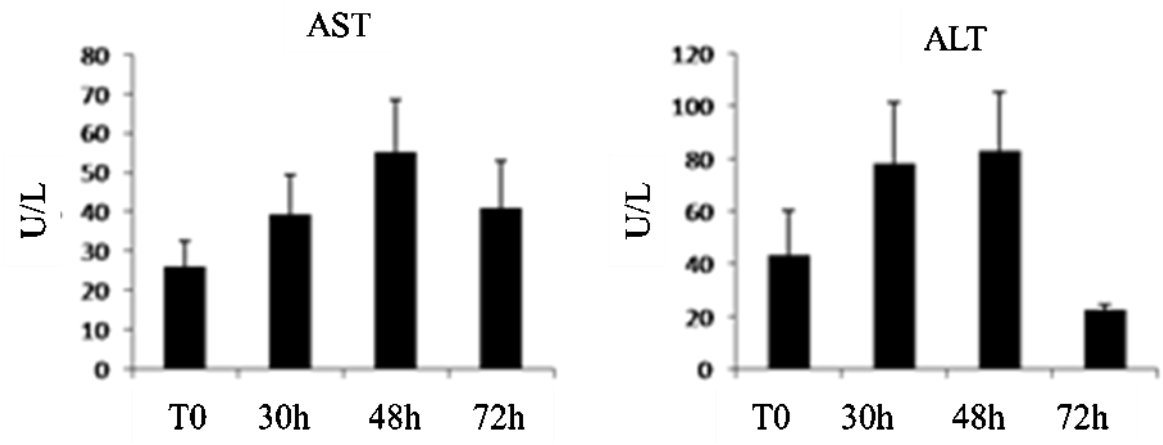


Figure 7. AST and ALT analyses. Graphs show fold change with respect to T0. T0 are mice prior to treatment with MCT and Gadolinium.

4.7 Hepatocytes engraftment

Liver function is primarily performed by hepatocytes, and their functions are supported by the niche of cells in which they reside. In order to reduce inflammation, GFP positive donors were excluded from the study, and at the same time, only hepatocyte engraftment was evaluated. In the following experiments, only hepatocytes DPPIV+ were transplanted alone or together with KCs and LSECs both DPPIV negative (hepatocytes 67% - LSECs 22% - KCs 11%), maintaining a balanced cell-cell communication. As shown by figure 7A, an increase of cell engraftment in all conditions was observed, however, only the hepatocytes alone did not improve their engraftment with respect to previous results. Remarkably, all the other new conditions (physiological) increased close to 3-fold; from 0.50 cells (total) per mm^2 to 1.45 hepatocytes (only hepatocytes) per mm^2. The average of the results after 1 week and 1 month was the same but with more variability, indicating that in presence of this pre-treatment there was no proliferation. (figure 8C). In figure 8B some rappresentative picture of cell engrafted and is clear that the distribution of the cells change in the different condition; hepatocytes with LSECs and togeder with all cell types is well distribuited in the parenchyma while with KCs we found cells positive only near the vessels.

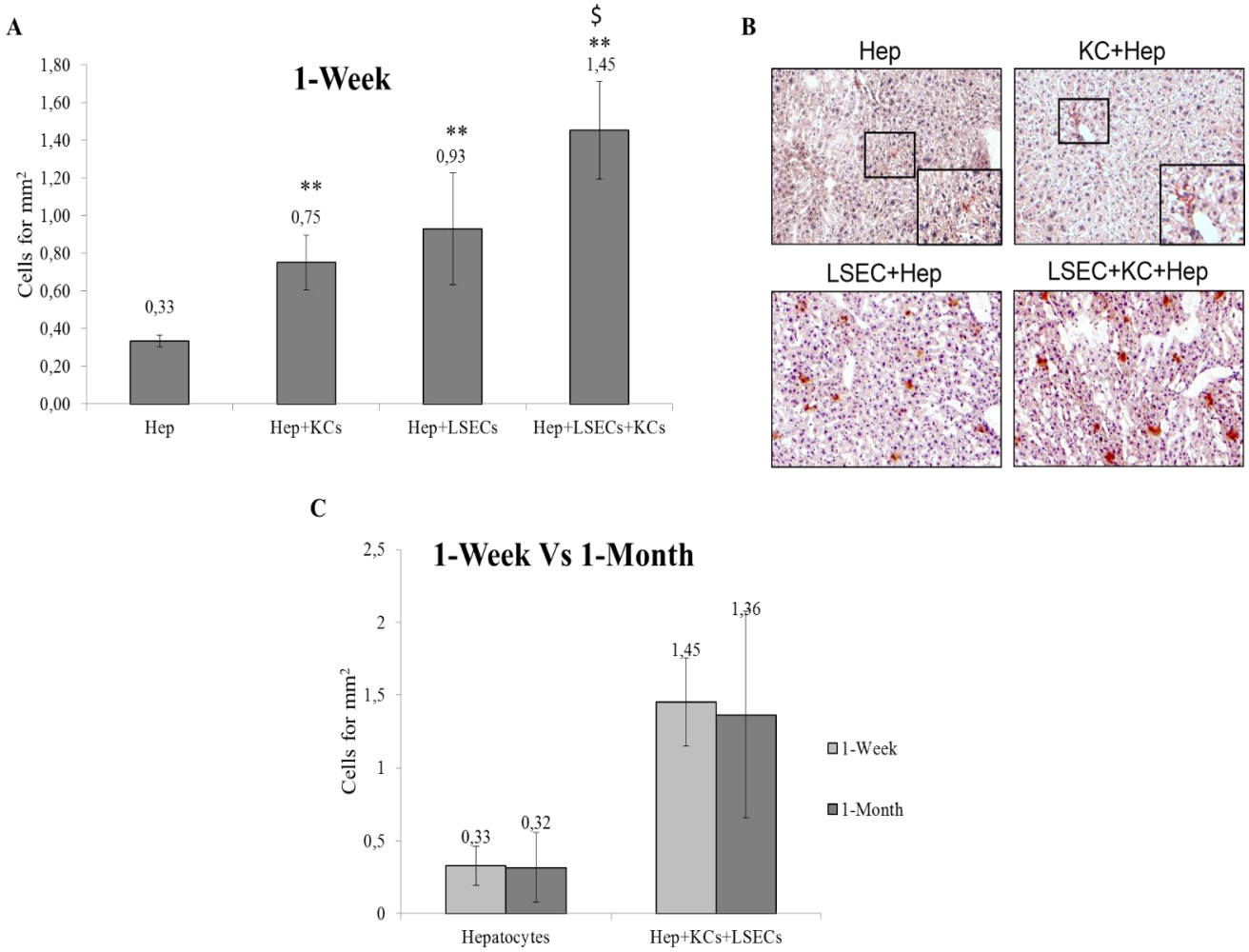


Figure 8. Co-transplantation after pre-treatment with gadolinium and MCT. A) All co-transplantation conditions. B) Representative DPPIV staining of the all conditions (magnification 400X). C) Co-transplantation after 1 week and 1 month. ** $p \leq 0.01$. n=5 mice/conditions used.

4.8 Proliferation at 6 weeks

The use of MCT, damages the fenestrate endothelium, however it is also essential to create a damage also in the parenchyma. As shown in literature, MCT+ requires a further treatment with phenitoin (30mg/kg) and rinfanpicin (75mg/kg) for the three days prior to MCT treatment. This treatment profile causes a greater damage to the parenchyma. We observed at 6 weeks after MCT+ pre-treatment and surgery, a substantial increase in engraftment for all the co-transplantation conditions. Hepatocytes increased 2-fold, while for all other conditions there was about a 3-fold improvement (fig. 9A). As shown in figure 10, after this pretreatment we can find groups of cells scattered in the parenchyma and close to the vessels, no longer single

cells. although when we transplant all cell types together, the engraftment increases significantly, it is to be noted that all conditions have a good increase even if the condition hepatocyti with KCs proves to be very different between a mouse and the other greatly increasing the deviation.

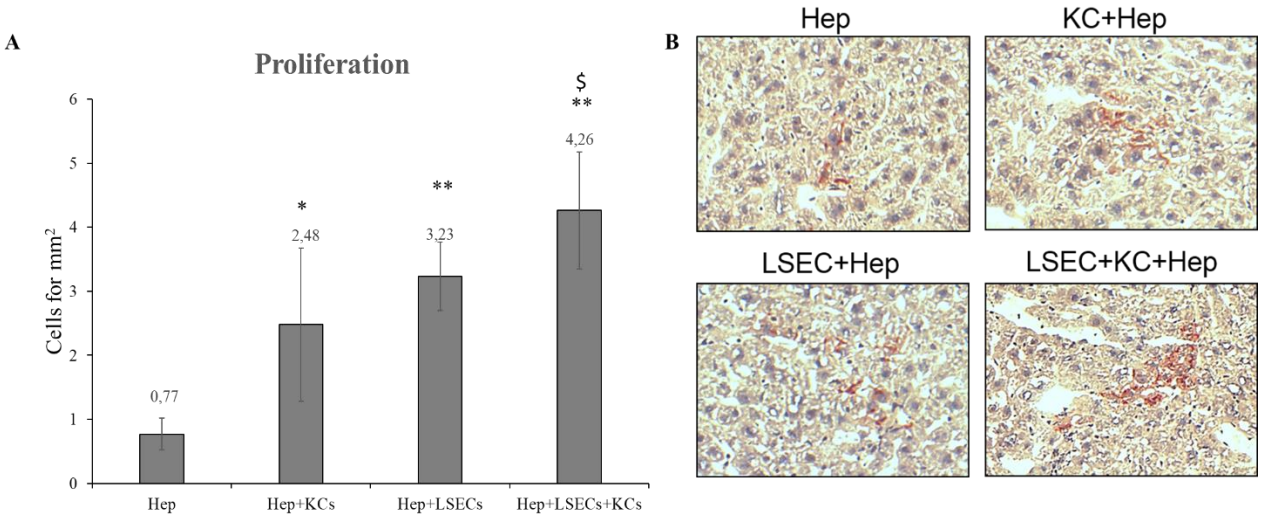
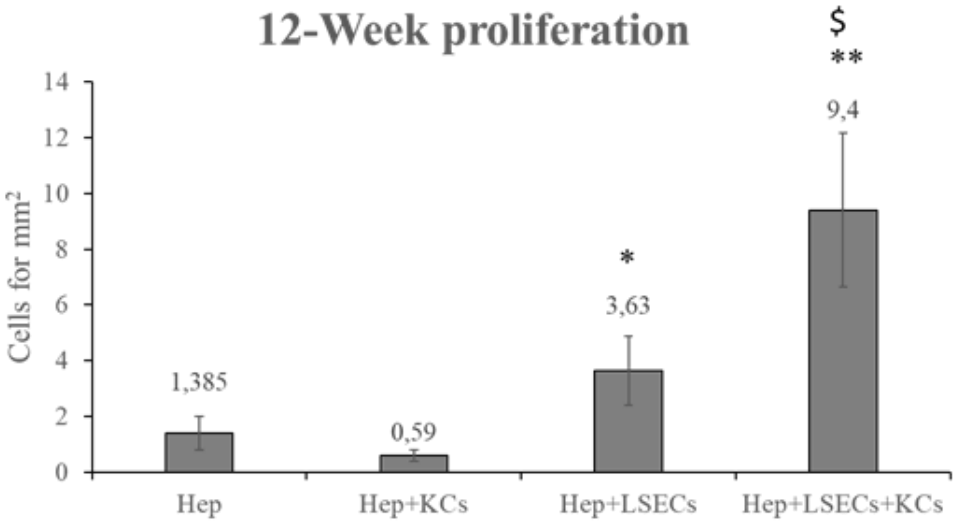


Figure 9. Cell engraftment and proliferation after 6 weeks. 1.2x10^6 hepatocytes, 4*10^5 LSECs, 2*10^5 for KCs were used. A) Analyses of DPPIV staining. B) Representative images of DPPIV staining (400X magnification). * indicates p ≤ 0.05, ** indicates p ≤ 0.01. N=5 mice for condition.

4.9 Proliferation at 3 months

Following the 6 weeks, a longer time point was performed (3 months). Unexpectedly, hepatocytes-alone slightly increased, and hepatocytes with KCs decreased substantially. Interestingly, the combination of all three cell types, increased further the number of positive hepatocytes detected, arriving up to a 2.2-fold increase when compared to the 6 weeks (fig. 10A). Unlike the six weeks, at three months, as shown in Figure 10B, the groups of positive cells we find are more open and distributed and with more positive cells. Overall, taking all 12 weeks results together, we can clearly see the effect of proliferation, when we transplant the hepatocytes alone, even if the increase is less than double but with all three cell types together the increase is significant. The fact that the combination of hepatocytes with KCs decreases close to zero can mean that at some point the liver rejects the transplanted cells, but when we go to look at the hepatocyte combination with LSECs nothing changes when compared with the six weeks, and this further demonstrates the effect of all cells together.

A



B

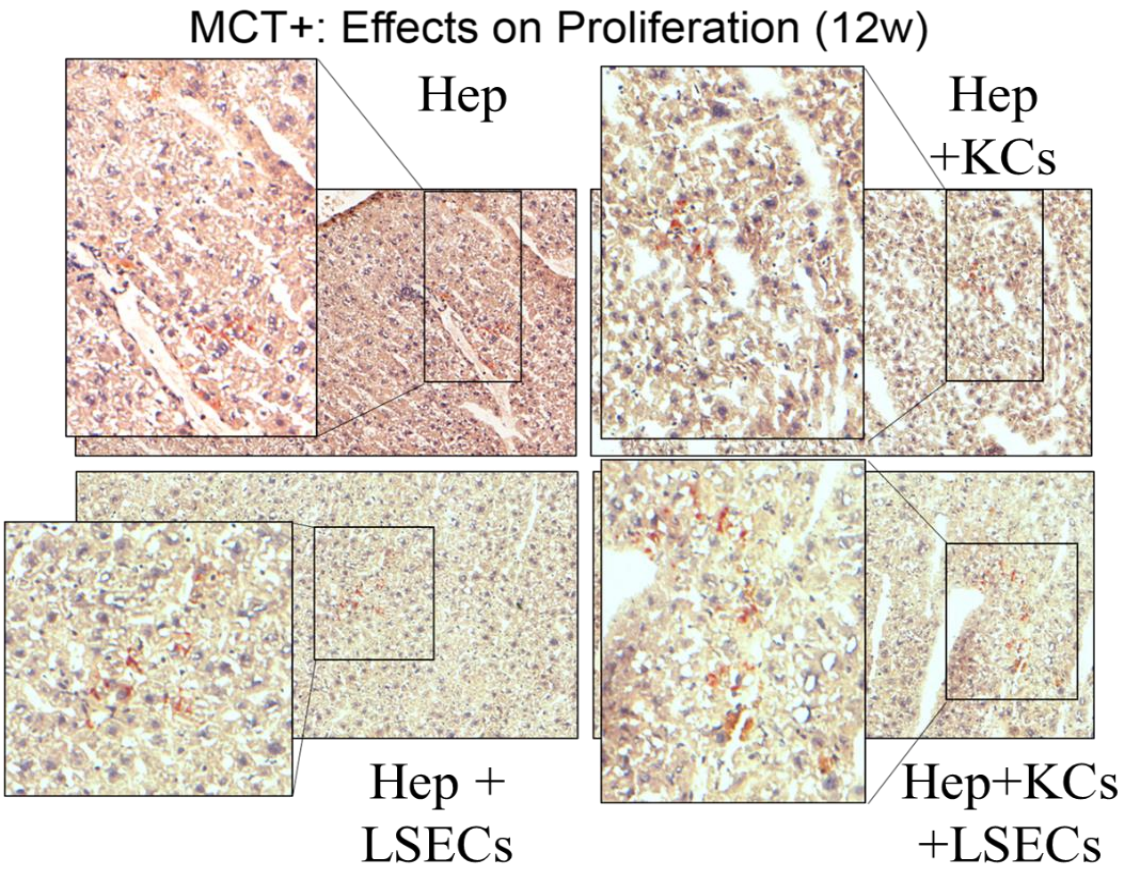


Figure 10. Cell engraftment and proliferation after 12 weeks. 1.2x10^6 hepatocytes, 4*10^5 LSECs, 2*10^5 for KCs were used. A) Analyses of DPPIV staining. B) Representative images of DPPIV staining (400X magnification). *p ≤ 0.05, ** p ≤ 0.01. n=5 mice for condition.

5. Discussion Aim 1

Discussion

The liver is the largest internal organ of the body (weighing up 1.5 kg in adults, Molina and Dimaio, 2012) and it supports many essential metabolic, exocrine and endocrine functions. Production of bile, the metabolism of dietary compounds, detoxification, the regulation of glucose levels through glycogen storage and control of blood homeostasis, are some of its vital functions and these are achieved through the secretion of clotting factors and serum proteins. (Jungermann and Keitzmann, 1996; Zorn, 2008). The liver's inability to perform its normal synthetic and metabolic functions, results in a pathological condition called liver failure. This dysfunction is due to several causes, including cirrhosis, hepatitis, cancer, drug overdoses, metabolic or autoimmune disorders, chemical toxins and trauma. Based on the permanence of the injury, two different forms of liver failure are recognized: acute and chronic failure. Liver replacement using the orthotopic technique (OLTx; normal, whole liver) represents the standard care for end-stage liver disease and many liver-based metabolic conditions (Gotthardt et al., 2007; Qu et al., 2009; Sotil et al., 2009). Organ shortage and patient instability, however, have emphasized the need for other non-traditional approaches for liver replacement, such as auxiliary liver transplantation, hepatocyte transplantation, xenotransplantation and bio artificial liver assist devices (Carpentier et al., 2009; Ekser et al., 2009; Fitzpatrick et al., 2009; Thamara et al., 2009; Waelzlein et al., 2009). Cell therapy represents an alternative method to overcome OLTx. For example, liver repopulation with hepatocyte transplantation has a substantial therapeutic potential, however multiple barriers still restrict engraftment and proliferation of transplanted hepatocyte (Joseph et al., 2006; Wu et al., 2008). For instance, 70–80% of transplanted cells are cleared from the liver within one or two days of transplantation. Subsequently, disruption of the sinusoidal endothelial barrier is required for transplanted cells to enter and integrate in the liver parenchyma (Joseph et al., 2006). During that period, multiple types of cell interactions take place, including hepatocytes with LSEC and KCs (Joseph et al., 2002; Kumaran et al., 2005). Some interactions are beneficial, and promote cell engraftment (Benten et al., 2005), while others are deleterious, such as KCs that phagocytose transplanted cells (Kumaran et al., 2005).

Herein, we show a new protocol involving a combination of mature cells to obtain a good liver regeneration, after conditioning. Injury was induced through the administration of a genotoxic pyrrolizidine alkaloid, monocrotaline (MCT), which disrupts the sinusoidal endothelial barrier and leads to the incorporation of transplanted cell (hepatocytes) into the liver parenchyma (Joseph et al., 2006; Witek et al., 2005; Wu et al., 2008). The rationale

behind this approach was based on the fact that MCT is a potent toxin for ECs, and causes a widespread disruption of the endothelia in animals (Copple et al., 2002; Deleve et al., 2003). This work established that co-transplanted cells engrafted and survived in the liver. Retention of the appropriate functions in transplanted KCs, LSECs and hepatocytes, indicates that replacement of these cells would provide important tools to address the biological and therapeutic potential for cell engraftments. To define the optimal cell combination, we isolated LSECs and KCs and hepatocytes from C57B/6l wt or GFP+ mice, and different transplantation or co-transplantation conditions were assessed in DPPIV- mice after 7 days. Hepatocytes were isolated by differential centrifugation, while KCs and LSECs were obtained through immunomagnetic selection. The cells were then characterized by cytometric analysis for sinusoidal endothelial marker (CD31 – CD146) and monocyte and macrophage markers (CD11b – F4/80), to establish the purity and the viability. For this purpose, co-transplantation had the best outcome when compared to the single cell type transplantations. Moreover, these results indicate that the presence of hepatocytes seems necessary in all situations. Specifically, when hepatocytes were present in the co-transplantation conditions, cell engraftment increased 3-fold compared to other cell combinations. In addition, LSECs and KCs together reached an appropriate level of engraftment, but it remained lower than co-transplantation of LSECs with hepatocytes. These results represent the basis for further investigation regarding the relationship between cell transplantation and whole organ transplantation. Based on this evidence, new experiments were evaluated including more conditions, i.e. a one-month, end-point and transplantation of complete NPCs, alone or in combinations with other cell types.

To obtain restocking and functionality of the liver, co-transplantation in the presence of hepatocytes was verified. In this context, co-transplantation was performed in MCT pre-treated DPPIV-mice and based on the previous results, hepatocytes in combination with KCs and LSECs were transplanted.

These data showed that Hep-KC-LSEC co-transplantation reached the best outcome when compared to the transplantation of hepatocytes alone. Moreover, co-transplantation of all the cell types together further increased the engraftment.

The exact mechanisms by which the inflammatory environment of the liver, after cell transplantation, may affect cell engraftment, are currently under investigation. Furthermore, inflammation plays a preponderant role, reducing cell engraftment. Therefore, to obtain a reduction of inflammation, we decided to avoid the use of GFP positive cells and pre-treat mice with MCT and $GdCl_3$. Specifically, we decided to use only DPPIV+ mice as donors of

hepatocytes to calculate, during the analyses, exclusively their engraftment and not false positive signals derived from other implanted cell types. All cell types were transplanted under physiological conditions (67% hepatocytes, 22% LSECs, and 11% KCs) to reproduce the niche generating the normal cell-cell interactions and cell signaling.

In MCT/$GdCl_3$-treated mice, qPCR showed lower IL-6, IL-1β, and IL-10 expression after 30h from MCT and GdCl3 injection. Similarly, TGF-β expression was upregulated at 30h and started to decrease after 30h. Overall, these findings indicate that 48h after MCT and $GdCl_3$ treatment is the optimal time point to start transplantation. As expected, in MCT and $GdCl_3$-treated mice, hepatocyte engraftment was superior. When hepatocytes, KCs and LSECs were transplanted together, however, hepatocyte engraftment increased significantly compared to the controls up to 2-fold, both after 1 week and 1 month.

We also determined the proliferation potential of transplanted hepatocytes over 6 weeks and 3 months, after MCT plus treatment (Benten et al., 2018). The engraftment was 3-fold higher in these conditions, and after 12 weeks, DPPIV+ hepatocytes were double with respect to 6 weeks. This demonstrates that proliferation of transplanted hepatocytes occurred despite MCT treatment, which does not allow cell proliferation, due to the absence of parenchymal damages. Overall, our findings indicate that co-transplantation of LSECs, KCs and hepatocytes was superior with respect to transplantation of hepatocytes alone, for liver repopulation.

The benefit of cell co-transplantation was maintained despite liver injury after the preconditioning treatments. This ability of LSECs, KCs and hepatocytes to better reconstitute the liver by co-transplantation will be helpful for basic studies of cell-cell interactions for the development of advanced protocols for liver cell therapy.

Future perspectives will focus on studies regarding cytokines and chemokines involved in cell-cell interactions and communication. Specifically, these mechanisms should be highly significant to define organ-specific perturbations after cell transplantation and to guide cell and gene therapy strategies. To this purpose, we will correlate studies on the administration of a stimulus (such as thyroid hormone, T3), to significantly increase hepatocyte proliferation after cell co-transplantation (PIBIRI, 2001). Moreover, an alternative source of transplanted cells such as hepatocyte-like cells derived from iPSCs will be evaluated, to decrease the risk of rejection.

6. Aim 2

Aim of the project 2 (Kevin B. et al. 2017)

"A long term, non-tumorigenic rat hepatocyte cell line and its malignant counterpart, as tools to study hepatocarcinogenesis".

Aim 2: Describe the isolation and characterization of two rat hepatocyte cell lines as tools to study liver carcinogenesis.

Rationale: Hepatocellular carcinoma (HCC) is the fifth most common cancer worldwide and the second cause of cancer-related death. Establishing those genes/proteins whose expression can discriminate between normal and neoplastic liver is fundamental for diagnostic, prognostic and therapeutic purposes. Currently, the most used *in vitro* hepatocyte models to study molecular alterations underlying transformation, include primary hepatocytes and transformed cell lines. Each of these models, however, presents limitations. Here we describe the isolation and characterization of two rat hepatocyte cell lines as tools to study liver carcinogenesis. Long-term stable cell lines were obtained from a HCC-bearing rat exposed to the Resistant-Hepatocyte protocol (RH cells) and from a rat subjected to the same model in the absence of carcinogenic treatment, thus not developing HCCs (RNT cells). The presence of several markers identified the hepatocytic origin of both cell lines and confirmed their purity. Although they are morphologically like normal primary hepatocytes, RNT cells were able to survive and grow in monolayer culture for months, they and were not tumorigenic *in vivo*. On the contrary, RH cells displayed tumor-initiating cell markers; they formed numerous colonies in soft agar and spheroids when grown in 3D and were highly tumorigenic and metastatic after injection into syngeneic rats and immunocompromised mice. Moreover, the RNT gene expression profile was like normal liver, while that of RH resembled HCC. In conclusion, the two cell lines described herein represent a useful tool to investigate the molecular changes underlying hepatocyte transformation and to experimentally demonstrate their role in HCC development. We aim to:

Aim 2.1. Characterize RH and RNT cell lines and their potential as disease cell models.

Aim 2.2. Define tumor markers between RH and RNT cell lines.

7. Materials and Methods

7.1 Animals and treatment

Guidelines for Care and Use of Laboratory Animals were followed during the investigation. The Ethical Commission of the University of Cagliari and the Italian Ministry of Health approved all animal procedures. Male Fischer F-344 rats (100-125 g) purchased from Charles River (Milan, Italy) and NOD.Cg-PrkdcscidIl2rgtm1Wjl/SzJ mice (γNull) from Jackson Laboratories (Bar Harbor, Maine, USA). Animals were kept on a laboratory diet (Ditta Mucedola, Milan, Italy) and given food and water ad libitum with a 12-hour light/dark daily cycle. The HCC was induced according to the Resistant Hepatocytes (R-H) model (Solt et al., 1977). Rats were injected intraperitoneally with the chemical carcinogen diethylnitrosamine (DENA, Sigma, MO) at a dose of 150 mg/kg body weight. After a 2-week recovery, rats were fed a diet containing 0.02% 2-acetylaminofluorene (2-AAF, Sigma, MO) for 1 week followed by a two-thirds partial hepatectomy (2/3 PH), and an additional week of 2-acetylaminofluorene diet. The animals were then returned to the basal diet and euthanized at 14 months (Supplementary Figure 1). Rats exposed to 2-AAF and 2/3 PH, but in the absence of carcinogen, were used as controls.

7.2 Cell isolation and culturing

The RNT (Rat Not Tumorigenic) and RH (Resistant Hepatocytes) cells were obtained from rats treated with 2-AAF + PH (rats with no tumors) or DENA + 2-AAF + PH (tumor bearing rats), respectively. Livers were perfused at 10 ml/min via portal vein for 5 minutes with Leffert's buffer (at 37°C) containing 1.9 mg/ml EGTA, for 2 minutes with buffer lacking EGTA, and for 10-15 minutes with buffer containing 0.03% (w/v) collagenase (Worthington Biochemical Corp.) and 5 mM CaCl2.2H2O, as described [35], with modifications made for rat livers. The perfusion buffer contained 10 mmol/l HEPES, 3 mmol/l KCl, 130 mmol/l NaCl, 1 mmol/l NaH2PO4. H2O, and 10 mmol/l d-glucose, pH 7.4). The livers were dissociated in Leffert's buffer, and cells were passed through Dacron fabric with 80-μm pores and centrifuged under 50 *g* for 5 minutes to recover hepatocytes. For livers containing tumors, digestion continued in the plastic dish for additional 10-20 min in the presence of collagenase: after incubation, tumor masses present in the remaining parenchyma were mechanically disrupted. Cells were recovered and washed several times in serum-free medium; then hepatocytes were maintained in petri dishes coated with rat tail collagen 0,2%, in 10% FBS DMEM. Murine peritoneal macrophages were collected by intraperitoneal injection of RPMI-1640 and maintained in 5% FBS RPMI-1640. Cultured

RNT and RH cells were maintained in 10% FBS RPMI with P/S (100U/ml Penicillin, 100mg/l Streptamicin), and L-Glutamine (2mM). C1C7 (murine HCC cell line), MS1 (murine endothelial cell line), U937 (human macrophage cell line) and HTC (rat hepatoma cell line) were maintained in 10% FBS DMEM.

7.3 RNT and RH spheroids

To obtain spheroids from RNT and RH cells, cells were seeded in low-attachment 24-well plates (104 cells/ well) in stem medium conditions (0% FBS DMEM-F12 medium, supplemented with EGF, bFGF, insulin and B-27 supplement). After the formation of spheroids, they were seeded in low-attachment 96-well plates and cell viability was evaluated at day 0, 3 and 7 after seeding, using CellTiterGlo assay (Promega).

7.4 Periodic acid-schiff (PAS) stain for glycogen

Culture dishes containing cells were fixed in ethanol and acetic acid (99:1) for 10 minutes at 4°C, incubated with Schiff reagent for 5 minutes and washed twice with periodic acid (Carlo Erba).

7.5 Immunofluorescence (IF) analysis

Cells were seeded on collagen-coated coverslips for 48h, subsequently rinsed with PBS and subjected to immunofluorescence. The RNT and RH spheroids were prepared by cyto-spin at 1000 rpm for 5 min. Cells were fixed with 4% paraformaldehyde (Sigma Aldrich) or with methanol (Sigma-Aldrich) at room temperature and permeabilized with PBS containing 0.1% Triton X-100 (Sigma-Aldrich). After blocking with 5% goat serum (Sigma Aldrich), cells were subjected to immunofluorescence staining with primary antibodies diluted in PBS containing 1% BSA, 0.1% Triton X-100 and 2% goat serum, as listed in Supplementary Table 2. Cells were finally incubated with either secondary anti-mouse, anti-rabbit AlexaFluor 488-conjugated or AlexaFluor 546-conjugated antibodies (dilution 1:500, Invitrogen, Carlsbad, CA) for 1 h in PBS containing 1% BSA and 0.1% Triton X-100. Nuclei were stained with DAPI (Sigma Aldrich). As a control, staining of RNT and RH was performed with the secondary antibody alone 15729 (Supplementary Figure 9). Cells were

examined by fluorescence microscopy (Olympus America Inc, Center Valley, PA). Images were acquired as color-images and prepared using Photopaint and Photoshop software.

7.6 Cytofluorimetric analysis

To analyze extracellular markers, cells were re-suspended in staining buffer (PBS, 1% FBS, 0.1% NaN3) followed by incubation with specific antibodies for 30 minutes on ice. For intracellular markers, cells were re-suspended in staining buffer containing 1% paraformaldehyde for 5 minutes, incubated with Perm wash buffer (BD Biosciences, San Diego, CA) for 5 minutes, followed by incubation with primary antibody in Perm/Wash buffer for 30 minutes on ice. Finally, cells were washed twice in Perm/Wash buffer (BD Biosciences) and re-suspended in staining buffer. Specific antibodies used are listed in Supplementary Table 3.

7.7 Lentiviral transduction

Four different lentiviral vectors expressing the green fluorescence protein (GFP) under the control of PGK (Phosphoglycerate kinase, ubiquitous), TTR (Transthyretin, hepatocyte specific), VEC (Vascular- Endothelial Cadherin, endothelial specific) and CD11b (Integrin alpha M, ITGAM, myeloid cells specific) promoters were used to transduce RH and RNT cells. As controls we used the following cell lines: C1C7 murine hepatocytes, MS1 murine endothelial cells, U937 human monocytes. Cells were transduced with a multiplicity of infection (MOI) of 1 and 0.1. Expression level and stability of the GFP expression was evaluated by flow cytometry 72 h after transduction. The RNT cells were stably transduced with an empty lentiviral vector (mock), with an NRF2 lentiviral construct (217EX-T3128-Lv157; GeneCopoeia, Rockville, MD), with the mutant forms of NRF2 (V32E and E82G), obtained using the QuickChange II XL Site-Directed Mutagenesis Kit (Agilent Technologies), and with a lentiviral construct expressing the activated form of KRAS G12V.

7.8 $CD24^+$ cells isolation

For CD24+ cell isolation, RH and RNT cells were first incubated for 20 min at 4°C with anti-rat CD24 PE-conjugated antibody (Miltenyi Biotec) followed by a second incubation with anti-PE Microbeads (Miltenyi Biotec) for 20 min at 4°C and finally

immunomagnetically separated using magnetic columns (Miltenyi Biotec). Cell purity was verified by flow cytometry. Freshly isolated cells were then counted and immediately injected s.c. into yNull mice.

7.9 Cell growth

RNT and RH cells were seeded in 96-well plates (4000 cells/well) at two different concentrations of serum (2% and 10%). Cells were fixed in 11% glutaraldehyde and stained with crystal violet at days 1, 3 and 6 after seeding. The dye retained by the cells was then solubilized in 10% acetic acid and the Optical Density (570nm) was measured using a Multilabel Reader (PerkinElmer, Waltham, MT, USA). For evaluation of anchorage-independent growth, 3000 cells/well were seeded in 10% FBS RPMI 0.5% soft agar and maintained in the presence of medium for 15 days. Grown colonies were visualized by staining with crystal violet and pictures acquired as black and white images by microscope.

7.10 RH and RNT expression profile by qRT-PCR

Total RNA from cultured RH and RNT at different culturing passages and from normal rat livers and HCCs was first extracted by using TRIzol® reagent (Invitrogen) and further purified using the RNeasy Micro Kit (Qiagen). The cDNA was obtained from 1 μg of RNA using the RT2 First Strand Kit (Qiagen). Gene expression profile of RH, RNT, normal livers and tumors was analyzed by real-time qPCR using the Rat Liver Cancer RT2 Profiler™ PCR Array (Qiagen), that evaluates 84 genes. Analysis was performed using the Web-based PCR Array Data Analysis Software available at SAB website (www.SABiosciences.com/ pcrdataanalysis.php). The B2m was used as housekeeping gene. Only mRNAs whose expression was dysregulated by at least 2-fold compared to their controls were considered modified.

7.11 RT-qPCR validation

Analysis of *Birc5, Igfbp1, Myc, Tert, Cnnd1* and *Met* expression was performed using specific TaqMan probes (Applied Biosystems); GAPDH was used as endogenous control. The results are the mean of three different samples/group.

7.12 Pathway and functional analysis by means of the DAVID bioinformatics resources software

The functional enrichment analysis of the differentially expressed genes was performed with the Database for Annotation, Visualization and Integrated Discovery (DAVID), including gene ontology (GO) function analysis and Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway analysis. Analysis of pathways and functions was based on the number of genes significantly dysregulated (fold difference cutoff + 2.0). In KEGG pathway analysis, enriched pathways were identified according to the hypergeometric distribution with a P-value < 0.01.

7.13 *In vivo* experiments

The rat *in vivo* experiments were performed by inoculating s.c.1.5 × 106 cells (suspended in sterile PBS/ Matrigel ratio 1:1) into the posterior flank of F344 male rats. For experiments performed in mice, 107 unselected RH or RNT cells or 5x105 – 3x106 CD24+ or CD24- RH or RNT cells were inoculated in the same manner in γNull mice. Tumor masses were measured with a caliper. Tumors were then resected and analyzed by H&E stain and IF. Lungs were surgically removed to histologically verify the presence of metastases.

8. Results
Aim 2

8.1 Isolation and characterization of RNT and RH cell lines

The R-H model consists of a single injection of DENA followed by a brief exposure to a promoting environment (2-AAF + PH). The HCCs arose 10-14 months after DENA treatment (the protocol scheme is shown in Supplementary Figure 1). Control rats exposed to 2-AAF + PH in the absence of DENA, do not develop tumors. The RH and RNT cells were obtained from a rat exposed to the full R-H protocol and from a rat not exposed to DENA, respectively. Briefly, cells were isolated from liver rats through collagenase perfusion by portal vein and maintained in culture. Both cell lines were vital after more than 50 passages in conventional 2D culture dishes and did not change their morphology and behavior. Therefore, they can be defined as "spontaneously immortalized" cells.

The RNT cells exhibit a clear hepatocyte morphology, as they show a typical polygonal architecture and big rounded nuclei. These cells are serum-dependent and show contact inhibition when growing in a monolayer (Figure 1A, 1C, 1E). In contrast, a more elongated morphology (fibroblast-like) characterizes the RH cells (Figure 1B, 1D). They can proliferate under suboptimal culture conditions (low serum, Figure 1E), losing cell-cell contact inhibition, continuing to divide, and forming multilayered foci.

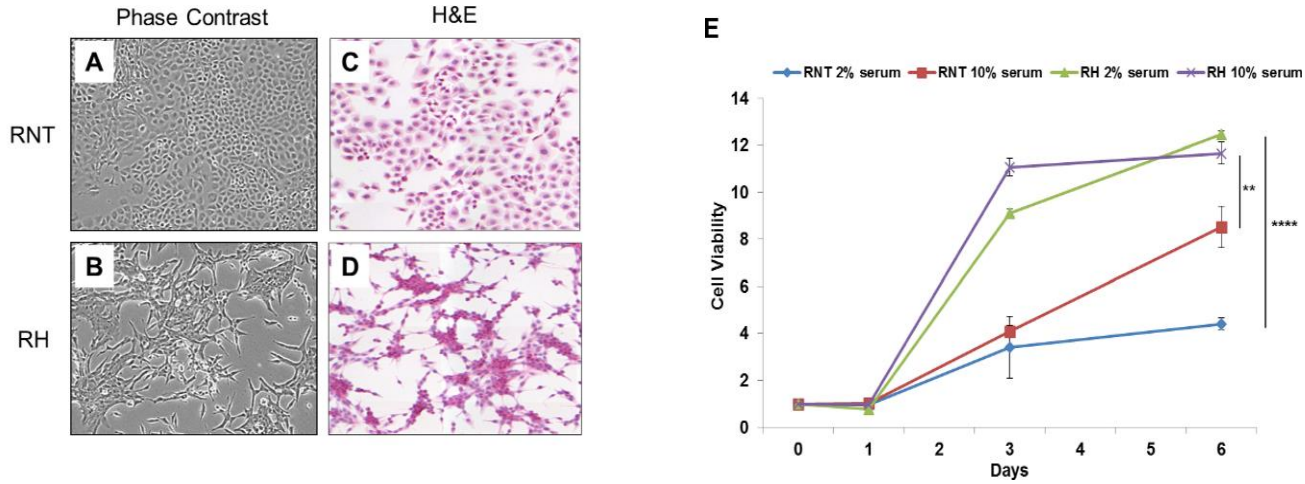


Figure 1: Morphological characterization and growth rate of RNT and RH cells. Phase-contrast microscopy and H&E staining of cultured RNT A., C. and RH B., D. cells. Magnification 20x. For the experimental procedure followed to obtain the cell lines, see Materials and Methods. E. The growth rate of the two cell lines in adherent conditions, in optimal (10% serum) and suboptimal (2% serum) growing conditions, was measured at the indicated times. Cells were fixed and stained with crystal violet; the dye retained by the cells was solubilized in 10% acetic acid and the Optical Density (570nm) was measured. On the X axis is shown the fold change increase of cell number, compared to time zero. ** P<0.01; ****P<0.0001.

We then went on to further characterize the RNT and RH cells for the expression of hepatocyte and non-hepatocyte markers. Both cell lines were positive for glycogen (as shown by PAS staining), a classical marker of hepatocyte function (Figure 2A). Immunofluorescence and flow cytometry analyses showed that both cell types were also positive for canonical hepatocyte cell markers, such as albumin (Alb, >90%) and cytokeratin-18 (KRT18, >95%; Figure 2A, 2B), Moreover, immunofluorescence for transthyretin (TTR), hepatocyte nuclear factor 4-alpha (HNF4A) and transferrin further confirmed the hepatocytic nature of the cells (Figure 3A-3C; Supplementary Figure 2). Performing the analysis for non-hepatocyte markers, we found that only the RH cell line displayed positivity for cytokeratin-19 (KRT19), a typical marker of bile ductular cells and of the so-called oval cells, emerging in pathological conditions (Moll et al., 1982; Strnad et al., 2008), including pre- and neoplastic stages (Figure 2A, 2B; Andersen et al., 2010; Govaere et al., 2014). Interestingly, the intermediate filament vimentin, a marker of mesenchymal origin/feature (Vuoriluoto et al., 2011) and involved in epithelial-mesenchymal transition (EMT), was strongly expressed in RH cells, while it was almost completely absent in RNT cells (Figure 4A).

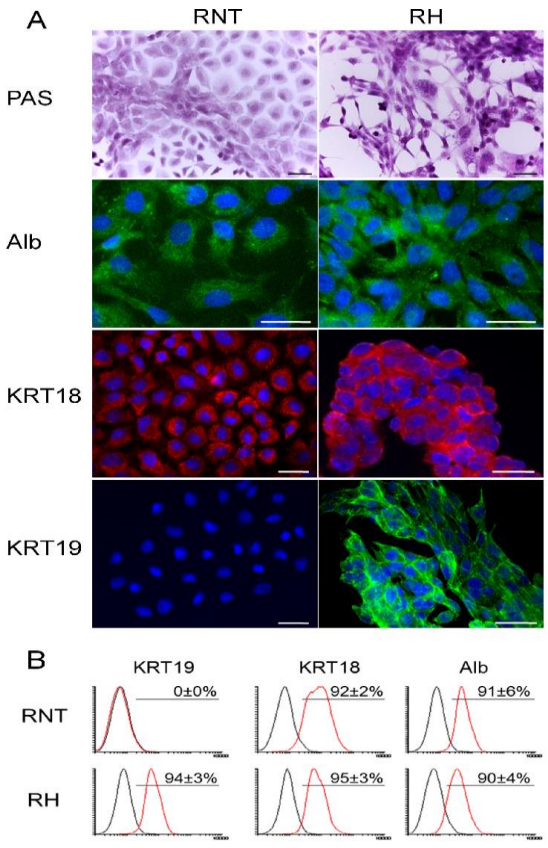


Figure 2: Profiling of typical hepatocyte markers in RNT and RH cells. A. Microphotographs showing positivity for glycogen (PAS staining); A., B. Staining for Albumin (Alb), Cytokeratin 18 (KRT18) and Cytokeratin 19 (KRT19) was assessed by IF (A) and flow cytometry B. analysis in both cell lines. Bars = 25 µm.

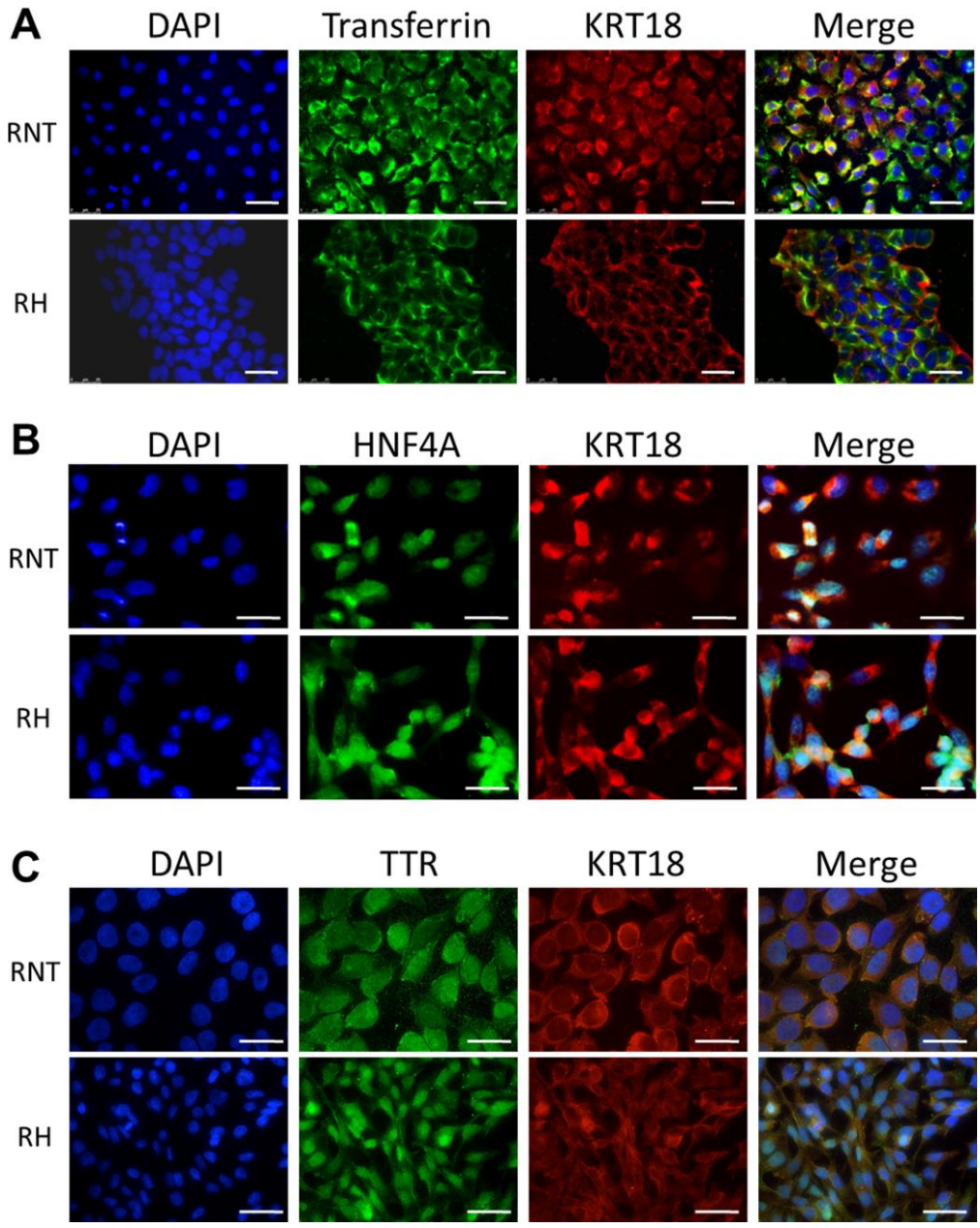


Figure 3: Expression of transferrin, hepatocyte nuclear factor 4 alpha and transthyretin in RNT and RH cells. Co-expression of Transferrin (green) A., hepatocyte nuclear factor 4 alpha (HNF4A) (green) B., transthyretin (TTR) (green) C. and cytokeratin 18 (KRT18; red) in RNT and RH cells. Nuclei were stained with DAPI. Merge of the different staining is shown in the last column on the right. Bars = 25 µm.

To rule out the presence of non-parenchymal cells, which could have grown together with hepatocytes, we performed immunofluorescent staining for alpha-smooth muscle actin (SMA) (Figure 4B), a marker of activated hepatic stellate cells (Kisseleva et al., 2006), and for desmin (Figure 4C), a typical intermediate filament in cardiac, skeletal and smooth muscles (Kisseleva et al., 2006). The results showed that neither RH, nor RNT exhibited positivity for these non-hepatocyte markers. Lack of desmin, α-SMA and vimentin

expression was confirmed by flow cytometry analysis (Figure 4E). Tie-2, the tyrosine kinase receptor for angiopoietin 1 (De Palma et al., 2005; Tang et al., 2010), is expressed almost exclusively in endothelial cells, in a fraction of monocytes and hematopoietic stem cells (De Palma et al., 2005; Tang et al., 2010). Immunofluorescent staining for this receptor showed that neither RNT nor RH cells expressed Tie-2 (Figure 4D). Additionally, flow cytometry analysis revealed no expression in either cell line of intracellular and extracellular CD68, a macrophage marker (Figure 4E).

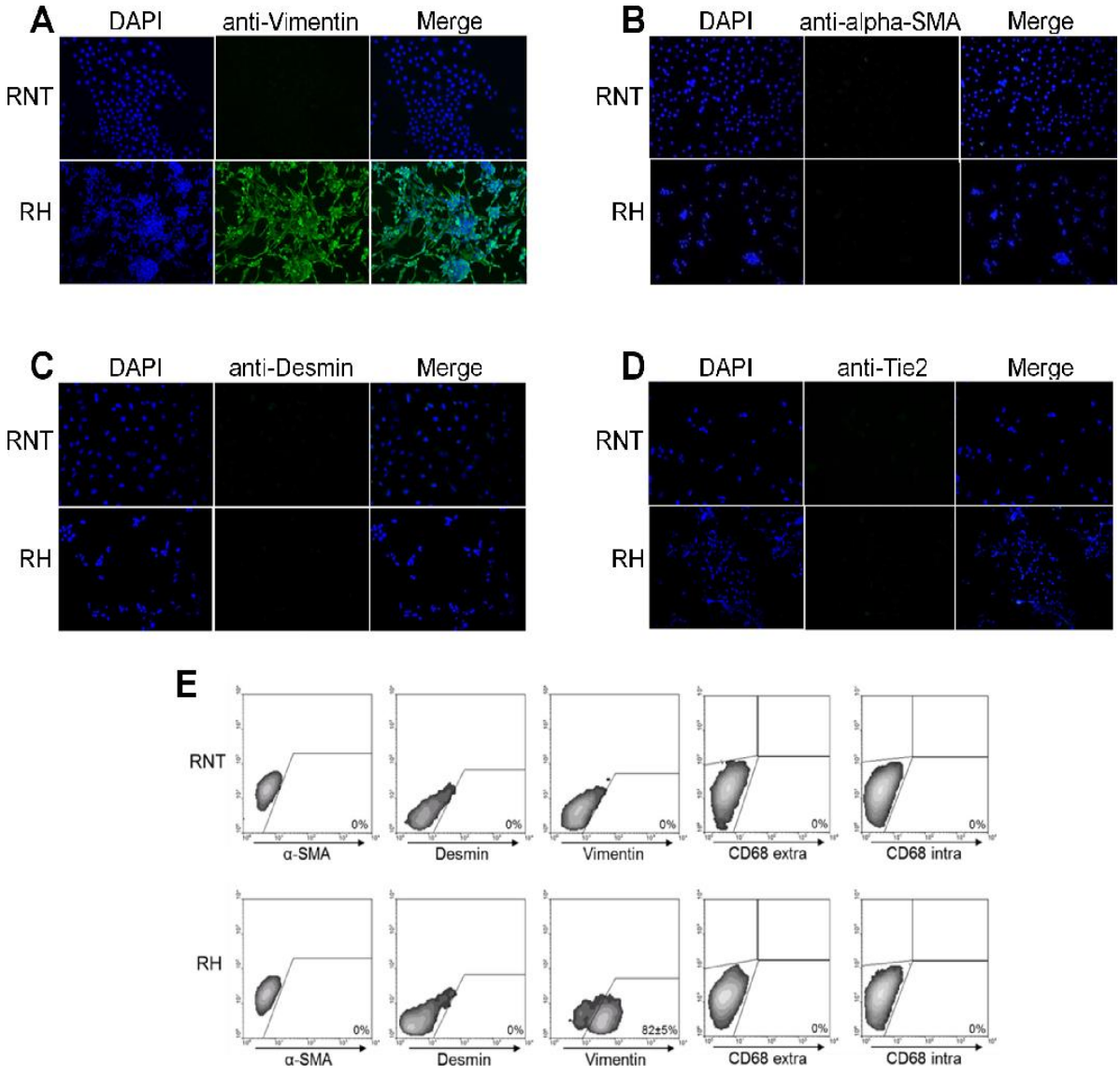


Figure 4: Panel of non-hepatocyte markers in RNT and RH cells. Immunofluorescent staining for Vimentin A., alpha-SMA B., Desmin C. and Tie-2 D. Magnification 20X. E. Flow cytometry analysis of the expression of alpha-SMA, Desmin, Vimentin and the intra/ extracellular macrophage marker CD68.

Moreover, to prove the hepatocytic nature of the two cell lines, we transduced them with lentiviral vectors expressing GFP under the transcriptional regulation of either hepatocyte-specific promoter or ubiquitous, endothelial- and myeloid-specific promoters. Since transthyretin (TTR) is a hepatocyte secreted protein, its promoter specifically drives transgene expression in hepatocytes (Vigna et al., 2005). At an MOI of 1 in cultured RH and RNT cells, the percentage of GFP positive cells was like those transduced with a lentiviral vector containing a ubiquitously expressed promoter, the phosphoglycerate kinase 1 (PGK1) promoter (respectively 76% and 91% for TTR, 69% and 83% for PGK1), confirming the hepatocytic origin of these cells (Figure 5). On the contrary, promoters specific for endothelial and myeloid cells showed low levels of GFP expression (15% and 26% for VEC and 11% and 15% for CD11b), further demonstrating that the isolated cells were hepatocyte-derived. At the 0.1 MOI, the differences were even more pronounced and were in line with the demonstration that these cells are indeed hepatocytes (Supplementary Figure 3A). To validate the specificity of expression of the different described Lentiviruses, we transduced hepatocytic (C1C7), endothelial (MS1) and monocytic (U937) cell lines as controls (Supplementary Figure 3B).

Taken together, these results demonstrate the hepatocytic nature of cultured RNT and RH cells and rule out a possible contamination by endothelial, macrophages or stellate cells.

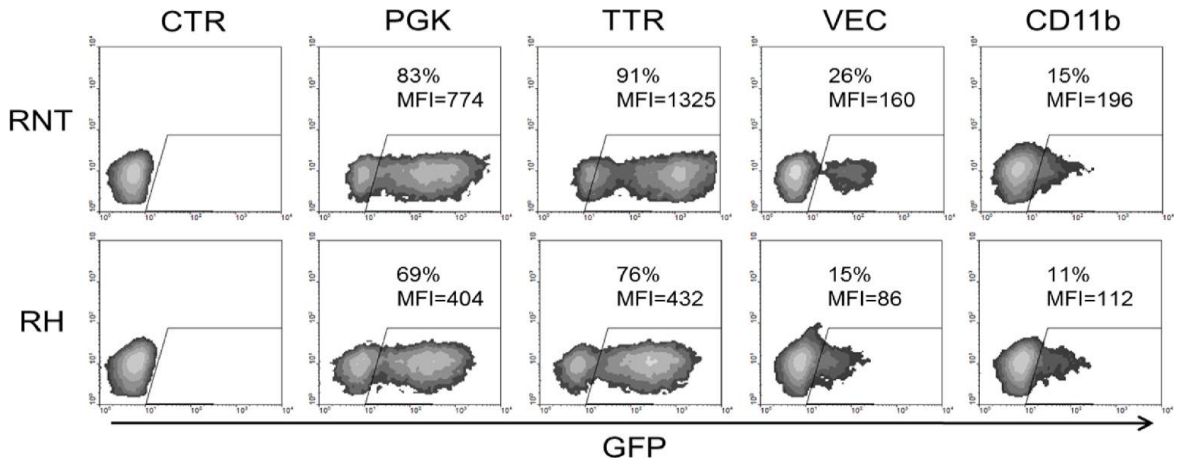


Figure 5: Lentiviral transduction and FACS analysis for promoter-characterization. RNT and RH cells were transduced with four different Lentiviruses (LVs) containing the GFP transgene under the control of ubiquitous (PGK) or cell-specific promoters (TTR, hepatocyte-specific; VEC, endothelial-specific; CD11b, myeloid cells-specific) at MOI 1. GFP expression was evaluated by FACS analysis 72 hours after transduction. GFP expression driven by the hepatocyte-specific TTR promoter in both cell types is comparable to the expression driven by the ubiquitous PGK promoter, while VEC and CD11b promoters were less active in both cell types, confirming the hepatocyte phenotype of these cells. MFI = mean fluorescent intensity.

8.2 RH but not RNT cells express tumor stem cell markers

Recently, putative liver tumor-initiating cells (T-ICs) have been identified by several cell surface antigens, such as CD90.1, EpCAM and CD24 (Ji and Wang, 2012; Yamashita et al., 2009; Yang et al., 2008). The T-ICs have been suggested to be critical for the maintenance, self-renewal, differentiation and the metastasis of tumors, as well as to significantly impact on a patients' clinical outcome (Lee et al., 2011). Therefore, we investigated by flow cytometry the presence of T-ICs-like cells in both rat cell lines.

As shown in Figure 6A–6D, a small percentage of these two cell lines expressed EpCAM (RNT 5% and RH 6%), CD24 (RNT 6% and RH 10%) and CD90.1 (RNT 0% AND RH 1%). Interestingly, while EpCAM- and CD24- cells co-expressed albumin and cytokeratin 18, EpCAM+ and CD24+ cells were negative for these differentiation markers, further supporting their stem-like cell nature. Moreover, only RH cells were double positive for CD90.1/CD24, (Figure 6D), as CD90.1 were not detected in the immortalized non-tumorigenic RNT cell line (Figure 6C). These findings show that only RH cells contain tumor stem-like cells, as defined by the double expression of CD90.1 and CD24, in agreement with the results obtained from human samples (Lee et al., 2011). To further characterize the two cell lines, we analyzed their growth ability in 3D cultures. We thus generated spheroids and found that RH cells gave rise to more numerous spheroids than RNT cells (Figure 7A). Notably, both cell lines maintained in 3D culture expressed hepato-specific markers, such as albumin and cytokeratin 18 (Figure 7B, 7D), and of the hepatic precursor marker CD24 (Figure 7C). In the RNT cells, CD24 was less represented and the staining for KRT19 (Figure 7B, 7C) was negative, confirming the results obtained in the 2D culture of RNT cells (Figure 2A–2B).

To evaluate the gene expression profile of these two cell types we used the Rat Liver Cancer RT² Profiler PCR Array and found that 44 out of the 84 genes present in the Array were differentially regulated between RH and RNT cells (Threshold = fold change +/-2) (Supplementary Table 1). As shown in Figure 8, while the Heat Map displayed only minimal differences in each cell type at different culturing passages (20, 40, 60), the dendrogram clearly separated RH from RNT. Furthermore, by comparing the expression profile of RH and RNT with normal livers and primary HCCs, we found that RNT clustered together with control livers, while RH were much closer to the HCC sub-cluster (Supplementary Figure 4). Quantitative RT-PCR validation performed on randomly selected genes (*Birc5*, *Igfbp1*,

Myc, *Tert*, *Cnnd1* and *Met*) confirmed the microarray expression data for all the examined genes (Supplementary Figure 5).

A DAVID Functional Analysis of genes altered in RH cells and HCC *vs*. their respective controls revealed that most of the dysregulated genes are involved in Ubiquitin conjugation, Apoptosis, Phosphoprotein, Glycoprotein and Signaling (Supplementary Figure 6A). Pathway analyses also underlined common modifications between RH cells and HCCs (Pathways in Cancer, MicroRNA in cancer, Hepatitis B, PI3K-AKT-signaling pathway) (Supplementary Figure 6B).

Collectively, these results show that RH and RNT cells not only exhibit a distinct expression profile, but they also maintain features displayed *in vivo* by transformed and normal hepatocytes, respectively.

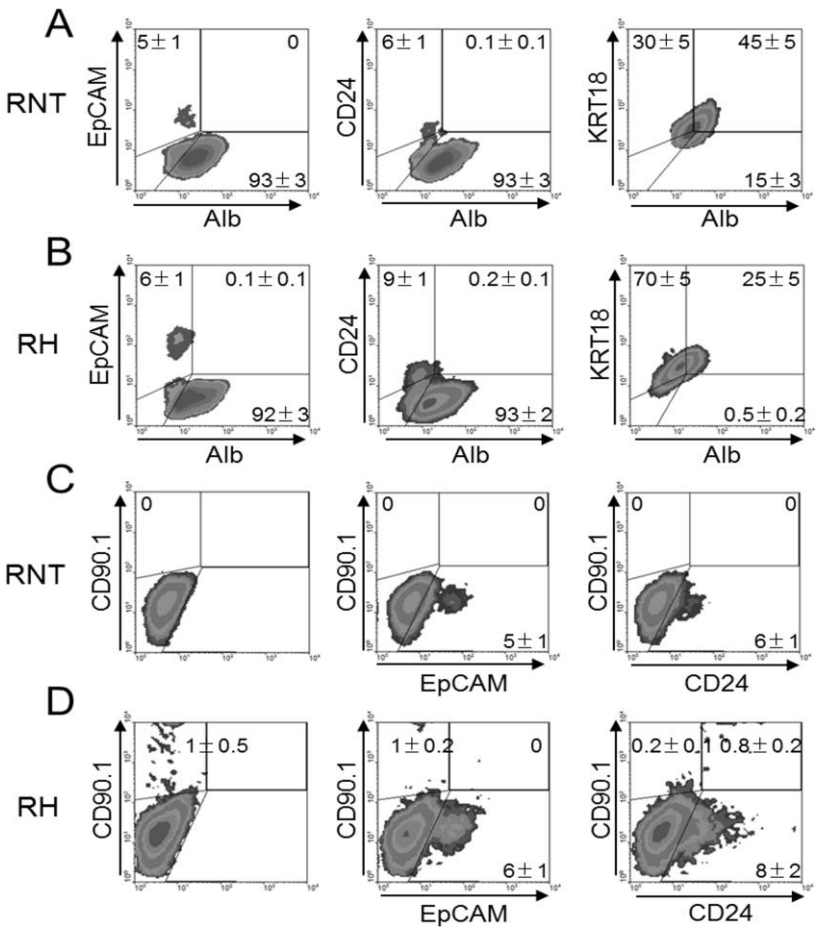


Figure 6: Identification of stem cell-like markers in RNT and RH cells. RNT A and C. and RH B and D. cells were analyzed by flow cytometry for the expression of mature hepatocyte markers (Alb and KRT18), hepatocyte precursor markers (EpCAM and CD24) or the cancer stem cell marker (CD90.1). Numbers indicate the mean of at least 5 different analysis performed ± SD.

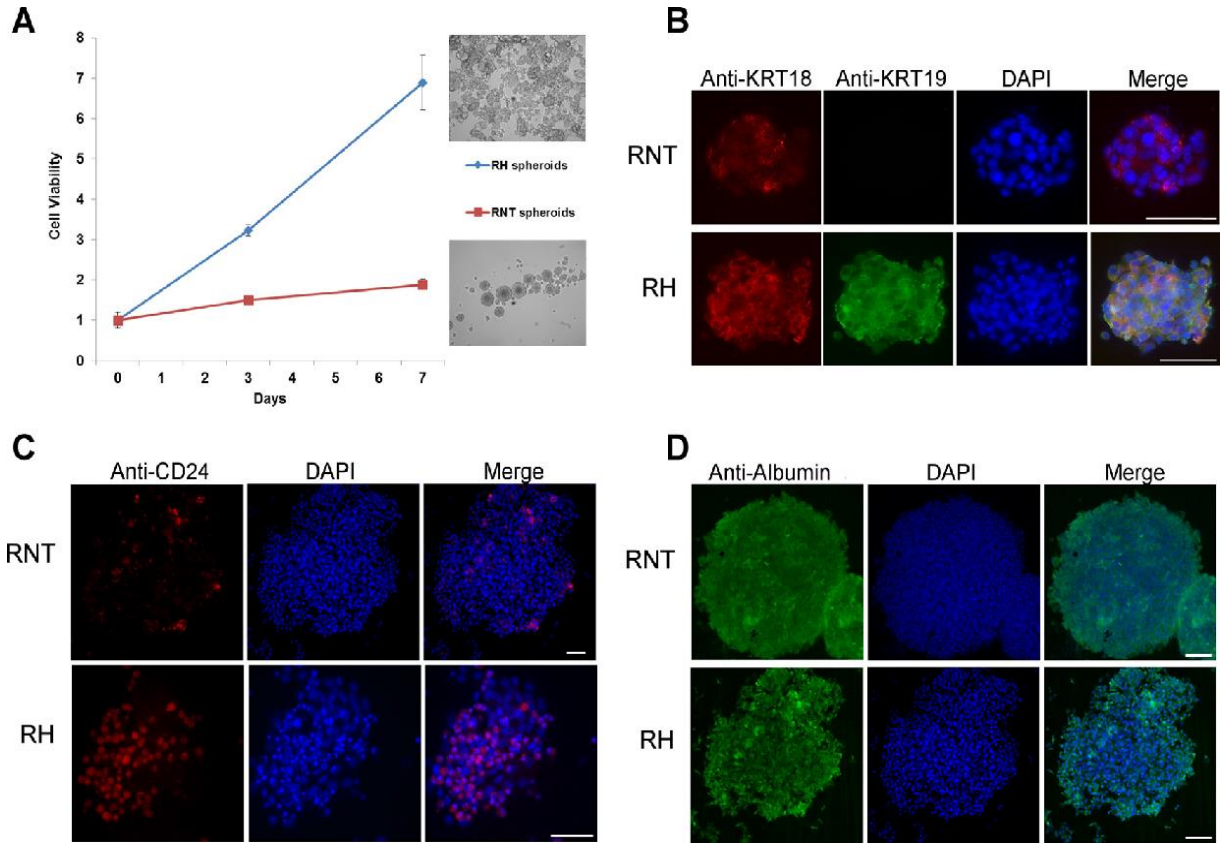


Figure 7: Generation of RNT and RH spheroids. A. The two cell lines were grown in stem medium and low attachment conditions; cell viability was measured at the indicated times. Pictures in the right part of the graph represent RH (upper figure) and RNT spheroids (lower figure) at day 7 (magnification 10X). The X axis shows the fold change increase in cell viability, compared to time zero. Immunofluorescence showing expression of hepatocytes markers KRT18 B. and Alb D. in spheroids of both cell types; on the contrary, KRT19 is present only in RH spheroids (B). A small percentage of RH and RNT spheroids are positive for CD24 C. the number of CD24+ spheroids is higher in RH cells (C). Scale bar = 25 µm.

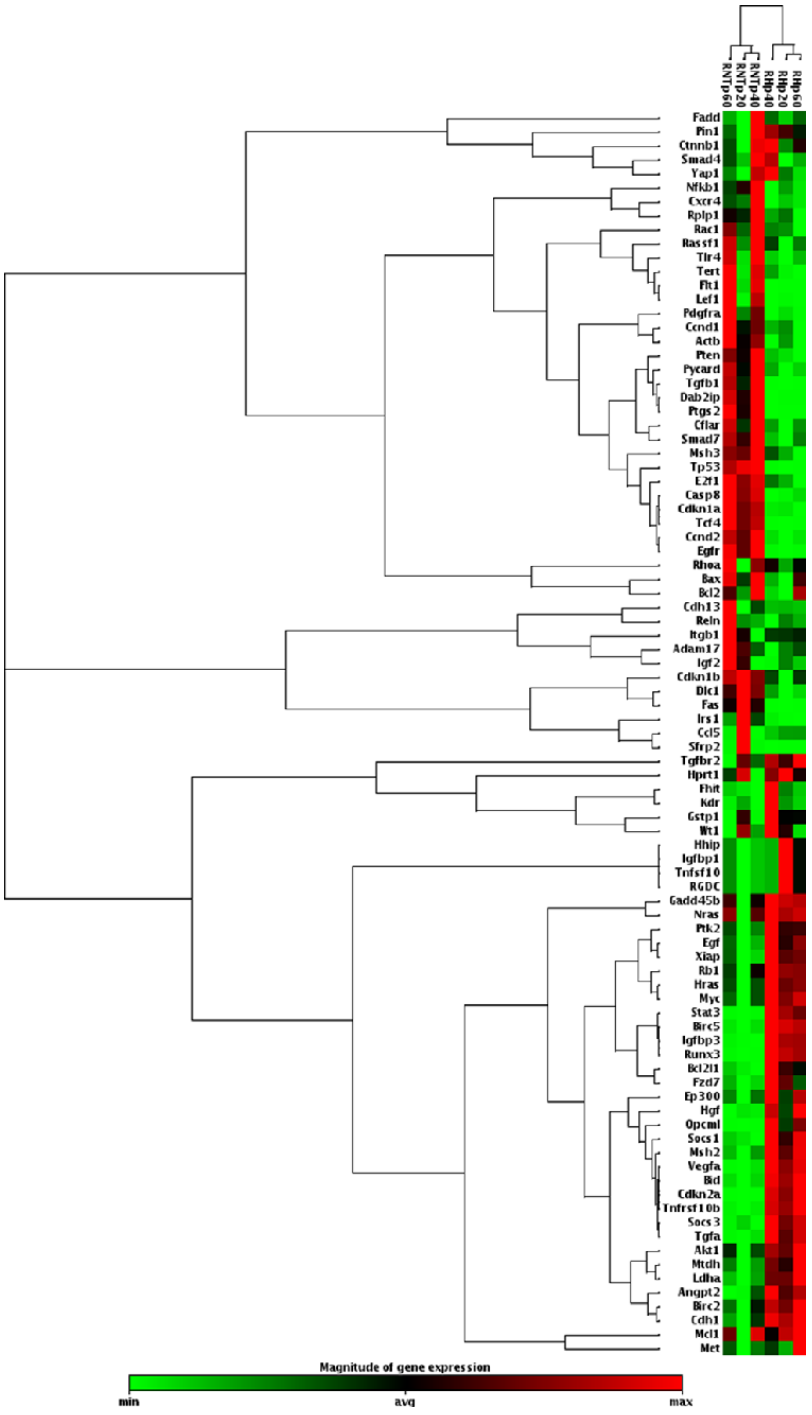


Figure 8: Hierarchical clustering of 84 genes in RNT and RH cells. Each row represents the expression profile of a gene. Only mRNAs whose expression was dysregulated at least by 2-fold were considered. Red and green colors represent higher or lower expression levels of the mRNA (median-centered), respectively.

8.3 RH cells, but not RNT, are endowed with a transformed/tumorigenic potential

Cell immortalization can be accompanied by transformation. Thus, for both the cell lines, we assessed the transformed/tumorigenic potential typically associated *in vitro* with the acquisition of anchorage-independent growth ability and *in vivo* with the capacity to generate tumors when grafted.

When plated in soft agar, RH cells were able to form numerous and large colonies within seven days, while RNT cells did not, even after 3 weeks. (Figure 9A). Moreover, while RH cells were able to form tumor masses in 30 days, when inoculated into the posterior flank of syngeneic rats, RNT cells were completely devoid of tumorigenic ability (Figure 9B, 9C); these tumors showed morphological features of HCC (Figure 9D) and were strongly positive for KRT19 (Figure 9E), thus maintaining the same features of the cells grown *in vitro*.

As RH cells display EMT features, we wondered if they were able to form metastases. Indeed, one month after grafting, we observed the appearance of lung macro-metastases (Figure 9F). H&E and immunohistochemically staining on serial sections of lung metastases showed the maintenance of primary HCC morphology and KRT19 positivity. As expected, RNT cells were unable to originate metastases up to 3 months after grafting (data not shown).

To investigate whether CD24+ stem-like cells are involved in tumor formation, $5x10^5$ to $3x10^6$ CD24+ and CD24- RH and RNT cells were injected s.c. into the posterior flank of NOD-SCID yNull mice (n=5), immediately after isolation. While both CD24 positive and negative RNT cells did not give rise to tumors up to 3 months, CD24+ RH cells generated tumors with a similar size within 14 days in all tested conditions (Figure 9G). As controls, we injected 10^7 unselected RH and RNT cells and only the RH cells formed tumors similar in size to those observed with CD24 positive RH cells. Interestingly, CD24- RH cells did not give rise to tumors, confirming that tumorigenic cells are in the CD24+ fraction (data not shown). Of note, we found that among the CD24+ RH cells, 7±2% were also CD90.1+, suggesting that this subpopulation could be responsible for the tumorigenic potential of RH cells. In agreement, the CD24+ RNT cells did not contain any CD90.1+ cell (Supplementary Figure 7).

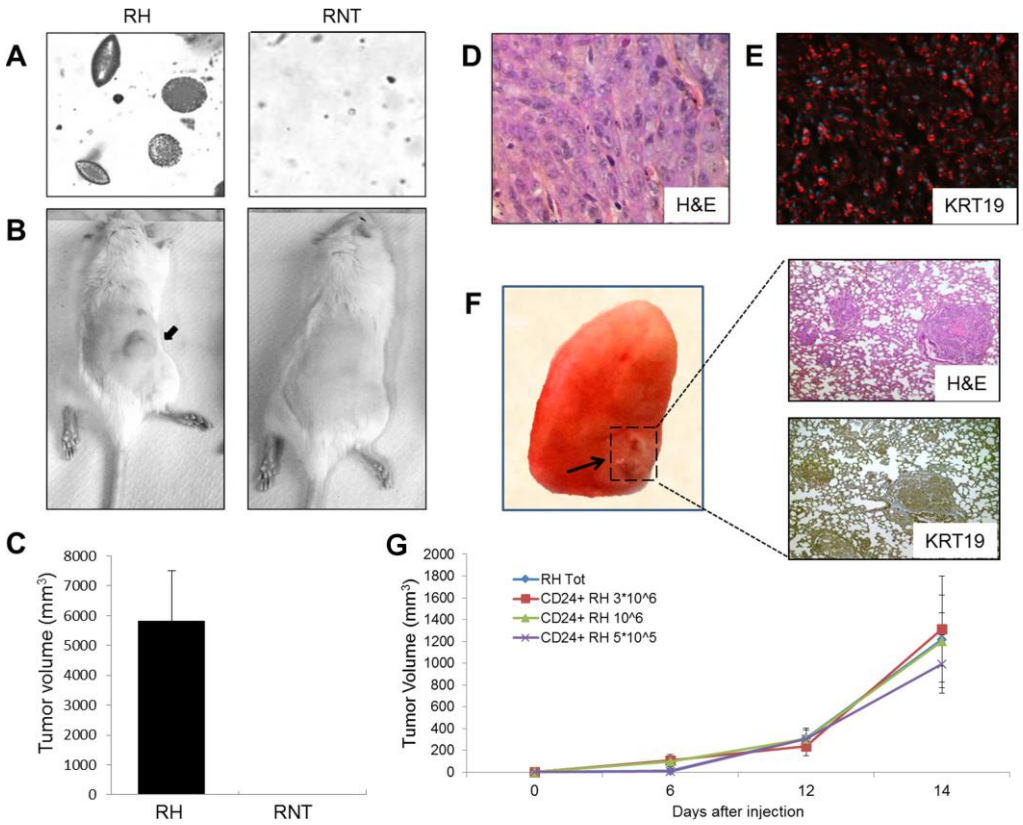


Figure 9: Biological assays and tumorigenesis. A. The ability to grow in anchorage-independent conditions was assessed by soft agar assay. Colonies were photographed after two weeks. B. Representative photographs of syngeneic rats injected with RH (left) or RNT (right) cells. RH and RNT cells were inoculated into the posterior flank of syngeneic rats (1.5*106 cells/injection); C. Graph showing the tumor volume of rats, 19 days after injection with RH or RNT. The results represent the mean ± SD of 5 animals/group; D. Microphotograph showing H&E staining of a HCC developed 19 days after injection of RH cells; E. Microphotograph illustrating a strong positivity of KRT19 in the same HCC; F. (Left) Macroscopic photograph of a lung from a rat injected with RH cells and killed 30 days after grafting. A metastasis is shown by the arrow. (Right) Microphotographs of the metastasis shown in F, stained for H&E (top) and KRT19 (bottom; Magnification 10x); G. Unselected (Tot) and CD24+ RH cells were inoculated into the posterior flank of NOD-SCID γNull mice (107 cells/ injection for unselected RH cells and 5x105, 1 x 106 and 3 x106 for CD24+ RH cells). The graph shows tumor growth in mice up to 14 days after injection. The results represent the mean ± SD of 5 animals/group.

8.4 RNT/RH cells as a tool to investigate the molecular mechanisms of hepatocarcinogenesis

The two cell lines we have established represent two different stages of the hepatocarcinogenic process. To provide a proof of concept that they can be used to study the role of candidate molecules in HCC progression, we focused our interest on NRF2. It is known that NRF2 is an integrated redox sensitive signaling system that regulates 1%-10%

of human genes and is negatively controlled by the ubiquitin ligase KEAP1, which promotes NRF2 proteasome-mediated degradation (Jaramillo and Zhang, 2013; Kobayashi et al., 2004).

As previously published (Petrelli et al., 2013), the NRF2 pathway is already activated in early preneoplastic lesions and along tumor progression. The silencing of NRF2 in RH cells inhibits their tumorigenic ability, both *in vitro* and *in vivo* (Zavattari et al., 2015), demonstrating that activation of this pathway is necessary to sustain the malignant phenotype. To investigate if NRF2 activation is "sufficient" to confer transforming ability to RNT cells, we transduced them with a lentiviral vector containing NRF2 cDNA and evaluated their *in vitro* transforming ability. As shown in Figure 10A, over-expression of NRF2 was not sufficient to confer to the cells the ability to grow in an anchorage-independent manner.

We previously showed that *Nrf2/Keap1* mutations are present in 59.3% of HCCs developed in the R-H model (Zavattari et al., 2015). Interestingly, while RNT cells are wild type for NRF2, RH cells display an activating mutation (Figure 10B). Therefore, to further stress the system, we transduced RNT cells with constitutively activated forms of this gene, as a consequence of activating mutations (Supplementary Figure 8). Transduction of RNT cells with two different NRF2 mutated forms did not confer them with a tumorigenic ability *in vitro.* The RNT cells, however, could be transformed by transduction of a well-known oncogene, such as the active (G12V) KRAS (Figure 10A).

All together, these experiments show that NRF2 activation is necessary to sustain the tumorigenic status but is not sufficient *per se* to induce it.

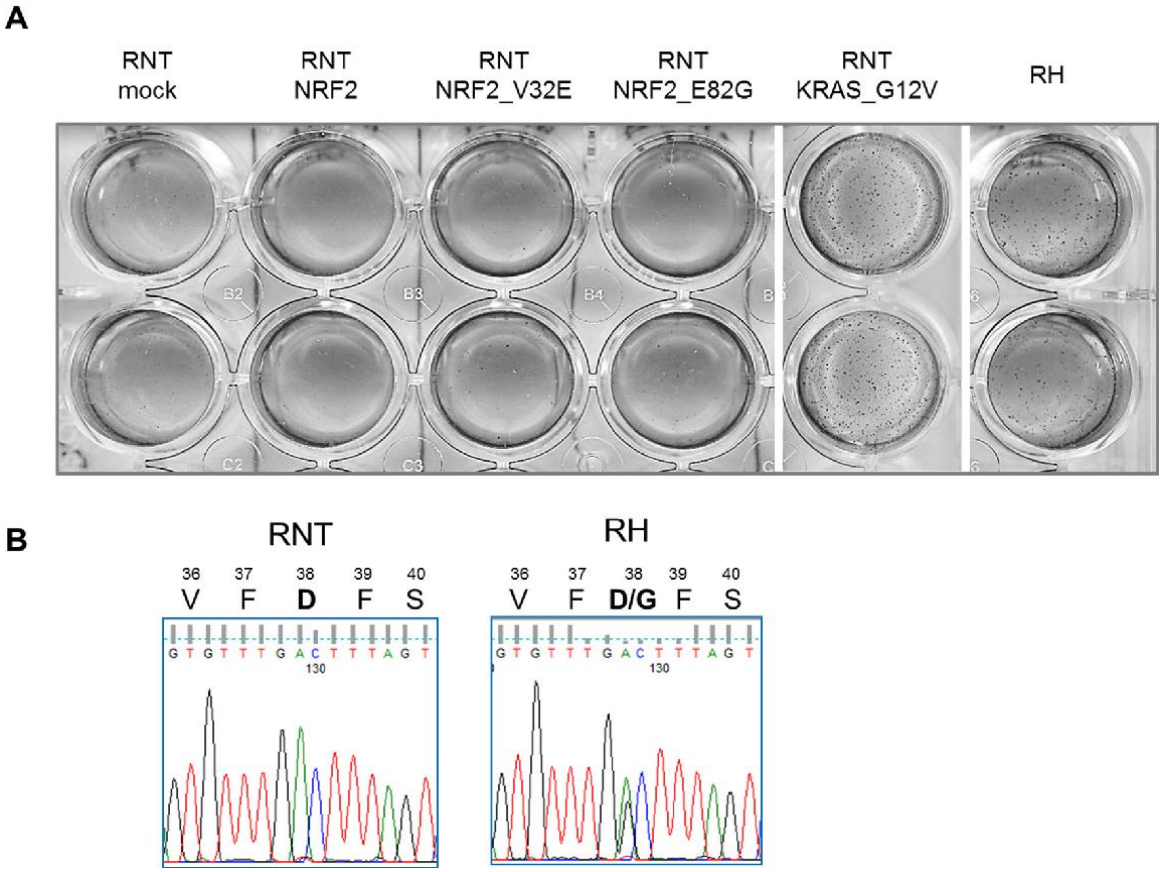
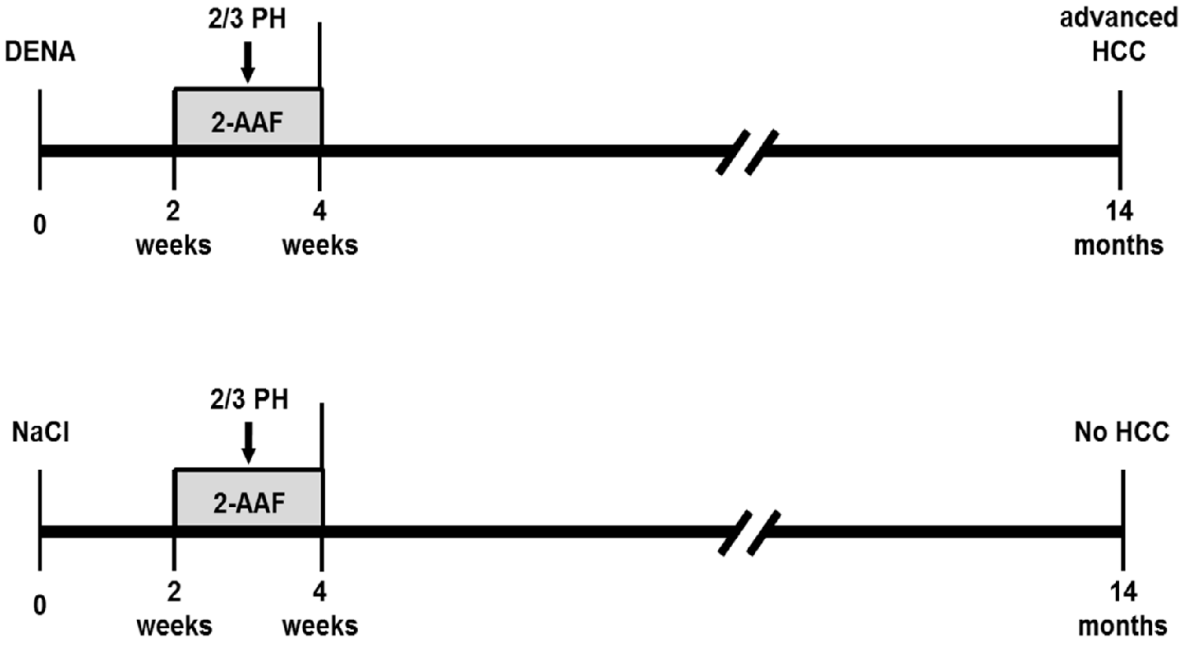
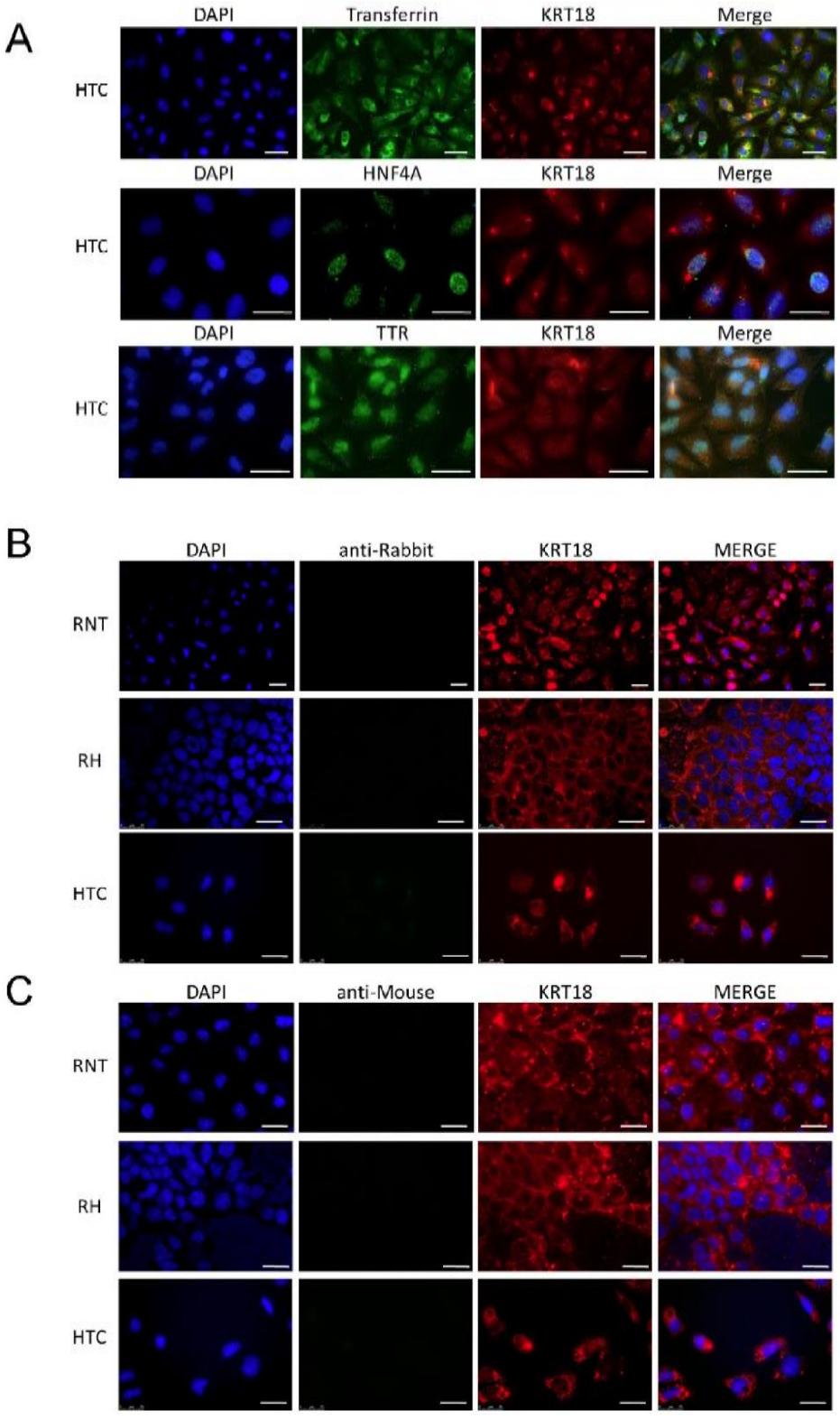


Figure 10: NRF2 transduction is not sufficient to transform RNT cells. A. RNT cells were transduced with the indicated constructs (mock vector, wild type NRF2, NRF2 constructs bearing activating mutations, activated KRAS). The ability to grow in anchorage-independent conditions was assessed by soft agar assay. Colonies were photographed after two weeks. RH cells were used as positive controls for the growth in soft agar. B. Pherograms showing the presence of a NRF2 activating mutation (D38G) in RH but not in RNT cells.

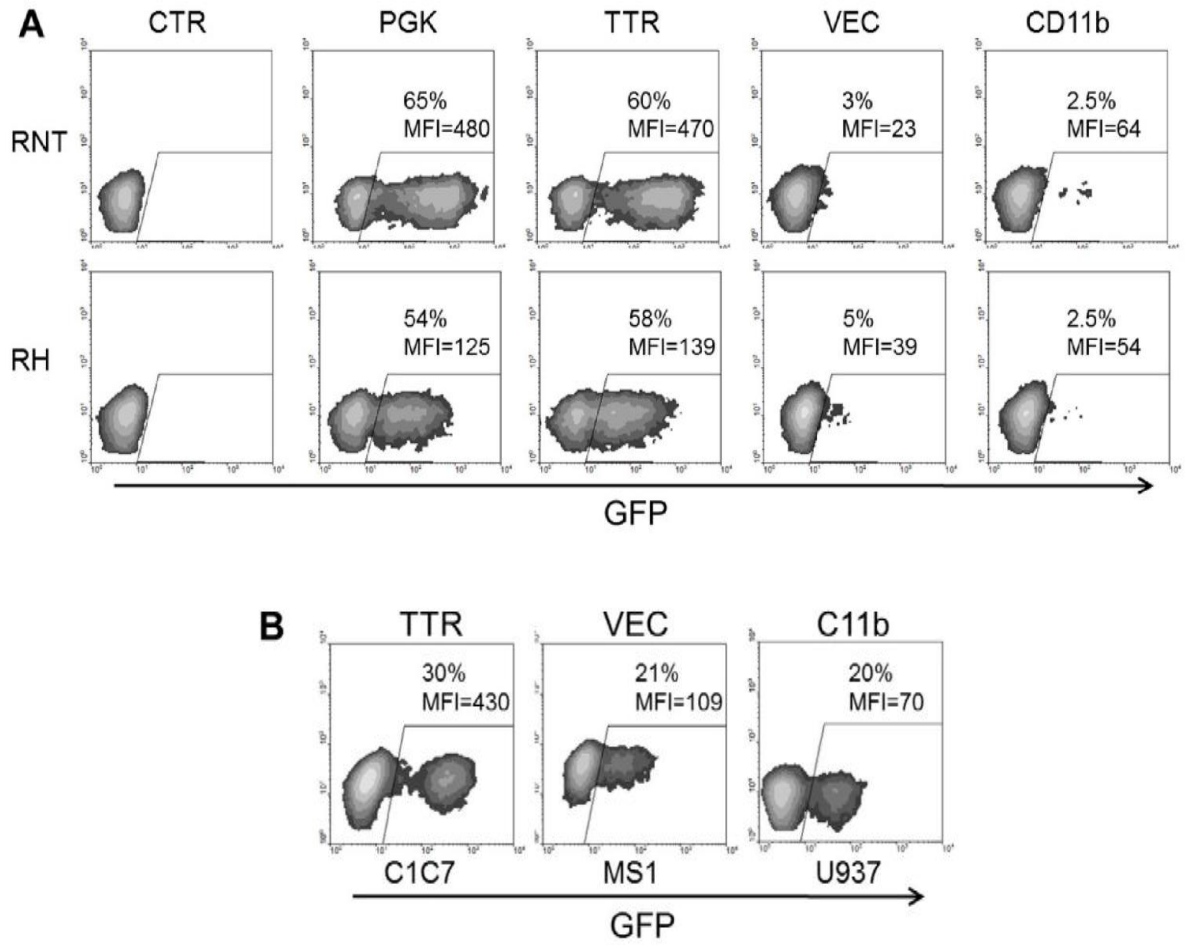
8.5 SUPPLEMENTARY FIGURES AND TABLES



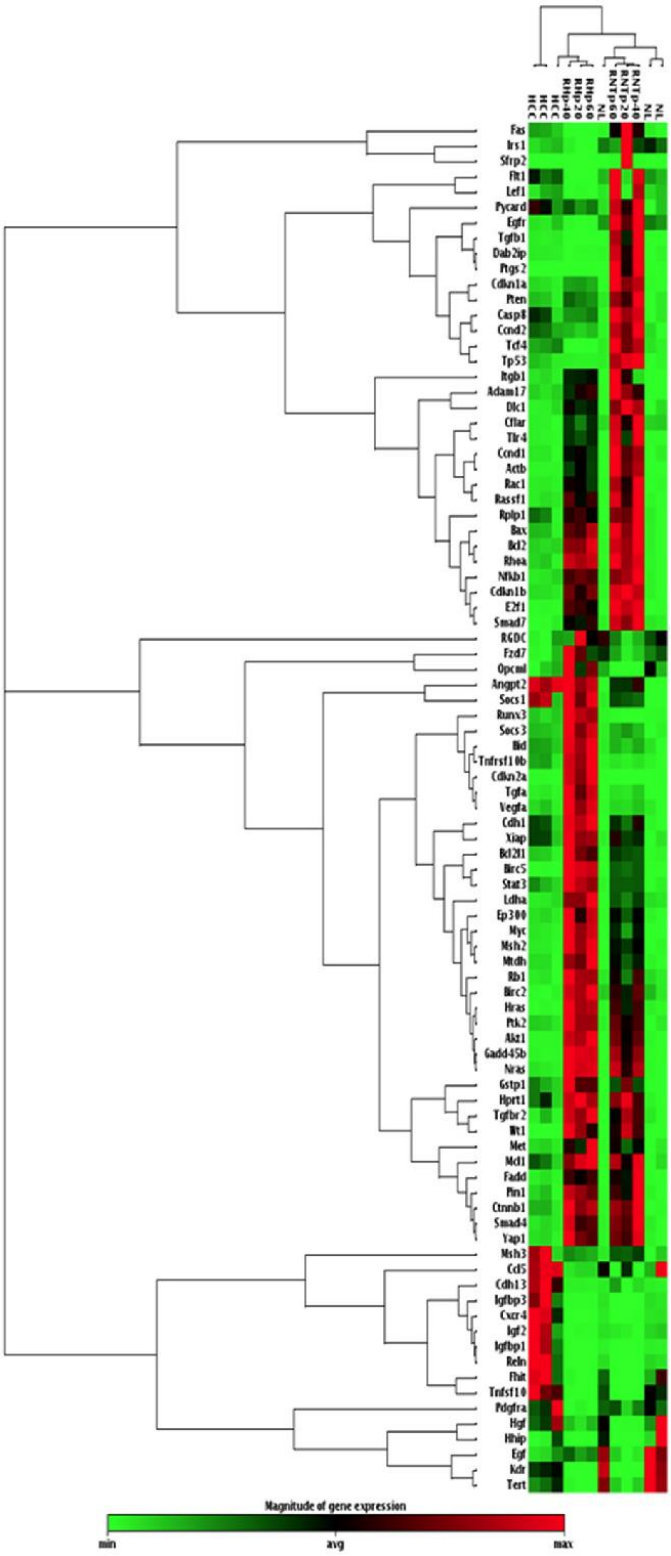
Supplementary Figure 1: Schematic representation of the experimental protocol. F-344 rats injected with a single intraperitoneal dose of diethylnitrosamine (DENA) or saline (NaCl) were subjected to the R-H protocol, consisting of a 2-week diet supplemented with 0.02% 2-acetylaminofluorene (2-AAF) and a two/thirds partial hepatectomy (2/3 PH). Rats were then switched to basal diet all throughout the experiment and killed 14 months after DENA administration.



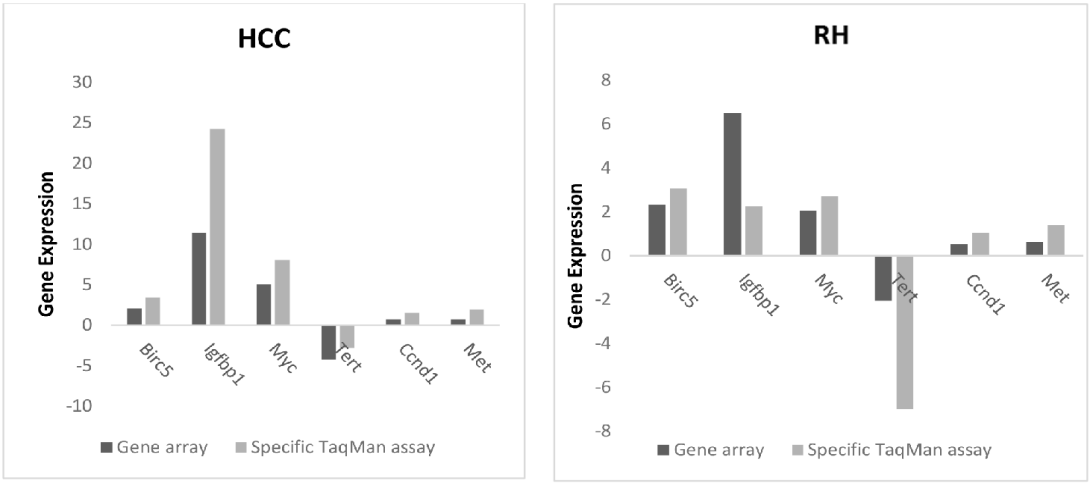
Supplementary Figure 2: Positive and negative controls for immunofluorescence on RNT, RH and HTC cells. A. HTC cells served as control cells for transferrin, HNF4A and TTR stainings. RH, RNT and HTC cells were stained with: i) DAPI for nuclear staining, ii) fluorochrome-conjugated secondary anti-rabbit B. or anti-mouse C. antibodies (Alexa Fluor® 488); iii) anti-KRT18 antibody. The right part shows the merged images of the different stains. Bars = 25 µm.



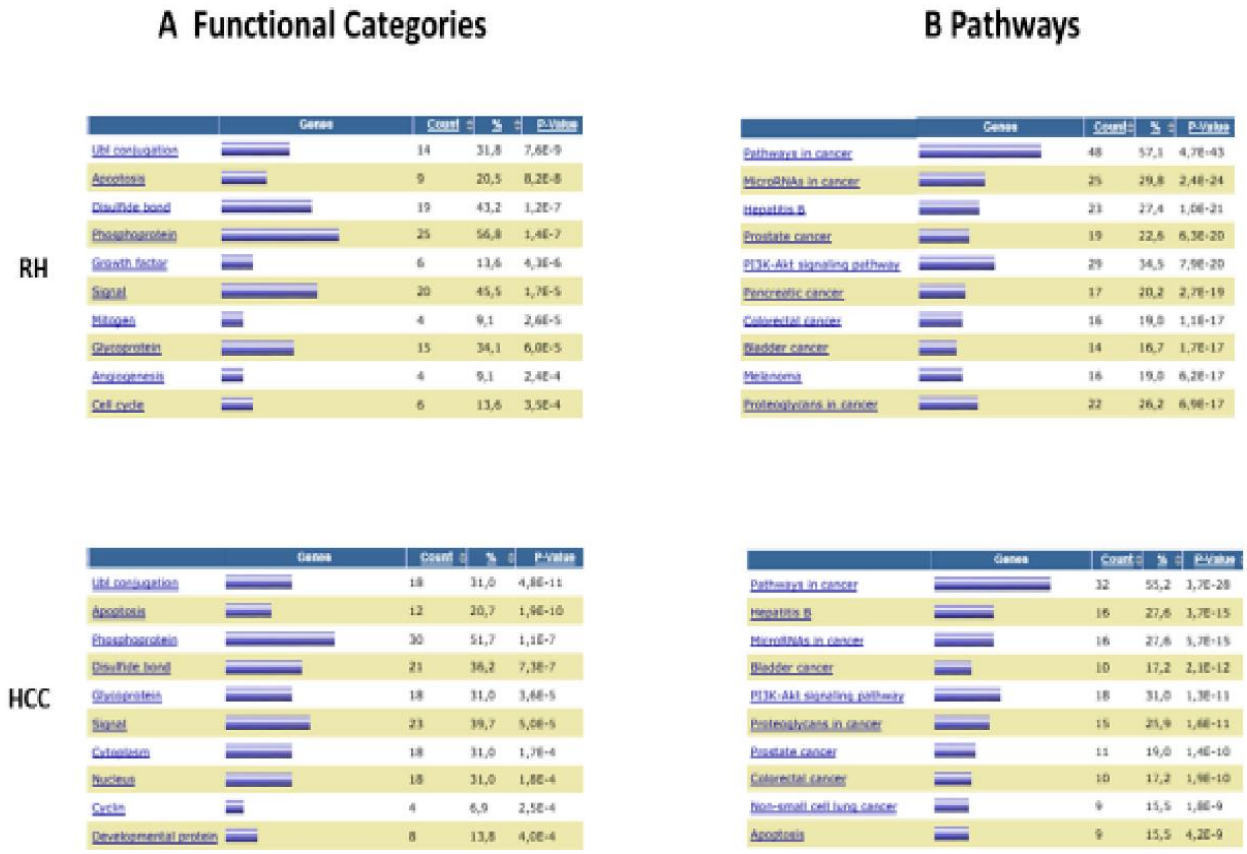
Supplementary Figure 3: Lentiviral Cell Transduction with cell-type specific promoters. Flow cytometry analysis shows RNT and RH cells (A) and control cells C1C7= murine hepatocytes; MS1= murine endothelial cells; U937= human monocytes). (B) transduced with four different LVs containing the GFP transgene under the control of ubiquitous (PGK) or cell-type specific promoters (TTR, hepatocyte-specific; VEC, endothelial-specific; CD11b, myeloid cells-specific) at MOI 0.1. After transduction, GFP expression was higher in cells transduced with LV containing the TTR promoter in both cell lines and comparable to the expression driven by the ubiquitous PGK promoter; GFP expression was very low in RH and RNT cells transduced with LVs containing VEC and CD11b promoters, confirming the hepatocyte phenotype of these cells. MFI = mean fluorescent intensity.



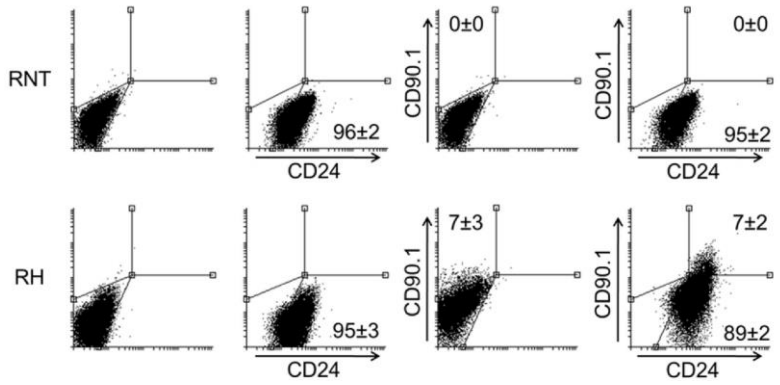
Supplementary Figure 4: Hierarchical clustering of 84 genes in RH and RNT cells, normal livers and HCCs generated 14 months after administration of DENA. Each row represents the expression profile of a gene. Only mRNAs whose expression was dysregulated at least by 2-fold were considered. Red and green colors represent higher or lower expression levels of the mRNA (median-centered), respectively.



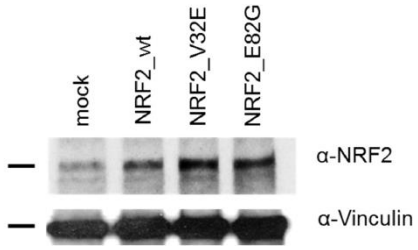
Supplementary Figure 5: QRT-PCR validation of randomly selected genes in rat HCC and RH cells. Gene expression is reported as log fold-change relative to control liver (left panel) and RNT (right panel), respectively.



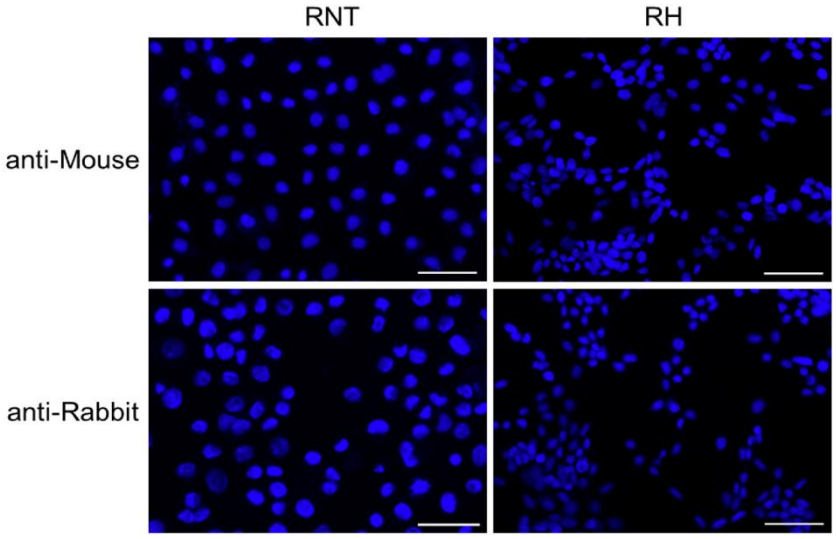
Supplementary Figure 6: Functional categories and Pathway analysis of differentially expressed genes. Top 10 enriched functions A. and pathways B. in RH cells and rat HCC. *P* values were determined using the DAVID's EASE Score and were judged significant at $P < 0.05$.



Supplementary Figure 7: Flow cytometry analysis of freshly isolated CD24+ RNT and RH cells. Isolated cells were highly positive for CD24 (≥95%). A subpopulation of CD90.1+ cells (7±2%) is present within the CD24+ RH cells, while no CD90.1+ cells were found in CD24+ RNT cells. Numbers represent percentage of positivity ± SD of 5 flow cytometry analysis from several isolations.



Supplementary Figure 8: Western blot analysis of RNT cells transduced with mock, NRF2 wild type or mutated NRF2. The blot was probed with NRF2 antibodies (upper panel). Vinculin staining (lower panel) was used as loading control.



Supplementary Figure 9: Negative controls for immunofluorescence on RNT and RH cells. RNT and RH cells were stained only with fluorochrome-conjugated secondary anti-mouse or anti-rabbit antibodies (Alexa Fluor® 488 or Alexa Fluor® 546). Nuclei were stained with DAPI. Bars = 50 µm.

Supplementary Table 1: Analysis of gene expression in RH cells

Symbol	Fold Change	Symbol	Fold Change	Symbol	Fold Change
Adam17	0,68	Fas	0,02	Ptgs2	0,00
Akt1	1,42	Fhit	3,26	Ptk2	1,40
Angpt2	1,80	Flt1	0,00	Pycard	0,41
Bax	0,79	Fzd7	3,86	Rac1	0,60
Bcl2	0,92	Gadd45b	1,36	Rassf1	0,72
Bcl2l1	2,05	Gstp1	1,71	Rb1	1,81
Bid	4,93	Hgf	41,94	Reln	0,58
Birc2	1,41	Hhip	2,26	Rhoa	0,97
Birc5	3,07	Hras	1,57	Runx3	411,03
Casp8	0,26	Igf2	0,45	Sfrp2	0,00
Ccl5	1,02	Igfbp1	2,26	Smad4	0,93
Ccnd1	0,57	Igfbp3	291,95	Smad7	0,59
Ccnd2	0,26	Irs1	0,27	Socs1	2,59
Cdh1	2,24	Itgb1	1,25	Socs3	5,36
Cdh13	0,67	Kdr	3,11	Stat3	2,57
Cdkn1a	0,22	Lef1	0,03	Tcf4	0,05
Cdkn1b	0,72	Mcl1	1,17	Tert	0,14
Cdkn2a	34819,92	Met	1,41	Tgfa	10,60
Cflar	0,54	Msh2	2,04	Tgfb1	0,02
Ctnnb1	1,06	Msh3	0,59	Tgfbr2	1,35
Cxcr4	0,16	Mtdh	1,91	Tlr4	0,55
Dab2ip	0,03	Myc	2,71	Tnfrsf10b	15,03
Dlc1	0,53	Nfkb1	0,78	Tnfsf10	2,26
E2f1	0,63	Nras	1,25	Tp53	0,04
Egf	2,64	Opcml	167,63	Vegfa	6,10
Egfr	0,02	Pdgfra	0,02	Wt1	1,14
Ep300	1,84	Pin1	1,20	Xiap	1,68
Fadd	0,93	Pten	0,37	Yap1	1,03

Gene expression is reported as fold change relative to RNT cells.

Supplementary Table 2: Antibodies used for Immunofluorescence

Primary antibody			
Name	**Conjugation**	**Brand**	**Incubation conditions**
anti-alpha-SMA	Purified	Sigma Aldrich	60 min at RT
anti-rat Tie2	Purified	Santa Cruz Biotec	60 min at RT
anti-Desmin	Purified	Abcam	60 min at RT
anti-Vimentin	Purified	Sigma Aldrich	60 min at RT
anti-KTR18	Purified	Santa Cruz Biotec	60 min at RT
anti-KTR19	Purified	Novus Biologicals	60 min at RT
anti-Albumin	Purified	Santa Cruz Biotec	60 min at RT
anti-Transferrin	Purified	Abcam	60 min at RT
anti-HNF4α	Purified	Abcam	60 min at RT
anti-TTR	Purified	Thermo Fisher Scientific	60 min at RT

Secondary antibody

Name	Conjugation	Brand	Incubation conditions
Goat anti-rabbit	Alexa Fluor488 or 546	Life Technologies	45 min at RT
Goat anti-mouse	Alexa Fluor488 or 546	Life Technologies	45 min at RT

Supplementary Table 3: Antibody used for flow cytometry

Primary antibody

Name	Conjugation	Brand	Incubation conditions
anti-rat CD24	PE	MiltenyiBiotec	30 min on ice
anti-rat CD90.1	APC	MiltenyiBiotec	30 min on ice
anti-rat EpCAM	PE	Santa Cruz Biotech	30 min on ice
anti-rat Albumin	FITC	Abcam	30 min on ice
anti-rat KRT18	PE	Santa Cruz Biotech	30 min on ice
anti-rat KRT19	Alexa Fluor 488	Novus Biologicals	30 min on ice
anti-rat CD68	FITC	MiltenyiBiotec	30 min on ice
anti-alpha-SMA	Purified	Sigma Aldrich	30 min on ice
anti-Desmin	Purified	Abcam	30 min on ice
anti-Vimentin	Purified	Sigma Aldrich	30 min on ice

Secondary antibody

Name	Conjugation	Brand	Incubation conditions
Goat anti-rabbit	Alexa Fluor488 or 546	Life Technologies	30 min on ice
Goat anti-mouse	Alexa Fluor488 or 546	Life Technologies	30 min on ice

9. Discussion Aim 2

In the present work, we report the *in vitro* establishment and the functional characterization of two novel rat immortalized hepatocytic cell lines: RH and RNT. Both cell lines were obtained by perfusion of rat livers exposed to the Resistant-Hepatocyte protocol, in the presence or absence of carcinogenic treatment, and could be maintained in culture for more than 50 passages, without signs of senescence.

Both cell lines display features typical of hepatocytes as they are glycogen-positive, produce albumin and express KRT18, and are not contaminated by other cell types usually present in the liver, such as macrophages, stellate cells, and endothelial cells.

Obvious differences, however, exist between them, as RNT cells display a typical hepatocytic morphology, while RH cells show a fibroblastic shape, with signs of EMT. The presence of EMT is a critical event in the induction of cell motility and invasion, both in physiological conditions, like wound healing or development and in malignant transformation when tumor cells acquire invasive/metastatic properties. Indeed, vimentin, an intermediate filament protein characteristically upregulated in cells undergoing EMT (Dong et al., 2016; Vuoriluoto et al., 2011; Zhai et al., 2014), was only weakly expressed in RNT cells, while it was strongly up-regulated in RH cells, in agreement with their fibroblastic shape and increased metastatic ability.

The performed gene expression profiling showed not only that many of the evaluated genes were differentially expressed, but also that RNT cells display a profile like normal livers, while RH are very similar to HCCs.

Another relevant difference concerns the expression of KRT19 that was observed only in RH tumorigenic cells. In normal liver, hepatocytes express KRT8 and KRT18, whereas biliary epithelial cells express KRT7 and KRT19 (Moll et al., 1982; Strnad et al., 2008). The presence of KRT19+ hepatocytes has, however, been demonstrated in a subset of human HCCs characterized with the worst prognosis among all HCC subclasses, suggesting that KRT19 is a negative prognostic marker (Govaere et al., 2014; Kawai et al., 2015).

Moreover, in the Resistant- Hepatocyte model, although KRT19+ lesions represent a minority of the total preneoplastic lesions, most HCCs are KRT19+, further suggesting that KRT19+ preneoplastic hepatocytes preferentially progress to malignancy (Andersen et al., 2010; Petrelli et al., 2013). Furthermore, KRT19+ HCCs show significantly increased EMT features and expression of invasion-related molecules, suggesting that they are endowed with higher invasive ability, compared to KRT19- HCCs (Kawai et al., 2015).

Another important difference between the two cell lines is the presence of tumor stem-like cells only in the RH cell line. We have investigated three markers that have been associated to

T-ICs, namely CD90.1, CD24 and EpCAM (Ji and Wang, 2012; Yamashita et al., 2009; Yang et al., 2008). The CD90.1 (Thy-1) antigen is expressed in bone-marrow derived stem cells and hepatic stem/progenitor cells (both in adult and fetal livers, but not in adult hepatocytes; Dennis et al., 2007). In the liver, CD90.1 expression was preferentially found in poorly differentiated HCCs and associated with a poor prognosis (Herrera et al., 2006). Moreover, only CD90.1+ cells obtained from HCCs displayed a tumorigenic and metastatic capacity when injected into immunodeficient mice (Lázaro et al., 2003). The CD24 marker, which is overexpressed in various human malignancies (Kristiansen et al., 2003, 2002), was expressed at higher level in RH than in RNT cells. More interestingly, the CD90.1 marker was present only in RH cells, where it is co-expressed within the CD24+ population. In fact, both markers have been reported to be involved in CSC differentiation in HCC (Yamashita et al., 2013). Interestingly, only the CD24+ RH population (containing also CD90.1+ cells) was endowed with tumorigenic ability, while neither CD24- RH cells nor CD24+ RNT cells (that do not display CD90.1+), gave rise to tumors after subcutaneous implantation in mice.

A critical difference between the two cell lines is that only RH cells displayed the typical behavior of malignant transformed cells, as *in vitro* they grew in an anchorage-independent manner and did not show contact inhibition, while *in vivo* they were strongly tumorigenic and metastatic. Interestingly, RNT cells are immortalized but not tumorigenic. Thus, they represent a critical "normal" counterpart of RH transformed cells. As these two cell lines epitomize two steps in the natural history of tumor development, they can therefore be utilized to study the molecular mechanisms underlying tumor progression. Indeed, we have shown that NRF2 silencing in RH cells reverted their phenotype toward that of RNT cells, demonstrating that activation of the NRF2 pathway is required to maintain the transformed phenotype (Zavattari et al., 2015).

The exogenous expression of NRF2 or even of activated forms of this gene, however, did not promote the progression toward a transformed phenotype. On the other hand, RNT cells could be transformed by transduction with an oncogenic form of KRAS, thus proving their susceptibility to becoming tumorigenic. All together, these results suggest that NRF2 constitutive activation is not sufficient to promote the transformation of the immortalized liver cells. The analysis of differentially expressed and mutated genes in RH vs. RNT cells can thus help in the identification of the genes that complement NRF2 in promoting transformation. Exogenous expression/silencing of selected genes will reinforce the results obtained *in silico*.

In conclusion, the two cell lines described herein, represent a useful tool for investigating the molecular changes underlying hepatocyte transformation and to experimentally demonstrate their role in HCC development.

10. Conclusion

In conclusion, basic and translational research in the field of hepatology has rapidly advanced. Areas of inquiry into basic mechanisms of liver injury, repair, regeneration, fibrogenesis, viral infection, ischemia-reperfusion injury and oncogenesis, among others, are expanding into molecular and sub-molecular areas with accelerating speed.

The increase of the knowledges in cell therapy, especially in the liver field, indicates that we will obtain great results in the near future. The evidence that we have more and more cell and animal models that reproduce more precisely what happens in nature, improves the possibility to better understand the natural and pathological processes, but, in the meantime, these models need to be improved.

In the second part of this work, we described the isolation and characterization of two rat hepatocyte cell lines as tools for the *in vitro* studies. The cancer cell line, here described, represents a useful tool to investigate the molecular changes underlying hepatocyte transformation, and to demonstrate their role in HCC development, in contrast the normal cell line can help to study the changes that take place during the communication among the different cell types (hepatocytes, KCs, and LSECs) in the co-transplantation experiments, from cellular and molecular point of view.

11. References

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