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ANNUAL REPORT

LIVER REGENERATION AND MECHANISMS OF HEPATOPROTECTIONS IN ANIMAL MODELS

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Project aim/objectives:

Rationale

Aim 1. To investigate cell engraftment after liver cell co-transplantation of different cells combination.

Liver is maintained in a healthy state by tightly regulated interactions among liver cell types. Thus, mature cells transplantation, alone or in combination, could be useful to get information on their interactions and cell-cell communication, to find the best conditions to optimize the engraftment. Moreover, activation or destruction of the endothelium in combination with cells transplant are the keys necessary for cell engraftment.

Aim 2. To study the activity of two proteins, SNX5 and RGN up-regulated after ischemia-reperfusion injury, in an endothelial cell lines (MS1) and in an hepato-carcinoma (HCC) cell line (C1C7), respectively.

Proteomic studies on liver cells that have suffered from ischemia, showed that two proteins, Sorting Nexin 5 (SNX5) in liver endothelial cells and Regucalcin (RGN) in hepatocytes, were up-regulated when compared to others proteins, suggesting an hypothetical protective role in this context. To

evaluate and demonstrate their in vitro protective capacity two lentiviral vectors (LVs) carrying shRNAs were used to silence these proteins. Then, after optimizing the transduction, we choose the most functional shRNA and we tested cells for cell growth or mithocondrial activity by Crystal Violet and MTT assay, respectively.

Aim 3. To characterize a stable non tumorigenic hepatocyte (RNT) cell line and its malignant counterpart (RH) isolated by induced tumors:

Primary hepatocyte cultures are limited by a short-term survival, while transformed cell lines cannot be compared with their untransformed counterparts. In this aim I will characterize two rat hepatocyte cell lines as a promising tool to study liver carcinogenesis. Long-term stable cell lines were obtained by Prof. Columbano's group from an 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, not developing HCCs (RNT cells).

Background:

Liver

The liver is the largest internal organ of the body. Roughly 70% of the liver blood is coming from the hepatic portal vein, carrying venous blood drained from the spleen, gastrointestinal tract and its associated organs. Each lobule is made up of thousands of hepatic cells which are the basic metabolic cells, called hepatocytes, separated one from each other by large capillary spaces called "liver sinusoids". The liver has many functions, such as detoxification, metabolism, synthesis of lipoproteins and cholesterol, synthesis of plasma proteins, digestive functions, biotransformation of pharmaceuticals and vitamins, storage. It is composed by several cell types, divided into parenchymal cells and non-parenchymal cells. The various functions of the liver are carried out by the parenchymal cells, or hepatocytes, that make contact with blood in sinusoids, vascular channels lined with highly fenestrated endothelial cells, also called LSECs (Liver Sinusoidal Endothelial Cells). Sinusoids are populated with phagocytic Kupffer cells, the resident hepatic macrophages, that filter harmful substances, removing large amounts of debris and bacteria. The space between endothelium and hepatocytes is called the Space of Disse and it is populated by stellate cells, also known as fat-storing cells or Ito cells, that communicate with hepatocytes and modify the extracellular space by secretion of extracellular matrix. The liver has an exceptional regenerative capacity: 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, that can differentiate either into hepatocytes or cholangiocytes (the latter are the cells that line the bile ducts) (Bird et al., 2008; Venter J. 2015). The liver inability to perform its normal synthetic and metabolic function results in a pathological condition called liver failure; this dysfunction is due to several causes, such as cirrhosis, hepatitis, cancer, drug overdoses, metabolic and autoimmune disorders, chemical toxins and trauma. On the basis of the permanence of the injury, two different forms of liver failure are known, acute and chronic: for this reason, liver transplantation remains the only proven treatment modality 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); however, because of organ shortage and patient instability, other non traditional 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;

Perera et al., 2009; Rosenthal et al., 1997; Waelzlein et al., 2009). In addition, in the case of liver transplantation, the organ moved from donor to recipient, undergoes a period of ischemia, due to the closing of the vessels, until its replacement. After replacement, at the opening of the vessels also undergoes damage, even more dangerous: reperfusion injury. To overcome this is necessary to resort to different types of drugs. During a study of proteomics, on LSECs and hepatocytes, after ischemia and reperfusion injury, two proteins were noted (SNX5 and RGN) to be greatly increased in translation, meaning that maybe they could be produced for protection. The study of these proteins and their molecular or intracellular mechanisms, that lead to this increment, might help the liver to be more protected (Mandili G. et al., 2015).

Hepatocytes

Hepatocytes account for up to 80% 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, and 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, only about 1-2 times/year. However, as a consequence of particular conditions, such as two-third partial-hepatectomy (PH) or acute toxic liver injury, hepatocytes turnover become faster and the liver can regenerate within 1-2 weeks (in rodents), increasing up to ~ 1 month in larger animal and humans (Font-Burgada J, 2015). The hepatocytes produce serum albumin, fibrinogen, and the prothrombin group of clotting factors (except for Factor 3, 4). These liver cells have the ability to metabolize, detoxify and inactivate exogenous compounds such as drugs (drug metabolism) and insecticides, and endogenous compounds such as steroids. One of the detoxifying functions of hepatocytes is to modify ammonia into urea for excretion. 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, or cryopreserved by freezing, even though they are sensitive to freezing procedure. 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 A, 2015). Hepatocytes transplantation, in fact, is less invasive than liver transplantation and the cryopreservation of these cells increase the possibility of immediate availability for the treatment of fulminant liver failure. Anyway, in the case of acute or chronic liver disease, the aim of hepatocytes transplantation is double: to replace the tissue functions and to allow the engraftment of transplanted cells for repopulating liver. Hepatocytes have a short life in culture so cellular model would be very helpful for in vitro studies requiring hepatocytes. For example, it would 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, or to study the expression of microRNAs involved in disease onset and eventually to develop molecular therapies for de-adjustment of these microRNAs and looked for new markers.

Kupffer cells

Kupffer cells (KCs), also known as Kupffer-Browicz cells, are specialized macrophages located in the liver and constitute part of the reticuloendothelial system (RES). KCs represent approximately 80% of the tissue-fixed macrophages in the body and account for 5-15% of all liver cells (Froh et al., 2003; Hijioka et al., 1992; Kolios et al., 2006). Their development begins in the bone marrow (BM) with the genesis of promonocytes and monoblasts into monocytes. Due to their localization in the liver sinusoids, KCs are the first barrier against antigens absorbed via the gastrointestinal tract and so these macrophages play a crucial role in identifying and detoxifying bacteria, endotoxins (Ruiter et al., 1981), apoptotic cells and immune complexes as well as toxic agents such as ethanol (Bilzer et al., 2006; Gregory et al.,1996). KCs have an essential role in maintaining liver homeostasis as well (Werner 2015). And represent an important component of the initial and rapid response to potentially dangerous stimuli, known as innate immunity. A recent study demonstrated

that hepatic macrophages derived from classical monocytes play a crucial role in promoting liver fibrosis, since mice with impaired this monocyte subset recruitment showed reduced hepatic stellate cells (HSCs) activation and diminished hepatic fibrosis (Karlmark et al., 2009). KCs mediate host resistance to infection releasing pro-inflammatory cytokines, such as IL-1, IL-6, IL-12, IL-18 and TNFα: IL-12 and IL-18 activate NK cells to produce anti-viral INFγ; TNFα promotes the neutrophilic granulocytes infiltration to eliminate bacteria and induces apoptosis in hepatocytes under pathological conditions (Schumann and Tiegs, 1999; Tiegs and Lohse, 2009). Although activated Kupffer cells 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 TGF-β (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 (Knolle et al., 1995), contributing to the ability of intrahepatic cells to induce tolerance (Tiegs and Lohse, 2009). Several studies reported the ability of KCs to induce tolerance in vivo after orthotopic liver transplantation (Chen et al., 2008; Liu et al., 2007) and the ability of these cells to inhibit DC-induced antigen-specific T cell activation in vitro (You et al., 2008). Furthermore, depletion of KCs compartment after gadolinium injection impairs the generation of systemic immune tolerance following portal injection of alloantigen 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., 2005a; Follenzi et al., 2008; Fontana et al., 2002; Gupta et al., 1999; Gupta et al., 1995), this raises the possibility that KCs can be replaced as well. Despite their long life span, KCs seem to have limited possibilities in therapy because their low or absent proliferating capabilities, but at the same time, their capacity of producing anti-inflammatory cytokines could promote the engraftment of other cells in co-transplantation.

Liver Sinusoidal Endothelial Cells

The blood vessels are the part of the circulatory system that transport blood throughout the body. Endothelial cells are a source of physiologically important molecules participating in 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). ECs also synthesize and secrete plasminogen activator and inhibitors, molecules regulating the growth of other cells and bind lipoproteins and hormones. Consequently, ECs are 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). Among vessels, the liver sinusoid is a specific capillary network system where a variety of metabolic substances are exchanged between hepatic blood flow and hepatic parenchymal cells. Liver sinusoidal endothelial cells (LSECs) comprise approximately 50 % of the non-parenchymal hepatic cells. These cells line the hepatic sinusoids, separating hepatocytes from the circulating blood and playing an important role in hepatic microcirculation (Oda et al., 2003). LSECs differ from endothelial cells (ECs) facing the blood vessels of other tissues for both structure and function: these cells lack a basement membrane and form a fenestrated monolayer organized in sieve plates, wich are about 0,1 μm in diameter (Aird, 2007; Braet and Wisse, 2002). 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. A particular aspect of liver sinusoids is the presence of resident hepatic macrophages, Kupffer cells (KCs), on the luminal side of the endothelium. LSECs and KCs constitute the most powerful scavenger system in the body (Enomoto et al., 2004; Smedsrod et al., 1990), they remove molecules via endocytosis (LSECs) or phagocytosis (KCs), contributing to the liver physiology and pathology (Aird, 2007; Carpenter et al., 2005). 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), acting in a paracrine manner to induce hepatocyte proliferation (LeCouter et al., 2003). Moreover, LSECs

synthesize and release Factor VIII, a critical co-factor in the intrinsic coagulation pathway (Do et al., 1999; Follenzi et al., 2008). Another relevant role of LSECs is connected to the immunity: in fact, they express major histocompatibility complex (MHC) class I and II molecules and costimulatory molecules CD40, CD80, CD86 and, 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). Studies demonstrated the possibility to isolate murine LSECs obtaining LSEC-enriched or high purified LSEC fractions from hepatic non-parenchymal cells (NPC) (Benten et al., 2005a; Follenzi et al., 2008; Kumaran et al., 2005); these studies demonstrated that LSECs can be transplanted and can engraft in recipient mice. Moreover, the therapeutic potential of these cells was demonstrated: since LSECs express factor (F) VIII of coagulation (Do et al., 1999; Follenzi et al., 2008; Kumaran et al., 2005), it has been demonstrated that they can correct the bleeding phenotype of Hemophilia A mice in cell therapy approaches (Follenzi et al., 2008; Kumaran et al., 2005). Thus, the opportunity to isolate and transplant liver ECs together with their ability in modulating immune tolerance to exogenous antigens open 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.

Experimental plan and methods:

Aim 1:
To investigate cells engraftment, initially donor DPPIV- mice (11 weeks old) were injected with $5x10^8$ TU of a LV containing the GFP under the ubiquitous PGK promoter (DPPIV-GFP). One week later, the recipients DPPIV- mice (9 - 11 weeks old) were treated with monocrotaline (MCT) 24h prior to surgery. (MCT is a pyrrolizidine alkaloid –PA- plant toxin that causes hepatotoxicity in humans and animals; intraperitoneal injection i.p. of 200 mg/kg MCT in mice produced time-dependent hepatic parenchymal cell HPC injury beginning at 12 h).

a) **Liver perfusion:** Mouse liver was perfused at 5 ml/min via portal vein for 15 minutes with buffer at 37°C containing 1.9 mg/ml EGTA, for 2 minutes with buffer lacking EGTA, and for 7–9 minutes with buffer containing 0.03% (w/v) collagenase and 5 mM $CaCl_2 2H_2O$. 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 (Sigma-Aldrich; collagenase from Worthington Biochemical Corp.).

b) **Cells isolation:** The liver was dissociated in perfusion buffer and cells were passed through Dacron fabric with 80-μm pores. Hepatocytes and LSECs were isolated from C57Bl/6-wt while KCs and LSECs were isolated from DPPIV-GFP mice. Hepatocytes were obtained by differential centrifugation (500 rpm for 5 minutes), LSECs by immunomagnetic positive selection from NPCs (non parenchimal cells) with an anti-CD146 antibody, and finally KCs were obtained by co-incubation with anti-CD11b and anti-F4/80 antibody.

c) **Transplant:** Mice were anesthetized with 5% isoflurane and maintained on 1.5% isoflurane anaesthesia during surgery. The portal vein was exposed by laparotomy, and injected with 1×10^6LSECs or $1x10^6$ KCs GFP+ or $1x10^6$ hepatocytes for the transplant group while for 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 injection site.

d) **Immunofluorescence:** After one week mice were killed and livers were sampled and fixed in PBS-Paraformaldeide (PAF 4%), than equilibrated in sucrose 30%, embedded in killik and conserved at -80°. Fixed samples were cryostat sectioned 4 μM thickness, incubated 5 minutes in PAF, rinsed in PBS, blocked in buffer containing 5% goat serum, 1% BSA, and 0.1% Triton X-100 in PBS and incubated with rabbit anti-GFP (1:300; Life Technologies) and with rat anti-mouse F4/80 (1:500; Serotec). Images were taken with Leica DM5500 microscope and analysed with LasX Software. All conditions were performed on two mice and then, two lobes each were analysed. For a statistical analysis, positive cells were counted in twenty fields.

e) **DPPIV staining:** Fresh samples, for DPPIV staining, were frozen directly in killik (OCT) and conserved at -80°. Samples were cryostat sectioned of 4 μM thickness, fixed in aceton-chloroform 1:1 for five minutes, incubated with DPPIV staining solution and countercolored with toluidine blue. Images were taken by Nikon microscope or Pannoramic Digital slide scanner and analysed through Pannoramic Viewer software 1.15.4 (3DHISTECH Ltd).

Aim 2:

MS1 and C1C7 cell lines were transduced with five different Lentiviral vectors leading shRNAs for Sorting Nexin5 or Regucalcin, respectively. Firstly to choose the best performing shRNA, cells were transduced with serial dilutions of each construct (1/2, 1:10, 1:100); then, as puromycin resistance was present in the expression cassette, we treated cells with 1,5μg/ml or 2 μg/ml (MS1 and C1C7 cell lines respectively) to select only the transduced ones.

a) **Western blot analysis:** Western blot analysis was performed on transduced cells and constructs appointed as sh98 (SNX5) and sh35 (RGN) were chosen for further experiments. Initially, western blot analysis showed only a partial reduction in transcription and both proteins were still present; it was than necessary to repeat the transduction in order to obtain a complete silencing of the two proteins. Lysates from untreated cells were used as a control.

b) **Mitochondrial activity:** These cells were tested for mitochondrial activity through MTT assay in low and normal level of FBS (2% and 10%), and in presence or absence of hydrogen peroxide. We choose a 24h time point for the H_2O_2 assay, while FBS reduction was carried on for 24, 48 and 72 hours to evaluate if any difference between direct and long-time protection might occur.

c) **Growth curve:** Cells growth was evaluated by Crystal violet assay on cells in presence or absence of hydrogen peroxide and with or without an A2aR receptor agonist, CGS21680 (CGS). All the experiment's conditions were performed in normal oxygen and in hypoxia. To generate an hypoxic condition in cultured plates, all the wells were filled to the top and plates were sealed to avoid the exchange of oxygen.

Aim 3:

To characterize a stable non tumorigenic hepatocyte (RNT) cell line and its malignant counterpart (RH) isolated from induced tumours, different assays were performed in order to assess the presence of specific hepatic markers

a) **Immunofluorescence staining for Tie-2 and Desmin:** Cells were seeded and cultured on 1cmØ cover glass, fixed in PAF 4%, permeabilized and blocked in buffer containing 5% goat serum, 1% BSA, and 0.1% Triton X-100 in PBS, and incubated with a polyclonal antibody anti Tie-2 (Santa Cruz) and a polyclonal antibody anti-desmin (AbCam). Cells were then incubated with Alexa Fluor 488-conjugated (Green) and DAPI-Antifade for nuclear staining (Sigma Aldricth). Peritoneal macrophages and C2C12 (myoblast cell line) were used as a negative control, respectively. Images were taken with Leica DM5500 microscope and analysed with LasX Software.

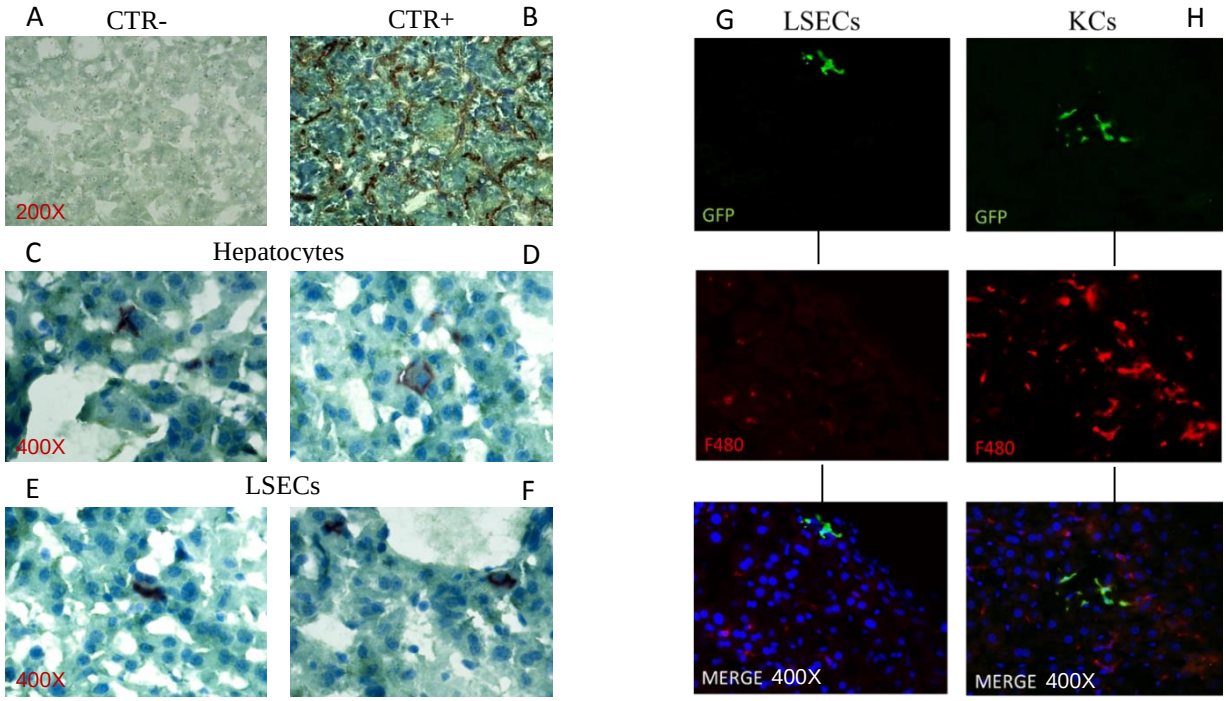
b) **Hepatocytes characterization by RT-PCR:** Cells were lysed in trizol (5 Prime), RNA extracted and treated with DNase I (RNase-free DNase; Qiagen) and retro-transcribed (Maxima H Minus Reverse Transcriptase, Thermo Scientific™). RT-PCR was performed using primers for Ceruloplasmin (For: ATGTGATGGCTATGGGCAATG, Rev: AGTTACCATCCCCGCATGAA), FVIII (For: GACGACGATGCTGTCACGG TG, Rev: GGCTGGAGTAGAAGGAGTAGG) and Albumin (For: AAGGCTGCCGA CAAGGATAA, Rev: TTGCGGCACAGAGAAAAGAA) as hepatocyte markers. RNA extracted from wild type rat liver was used as positive control.

c) **Cell markers characterization by cytofluorimetric analysis:** Cells were detached, resuspended in staining buffer and incubated with different antibodies: CK18 (hepatic receptors for thrombin-antithrombin "TAT" complexes), Albumin, EpCam (Epithelial cell adhesion molecule) e CD24 (heat stable antigen, cell adhesion molecule). Two negative markers represented by CD90.1 (thymocyte antigen, Thy-1: Oval and stellate cells) and CD68 (glycoprotein which binds to low density lipoprotein, Macrophages) were used. For each sample $2x10^5$ events were acquired by BD FACSCalibur™. Data were analysed by WinMDI.

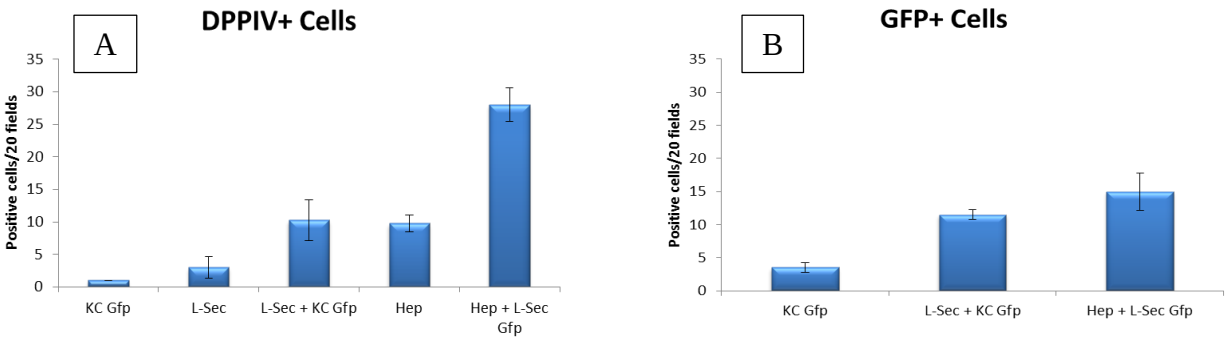
d) **Cell transduction LV-GFP under the control of four promoters:** Cells were transduced with MOI (multiplicity of infection) = 0.5, with lentiviral vectors carrying GFP under the control of different promoters: the endothelial specific VE Cadherin promoter (VEC), the myeloid cells specific promoter, (Integrin alpha M, ITGAM or CD11b), an ubiquitous promoter, Phosphoglycerate kinase (PGK) and the hepatocyte specific promoter, Transtiretin (TTR). In this way it was possible to verify the activity of a specific promoter for hepatocytes (TTR) and compare it with a positive control (PGK) and two non-specific (CD11b and VEC) or "negative" controls. For each sample $2x10^5$ events were acquired by BD FACSCalibur™. Data were analysed by WinMDI.

Results

Aim 1. To investigate cell engraftment after liver cell co-transplantation of different cells combination.



DPPIV and immunofluorescence staining: Representative images for both stainings. DPPIV staining represents a good tool to quantify cells that made engraftment in DPPIV- liver after transplantation. Some positive cells are detectable in slices showed: A) liver from DPPIV+ mice used as a positive control. B) liver from DPPIV- mice, untreated, used as a negative control. C-D) Examples of engraftment of hepatocytes wt, and E-F) LSECs wt within the liver. As the KCs did not express DPPIV, the transplant combination of LSECs and KCs was analysed by immunofluorescence staining for the GFP, that allows us to identify and quantify the cells that made engraftment. G) LSECs in immunofluorescence did not show co-staining between GFP and F4/80, demonstrating that engraftment did occur without interference by macrophages; H) transplanted KCs that are positive for F4/80 showed co-staining with GFP, demonstrating the specific isolation and transplant of the cells.

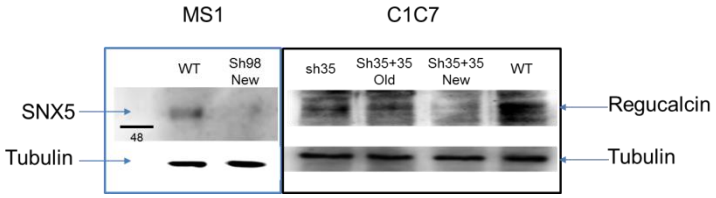


Analysis of DPPIV and immunofluorescence staining: analysis was performed counting 20 fields per slice, for each of the two lobes analyzed, for each mouse. A) KCs (macrophages) did not express the DPPIV, so that one who was represented graphically, probably is derived from a

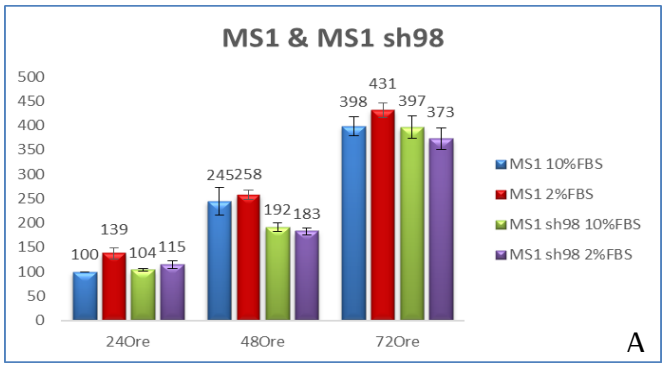
contamination of LSECs during their isolation. Co-transplantation of LSECs wt and KCs showed LSECs engraftment three fold higher than LSECs alone. The same happened with co-transplantation of Hepatocytes wt and LSECs GFP+, where the engraftment was again three fold higher instead of hepatocytes alone, nine fold up LSECs and more than two fold higher of them taken together. B) Moreover, IF analysis showed that co-transplantation of LSECs wt and KCs was three fold up of only KCs. Co-transplantation of Hepatocytes wt and LSECs GFP+ increases engraftment of the LSECs when compared with the graph for DPPIV staining.

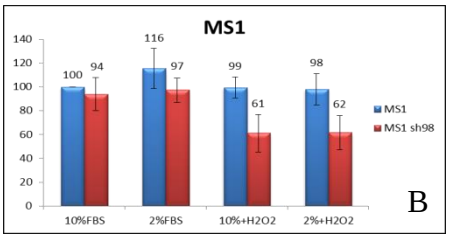
Aim 2

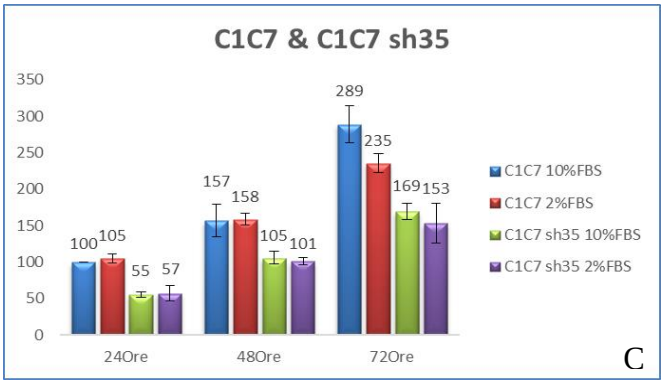
To study the activity of two proteins, SNX5 and RGN, in endothelial cell lines (MS1) and hepato-carcinoma (HCC) cell lines (C1C7), respectively

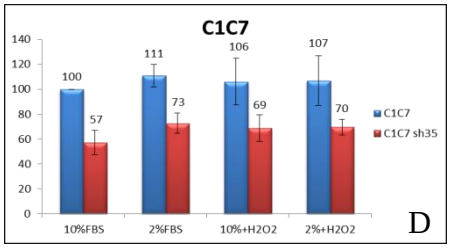


Western blot analysis: Cells were transduced with serial dilution of each construct and, based on the first western blot analysis, the best performing shRNAs were chosen, resulting in shRNA 98 on MS1 and shRNA 35 (both at dilution 1:2) Although they worked well, silencing was only partial, so it was necessary to transduce cells again. The following western blots, finally showed the almost complete silencing of the expression of SNX5 in MS1 cell lines and RGN in C1C7 cell lines.



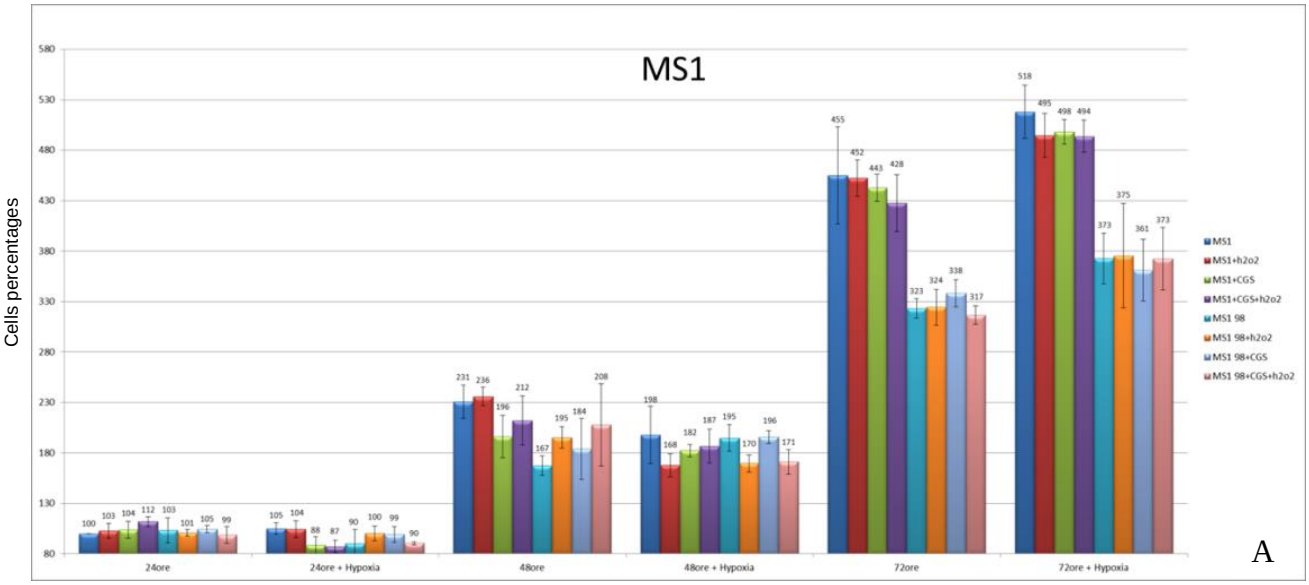


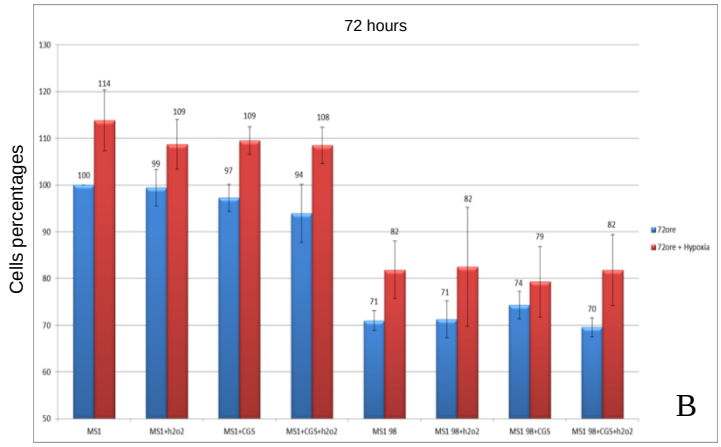


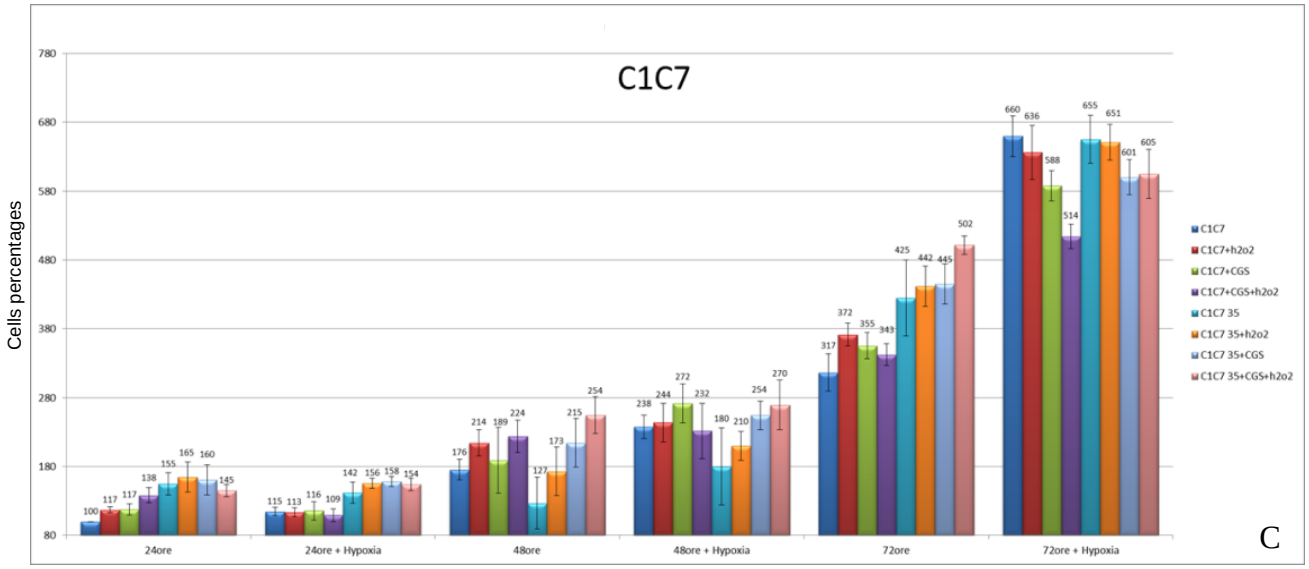


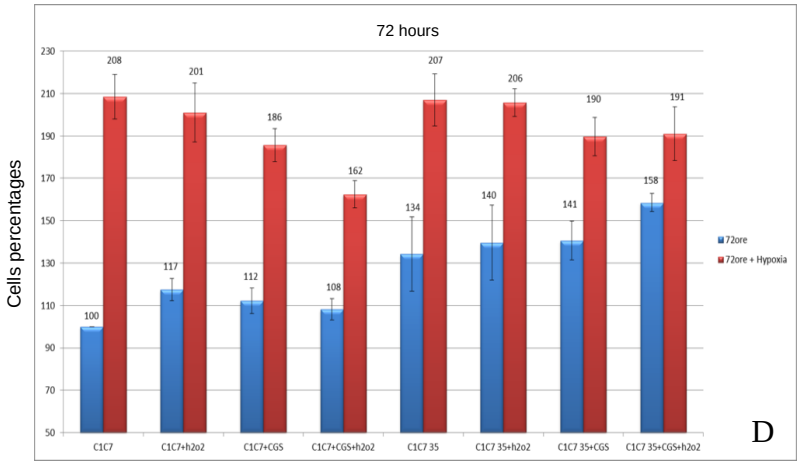
Mitochondrial activity: To check if the proteins provide protection on endothelial and hepatocytes cell lines, we performed MTT assays. A) MS1 cells did not show any statistic difference between normal and low serum concentration, nor between transduced and untransduced cells, in all the time

points. B) At 24 hours, when cells were treated with hydrogen peroxide, the absence of SNX5 resulted in a reduction of mitochondrial activity (~40%). C) in C1C7 cells, despite a reduction of their mitochondrial activity at all the time points (~50% at 24 and 48 hours, ~60% at 72 hours), D) when treated with hydrogen peroxide, no differences did occur.





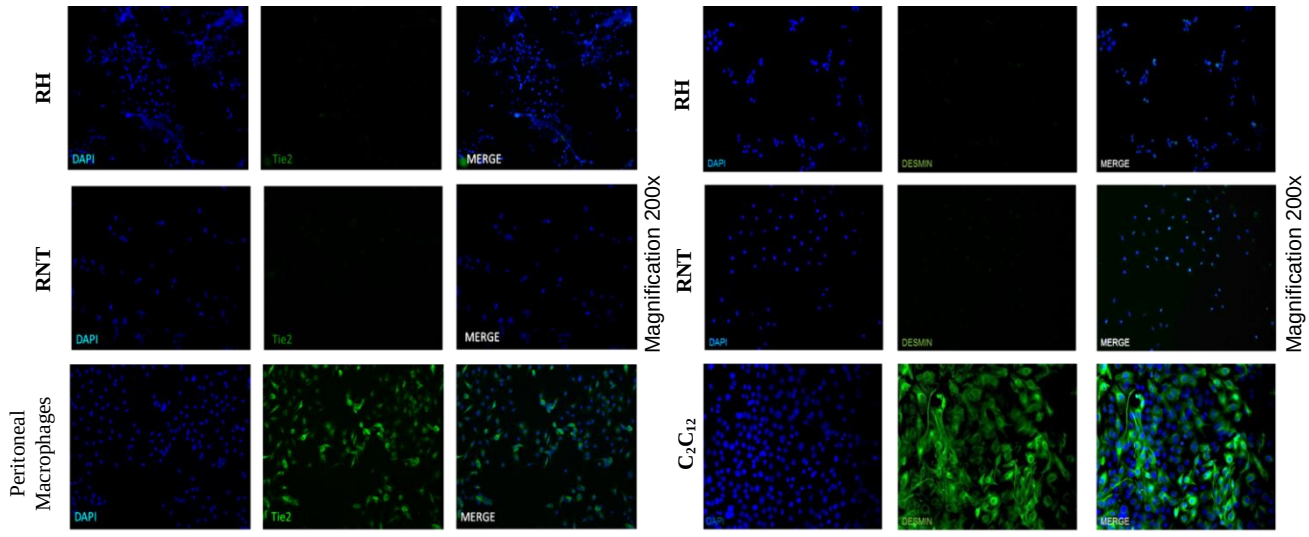




Growth curve: Crystal Violet assay on MS1 cells (A,B) at 72 hours showed a growth reduction in cells transduced but no difference was revealed among the treatments; as expected from endothelial cells, they grew better in hypoxic conditions, both transduced or non-transduced. (C, D) C1C7 cell line, again, did not show significant reduction in growth in any case.

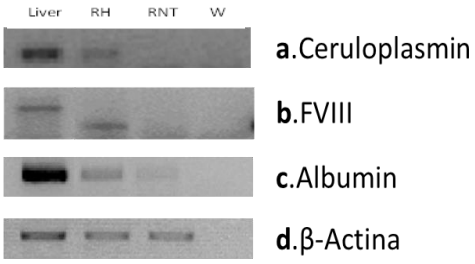
Aim 3.

To characterize a stable non tumorigenic hepatocyte (RNT) cell line and its malignant counterpart (RH) isolated by induced tumors.



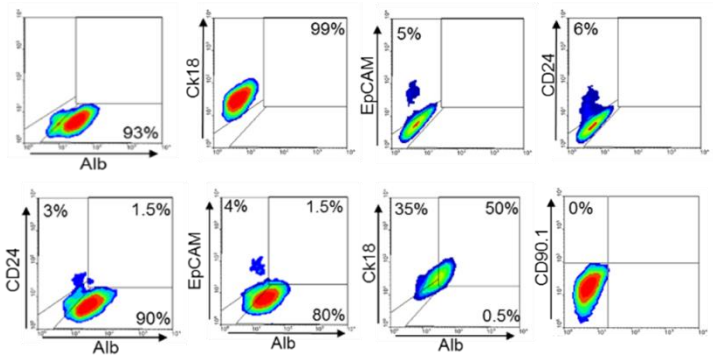
Immunofluorescence staining for Tie-2 and Desmin: We performed immunofluorescence staining for desmin, a marker for hepatic stellate cells and intermediate filaments in cardiac muscle, skeletal muscle and smooth muscle tissue. Our cells were negative instead the control with C_2C_{12} cells espressed desmin. Tie-2 is a receptor tyrosine kinase of the Tie family, receptor for angiopoietin 1, It is expressed almost exclusively in endothelial cells, in a fraction of monocytes and hematopoietic stem cells. Immunofluorescent staining against this receptor in figure 5 showed

that both cultured RNT and RH cells did not express this endothelial marker. Instead peritoneal macrophages resulted positive for Tie-2.

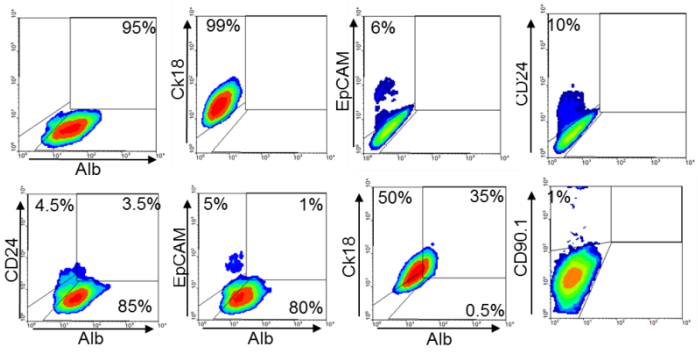


Hepatocytes characterization by RT-PCR: RNA was isolated from cultured RH and RNT cells and analyzed for gene expression by RT-PCR. RNA from rat liver was used as positive control. This analysis revealed that: a) RNT cells did not express both Ceruloplasmin and FVIII**.** On the contrary, RH cells showed a lower expression of either Ceruloplasmin and a low molecular weight isoform of FVIII.

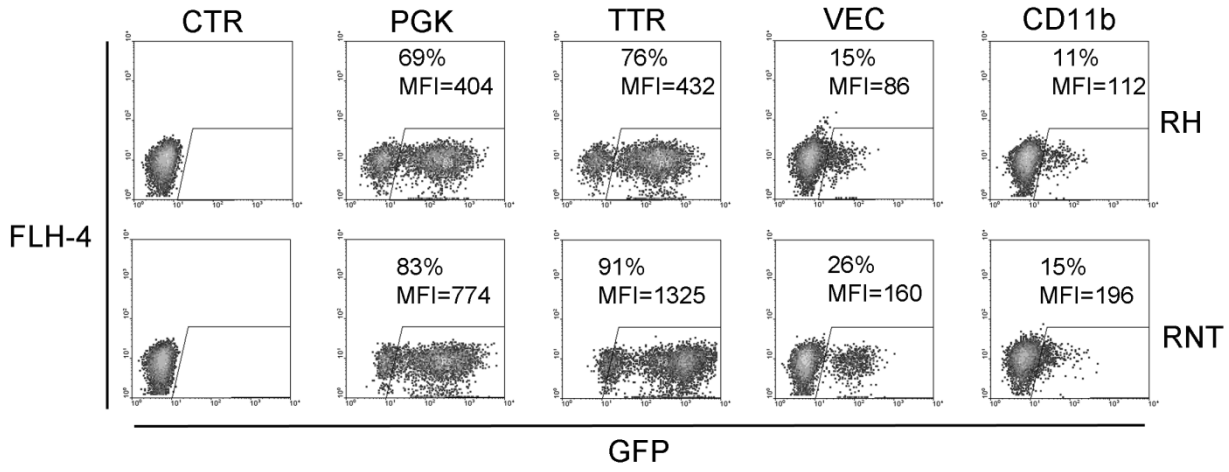
RNT



RH



Cell markers were analysed by cytofluorimetric analysis: Cultured RNT and RH cells were characterized by FACS analysis for the expression of different markers (hepatic and not). Both cell types were positive for albumin (90% RNT, 85% RH) and cytokeratin-18 (99%). A small percentage of these two cell types expressed EpCAM (RNT 18% and RH 9%) and CD24 (RNT 17% and RH 21%). Both cell types co-expressed albumin and cytokeratin-18 (RNT 50% and RH 35%), while the EpCAM+ and CD24+ cells did not co-express albumin. All these data indicated that cultured RNT and RH cells maintained the expression of hepatic markers and no contaminating cells, such as macrophages or stellate cells, were present.



The two cell types were transduced with LV-GFP under the control of four promoters: Since transtiretin is a protein secreted by hepatocytes, TTR is a promoter specific for hepatocytes. As expected, at MOI 0.5, cultured RH and RNT cells, showed an higher percentage of GFP positive cells, compared to cells transduced with lentiviral vector containing PGK promoter, our positive control (respectively 76% and 91% for TTR, 69% and 83% for PGK); on the contrary, other promoters showed low level of GFP expression. Cell lines specific for each promoter were used as control: C1C7 (HCC) for TTR – promoter, MS1 (endothelial) for VEC - promoter and U937 (Lymphocytes) for CD11b – promoter. (data not show).

Discussion

1) LSECs and KCs GFP+ or hepatocytes were isolated and different transplant or co-transplantation were performed in DPP IV- mice, in order to study the engraftment after 7 days and to define the best combination for our aims. Data obtained showed that co-transplantation had the best outcome if compared to the single cell type and, moreover, the presence of hepatocytes would seem to be necessary in all the situations because they are able to engraft three times more than other cells in the liver. In addition, LSECs and KCs together reach the same level of engraftment, but it remains lower than co-transplantation of LSECs + hepatocytes. These preliminary results represent the basis to further investigate relations between cells transplanted in a whole organ: to date, new experiments including more conditions i.e. longer end-point time (up to three weeks) and transplantation of complete non-parenchymal cells, alone or in combinations with other cell types, are ongoing.

2) Previous proteomic studies on liver cells that have suffered from ischemia-reperfusion injury showed that two proteins, Sorting Nexin 5 (SNX5) in liver endothelial cells and Regucalcin (RGN) in hepatocytes, were upregulated when compared to others. We generated lentiviral vectors with the specific shRNAs sequences for the two proteins and we tested different constructs in order to obtain the complete protein silencing through transduction of the endothelial cell line MS1 and the HCC cell line C1C7, to study respectively SNX5 and RGN. Western blots and MTT assays were performed to assess the silencing and to verify the integrity of mitochondria, revealing a possible involvement of these two proteins in the apoptosis. Results obtained showed that MS1 cells silenced for SNX5 did not vary their mitochondrial activity, nor in the case of lower serum conditions; in addition, the treatments with CGS, hydrogen peroxide or hypoxia did not affect cell growth of MS1 wt, while silenced cells showed a reduction of about 25% compared to the controls. Similarly, C1C7 did not suffer from lower serum presence, but silencing of RGN led to a strong general decrease of 45% in vitality when compared to untreated cells. Summarizing,

all these data, resulted in a reduction of cell growth when the proteins are silenced but, interestingly, in both cell lines, cell vitality seems to increase in hypoxic conditions. Next step would be to silence the SNX5 and RGN proteins directly in vivo.

3) Long-term stable cell lines were obtained by Prof. Columbano's group from an 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, not developing HCCs (RNT). Characterization under different point of view was carried on. The absence of muscle or endothelial cells contaminants was assessed by immunofluorescence staining, that resulted, as expected, negative for Desmin and Tie2 respectively. The transcription of other possible markers has been verified via RT-PCR, such as albumin, FVIII and ceruloplasmin.
Later, surface markers typical of hepatocytes and controls have been verified by FACS. Cells, as expected from hepatocytes, showed high level of CK18 e Albumin. RNT were negative for stem cell marker while RH that were, isolated from tumor, expressed stem cell marker (CD90.1) but not more than 1%. EpCAM is a marker for Hepatocytes/progenitor, this may represent aberrant EpCAM expression in injured hepatocytes or, as hypothesize, persistence of EpCAM in hepatocytes derived recently from precursors (Yoon SM1, et al. 2011). Taken together, these data confirmed their hepatocytes-derivation and they could be candidates, as an excellent models of study, such as tumor and non-tumor rat hepatocytes. With these properties, the cells could be used for studies of toxicity or selectivity of drugs for apoptosis and necrosis of tumor cells, or analysis of drugs to block proliferation of cancer cells. Moreover, they could be used as a model to carry out studies on the expression of microRNAs related to the development of the disease, and consequently, studies aimed at finding new markers or molecular therapies directed to deregulate these microRNAs.

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Poster and Congress

Publication (articles, poster, abstract)	**EASL 50th The International Liver Congress 2015.** April 22-26, Vienna, Austria. **<u>Poster Presentation</u>** **"Altering Transplanted Cell Engraftment and Proliferation in the Liver through Paracrine Signaling with Cotransplantation of Liver Sinusoidal Endothelial Cells and Hepatocytes in Mice"** Simone Merlin, **Kevin Bellofatto**, Ekaterine Berishvili, Brigid Joseph, Ralph Badhe, Sanjeev Gupta, Antonia Follenzi.
Congress partecipation	**Candiolo Cancer Institute** June 6th-7th, 2015, Candiolo, TO **"From Signal Transduction to Cancer Precision Medicine".** Ido Amit - Weizmann Institute of Science, Laboratory for Immuno-Genomics, Rehovot (Israel), Alberto Bardelli - Candiolo Cancer Institute and University of Turin School of Medicine, Candiolo (Italy), Walter Birchmeier - Max Delbrück Center for Molecular Medicine (MDC), Berlin (Germany), Johanna Chiche - Centre Méditerranéen de Médecine Moléculaire, INSERM U1065, Nice (France), Paolo Comoglio - Candiolo Cancer Institute and University of Turin School of Medicine, Candiolo (Italy), Michele De Palma - The Swiss Institute for Experimental Cancer Research (ISREC), Lausanne (Switzerland), Annika Fendler - Max Delbrück Center for Molecular Medicine (MDC), Berlin (Germany), Douglas Hanahan - Swiss Institute for Experimental Cancer Research, ISREC, EPFL, Lausanne (Switzerland), Carl Henrik Heldin - Ludwig Institute for Cancer Research, Uppsala Branch, Uppsala (Sweden), David Livingston - Harvard Medical School, Dana-Farber Cancer Institute, Boston (USA), Daniel Louvard - Institut Curie, Paris (France), Stefano Piccolo - University of Padua, Department of Molecular Medicine, Padua (Italy), Kristian Pietras - Lund University, Division of Translational Cancer Research, Lund (Sweden), Jacques Pouysségur - Institute of Research on Cancer and Aging (IRCAN), Nice (France), Tuomas Tammela - Massachusetts Institute of Technology, Koch Institute for Integrative Cancer Research, Cambridge (USA), Livio Trusolino - Candiolo Cancer Institute and University of Turin School of Medicine, Candiolo (Italy), Yosef Yarden - Weizmann Institute of Science, Department of Biological Regulation, Rehovot (Israel)

Lessons

- **"Tissue engineering: the state of the art"** – 14 November 2014 – Dott.ssa Francesca Boccafoschi - Department of Health Sciences, Università del Piemonte Orientale.
- **"Regenerative Medicine"** – 21 November 2014 – Prof. Maria Prat - Department of Health Sciences, Università del Piemonte Orientale.
- **"Ribosomopathies"** – 25th May 2015 – Prof. Steve Ellis – Medical School, University of Louisville (Kentucky)
- **"Basis of scientific research"** – 10th June 2015 – Prof. Nicoletta Filigheddu – Università del Piemonte Orientale.

Attended seminars

1. **"Dysregulated antigen receptor signaling: molecular lessons from two congenital lymphoproliferative disorders"** – 06 November 2014 - Prof. Andrew L. Snow - Department of Pharmacology Uniformed Services University of the Health Sciences Bethesda (Maryland, USA).
2. **"Optical coherence tomography from bench to bedside shening the light during percutaneous vascular intervention"** – 17 November 2014 - Dott. Secco Gioel Gabrio – Department of Health Sciences, University of Eastern Piedmont.
3. **"La scoperta del bosone di Higgs"** – 25 November 2014 - Dott. Roberta Arcidiacono - DiSCAFF, University of Eastern Piedmont - Dott. Marta Ruspa - Department of Health Sciences, University of Eastern Piedmont.
4. **"Nuove sfide ed opportunità dell'epidemiologia molecolare per lo studio dei tumori"** – 27 November 2014 - Prof. Laura Baglietto - Inserm - Centre for Research in Epidemiology and Population Health, Unit: Nutrition, Hormones and Women's Health, Paris.
5. **"Humoral responses to HCV infection and clinical outcomes"** – 28 November 2014 - Dott. Arvind Patel - Programme Leader, MRC Centre for Virus Research, University of Glasgow (UK).
6. **"Uncovering the role of β-HPV in field cancerization: a collaboration in progress"** – 4 December 2014 – Dott. Girish Patel - European Cancer Stem Cell Research Institute, Cardiff (UK).
7. **"Focus on the liver: from basics of NAFLD to hot topics in HBV & HCV infections"** – 5 December 2014 – Prof. Rifaat Safadi M.D, Chairman of the Israeli Association for the Study of Liver. Director of Liver Unit. Institute of Gastroenterology and Liver Diseases. Division of Medicine. Hadassah Medical Organization, Hadassah Hebrew University Medical Center, Jerusalem.
8. **"From the legend of Prometheus to regenerative medicine"** – 16 December 2014 – Prof. Antonio Musarò, DAHFMO-Unit of Histology and Medical Embryology Sapienza University of Rome

9. **"Microglia microvescicles: messengers from the diseased brain"** – 17 December 2014 - Dott. Roberto Furlan, San Raffaele University, Milan.
10. **"Anticancer strategy Targeting cancer cell metabolism in ovarian cancer"** – 19 January 2015 - Prof. Dr Yong-Sang Song, MD, PhD Director Cancer Research Institute, Gynecologic Oncology Chariman, Cancer Biology Interdisciplinary Program Professor, Obstetrics and Gynecology, College of Medicine Seoul National University.
11. **"Different molecular mechanisms regulate hepatocyte differentiation during the transitions between epithelial and mesenchymal states"** – 20 January 2015 - Dott. Tonino Alonzi, PhD, Lab. Of Gene Expression and Experimental Hepatology, Istituto Nazionale per le Malattie Infettive "L. Spallanzani" IRCCS, Rome.
12. **"Targeting the liver to cure myocarditis: a lesson from a model of STAT3-dependent auto-immune myocarditis"** – 21 January 2015 - Prof. Valeria Poli - Department of Molecular Biotechnology and Health Sciences, Molecular Biotechnology Center, University of Turin.
13. **"Myeloid cells as therapeutic target in cancer"** – 27 January 2015 - Prof. Antonio Sica - DiSCAFF, UPO, Novara.
14. **"Proof of principle for cell therapy: from autologous transplantation of tissue specific progenitors to gene corrected patient specific injured pluripotent stem cells"** – 11 March 2015 – Prof. Darko Bosnakovski - Associate Professor, University "Goce Delcev" Stip, Faculty of Medical Sciences, Krste Misirkov bb, 2000 Stip R. Macedonia.
15. **"Signal control in iNKT cell development and function"** – 09 April 2015 - Prof. Xiaoping Zhong, MD, PhD - Associate Professor, Department of Pediatrics-Allergy and Immunology Duke University, Medical Center, Durham (North Carolina, USA).
16. **"Actin-based mechanisms in the control of gene expression and cell fate"** – 21st April 2015 – Prof. Piergiorgio Percipalle – Associate Professor, Department of Cell and Molecular Biology, Karolinska Institutet (Solns, Sweden).
17. **"An integrated approach to the diagnosis and treatment of ovarian cancer"** – 7th May 2015 – Prof. John McDonald, MD, PhD – Integrated Cancer Research Center, School of Biology and Parker H. Petit Institute of Bioengineering and Bioscience, Georgia Institute of Technology, Georgia Tech University, Georgia (Atlanta, USA).
18. **"Conflicting interests and scientific communication"** – 14th May 2015 – Prof. Kathleen Ruff – RightOnCanada Founder, Senior Advisor to the Rideau Institute (Ottawa, Canada).
19. Workshop on **"Stem cells in biomedical research"** – 22 May 2015 – Prof. Antonia Follenzi - Department of Health Sciences, Prof. Mariagrazia Grilli – Department of Pharmaceutical Sciences, Prof. Michela Bosetti - Department of Pharmaceutical Sciences, Prof. Giuliana Pelicci- Department of Traslational Medicine (Università del Piemonte Orientale), Prof. Antonella Consiglio – (Institute For Research on Biomedicine IBUB, University of Barcelona, Prof. Patrizia Dell'Era (Department of Molecular and Traslational Medicine, Università di Brascia), Prof. Letizia Mazzini (Centro Esperto SLA, Clinica Neurologica, AOU Maggiore Della Carità,Novara)
20. **"Recent developments in (cutaneous) Human Polyomavirus research"** – 5th June 2015 – Mariet C.W. Feltkamp – Associate Professor of Medical Virology, Department of Medical Microbiology, Leiden University Medical Center (Leiden, The Netherlands).
21. **"High-tech product preservation and operator protection: two apparently opposite requirements in different fields of medicine and biotechnology: the emerging glove box approach"** – 15th July 2015 - Dr. Ing. Marco Fatta, Phd – COMECER Group (Italy).

22. **“Le cellule staminali nel danno renale acuto e nel trapianto di rene”** – 28th July 2015 - Dr. Vincenzo Cantaluppi, MD – Facoltà di Medicina e Chirurgia, Università di Torino (Italy).
23. Miniworkshop on **“Biotechnology for Dermatology”** – 9th July 2015 - Dr Gwenaël ROLIN, PhD - Clinical Research Engineer - Thomas LIHOREAU - Ingénieur hospitalier, Research and Studies Center on the Integument (CERT), Department of Dermatology, Clinical Investigation Center (CIC INSERM 1431), Besançon University Hospital; INSERM UMR1098, FED4234 IBCT, University of Franche Comté, Besançon, France.
24. **“Cell based models for studying molecular mechanisms of Facioscapulohumeral Muscolar Distrophy (FSHD)” , “Toward animal model for Facioscapulohumeral Muscolar Distrophy (FSHD)”** – 3rd September 2015 - Prof. Darko Boshnakovski, PhD – University Goce Delcev Stip, Faculty of Medical Sciences (Stip, R. Macedonia).