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NRF2 INHIBITION IS REQUIRED TO ACTIVATE HEPATIC PROGENITOR CELLS

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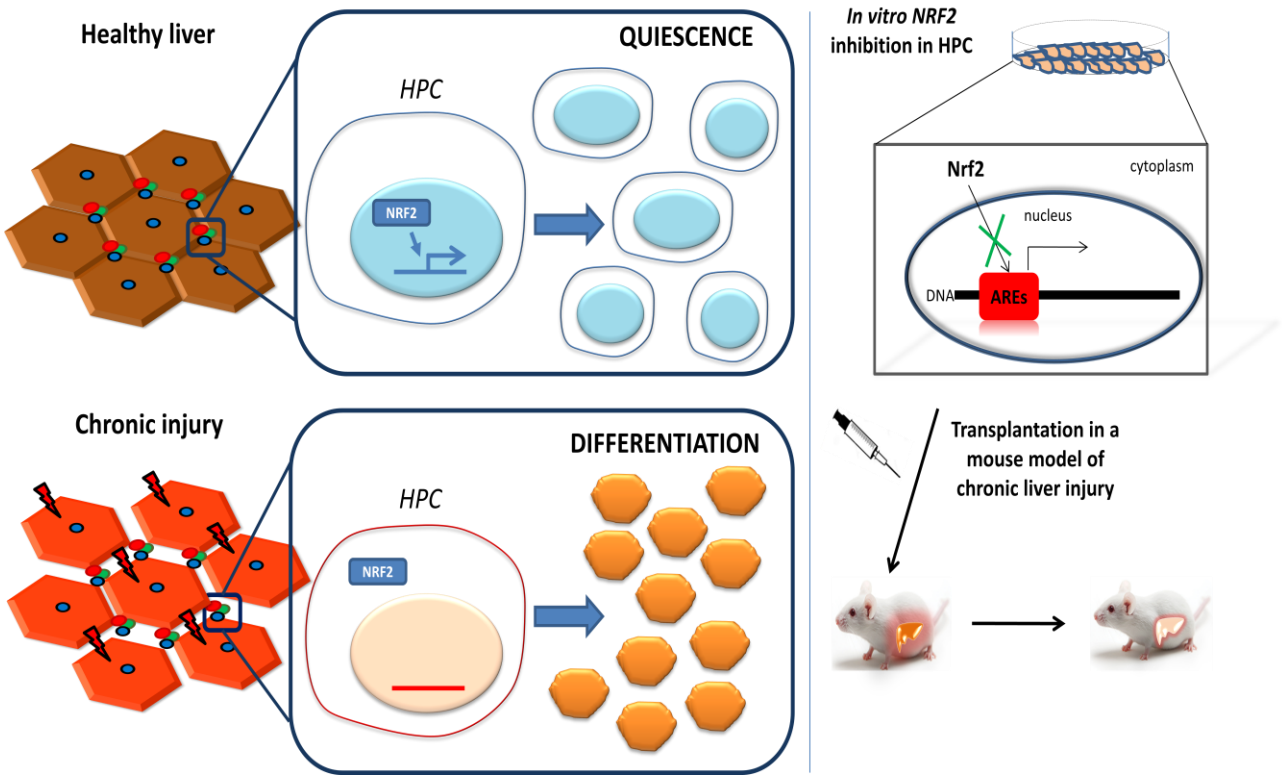
ABSTRACT

The current treatment of liver failure is organ transplantation. Nevertheless, the high costs, lack of donors, treatment-related mortality and long-term immunosuppression make this option possible only for a limited number of patients. Liver stem cell transplantation has been recently proposed as an alternative treatment. The identification of key regulators in hepatic progenitor cell differentiation is determinant for organ regeneration and may improve stem cell transplantation for end-stage liver disease.

The present investigation studied the role of the transcription factor Nuclear factor (erythroid-derived 2)-like 2 (Nrf2) in the regulation of hepatic progenitor cell fate. Our data show that Nrf2 is constitutively activated in the hepatic stem cell niches to maintain progenitor stemness, but it is down-regulated in chronic liver injury. The *in vitro* inhibition of Nrf2 induces morphological, phenotypical and functional modifications typical of differentiated elements. We thus inhibited Nrf2 via ARE expression modulator 1 (AEM1) in the human-derived HepaRG cell line; these cells were transplanted into SCID/beige mice administered with anti-Fas antibody to induce hepatocellular apoptosis, resulting in effective human hepatocyte repopulation with restoration of liver function.

To conclude, the present study shows that Nrf2 inhibition leads to the activation and differentiation of liver progenitor cells. This redox-dependent transcription factor may represent a potential target to regulate the commitment of undifferentiated hepatic progenitor cells into specific lineages.

GRAPHICAL ABSTRACT



1. INTRODUCTION

1.1. Reactive species and regulator factors

Cells produce two different types of oxidants: reactive oxygen and nitrogen species, also known as ROS and RNS, respectively. These include molecules characterised by one or more unpaired electrons, such as hydroxyl radicals ($HO^{\cdot}$), superoxide anions ($O_2^{\cdot -}$), nitric oxide ($NO^{\cdot}$), nitrogen dioxide ($NO_2^{\cdot}$), and non-radical compounds, such as hydrogen peroxide (H_2O_2) and dinitrogen trioxide (N_2O_3) (Sies, 2017).

Oxidants are mostly generated in mitochondria by the electron transport chain (ETC) (Sohal, 1990; St-Pierre, 2002). Electrons derived from metabolites are transferred to the ETC, which are in turn driven from Complex I and Complex II, by ubiquinone (coenzyme Q), to Complex III accompanied by the transient generation of ubisemiquinone (Sabharwal and Schumacker, 2014; St-Pierre, 2002); this in turn donates the electron to O_2 at Complex IV, with consequent formation of superoxide. Complex V does not participate in the electron transport (Sabharwal and Schumacker, 2014).

In physiological conditions, reactive species are counteracted by antioxidant factors, which include enzymes such as superoxide dismutases (SODs) and glutathione peroxidases (GPxs), and non-enzymatic scavengers such as glutathione (GSH), which is a co-factor/co-substrate of GPxs (Sies, 2017, di Bello, 2018) (Fig. 1).

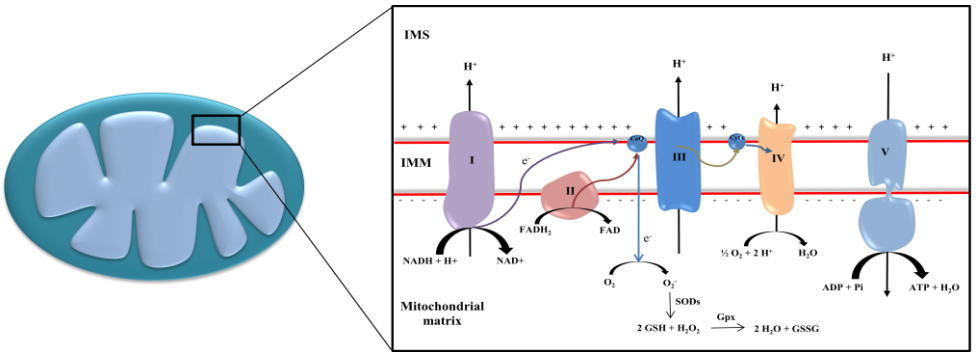


Figure 1: ETC and generation of reactive species. The mitochondrial ETC is the main source where oxidants are produced. Electrons from the Complex I and II are transferred to complex III through coenzyme Q (CoQ), causing the transient generation of the free radical ubisemiquinone; when electrons are donated to oxygen (O_2), superoxide anion (O_2^{-}) is generated. ROS are usually kept at low levels by the mitochondrial antioxidant enzymes, such as SODs which convert O_2^{-} into H_2O_2. In the reaction catalyzed by peroxidases (GPX), hydrogen peroxide and reduced glutathione (GSH) are converted into H_2O and disulphure glutathione (GSSG). IMS = intermembrane space; IMM = inner mitochondrial membrane.

At low concentrations, reactive species act as second messengers, since they are involved in numerous processes, including cellular proliferation, apoptosis, modulation of metabolism (Laloi, 2004; Holmstrom and Finkel, 2014; Liang and Ghaffari, 2014), but their excess causes metabolic alteration and macromolecules damage (Mates and Sanchez-Jimenez, 1999; Ott, 2007; Bigarella, 2014), influencing cell function and viability (Ott, 2007). Particularly, high amounts of free radicals induce both single and double-strand DNA breaks with consequent arise of cancer, premature aging, and neurodegenerative diseases (Ott, 2007); oxidized proteins are degraded by proteases (Laloi, 2004; Ott, 2007), while lipid peroxidation alters mitochondrial metabolism, respiration, oxidative phosphorylation and mitochondrial membrane potential (Orrenius, 2003; Ott, 2007).

Oxidants modulate and are modulated by several factors including the nuclear factor (erythroid-derived 2)-like 2 (NRF2), Forkhead box O (FoxO) family, Glycogen Synthase Kinase (GSK-3β), the PR domain containing 16 (PRDM16), Peroxisome proliferator-activated receptor gamma coactivator 1-alpha (PGC1α), the p53 (TRP53) tumour suppressor, Wnt and Nucleoredoxin (Nrx) (Motohashi and Yamamoto, 2004; Liu, 2005; Sablina, 2005; Funato, 2006; Tomko, 2006;

Miyamoto, 2007; Tothova and Gilliland, 2007; Kaspar, 2009; Chuikov, 2010; Kajla, 2012; Bigarella, 2014; Zheqiong Tan, 2016; Klotz and Steinbrenner, 2017; Tonelli, 2017).

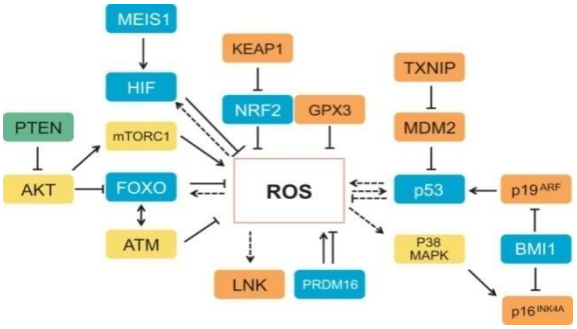


Figure 2: ROS regulator factors. ROS generation and inhibition is regulated by several factors which drive cell fate (Bigarella, 2014).

Nrf2 binds the antioxidant response elements (AREs) in the nucleus, but it is usually located in the cytosol, where it is inhibited by the Kelch-like ECH-associated protein 1 (Keap1) in the form of a dimer. Keap1 promotes the ubiquitination and the degradation of Nrf2 through the 26S proteasome, but reactive species modify two Keap1 cysteine residues, inducing its conformational change, with consequent Nrf2 dissociation and translocation into the nucleus (Canning, 2015; Kaspar, 2009; Soares, 2016; Suzuki and Yamamoto, 2015).

Nrf2 is a master regulator of redox balance, modulating both GSH and thioredoxin (Trx), involved in phase I and phase II detoxification of exogenous and endogenous products, NADPH regeneration, and heme metabolism (Dinkova-Kostova, 2002). Nrf2 is also involved in other cellular processes, such as autophagy, intermediary metabolism, liver toxicity and carcinogenesis, and stem cell quiescence (Motohashi and Yamamoto, 2004; Tonelli, 2017).

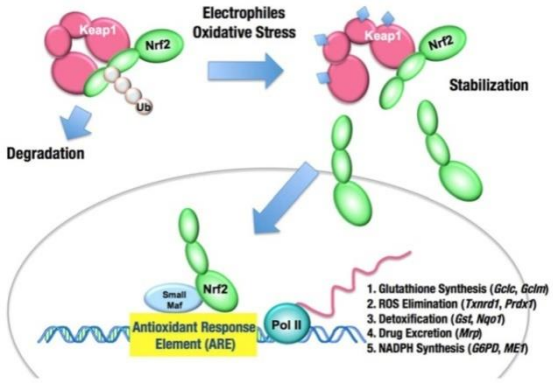


Figure 3: Keap1-Nrf2-AREs system. Nrf2 is normally located in the cytoplasm, where it is inhibited by Keap1 which induces its degradation. When oxidative stress occurs, Keap1 structure is altered, so that Nrf2 is free to migrate into the nucleus, where it binds ARE sequences and promotes the transcription of genes encoding antioxidant factors (Mitsuishi, 2012).

Members of FoxO family are involved in proliferation, cell metabolism and apoptosis (Klotz, 2015). In humans, 4 isoforms are known, called FoxO 1, 3, 4 and 6, respectively (Klotz and Steinbrenner, 2017), which upregulate two antioxidant factors, catalase (CAT) and SOD2 (Gomez-Crisostomo, 2014; Klotz and Steinbrenner, 2017).

It is also known that FoxO3 drives the transcription of Keap1, regulator of Nrf2. Thus, FoxO3 may control Nrf2 activity; on the other side, Akt stimulates Nrf2 by blocking FoxO activity, linking the two transcription factor systems (Klotz and Steinbrenner, 2017).

GSK-3β is a serine/threonine kinase phosphorylating the tyrosine kinase Fyn, which in turn promotes Nrf2 migration from nucleus to cytoplasm (Kaspar, 2009). GSK-3β may be inhibited by c-Jun N-terminal kinase 1 (JNK1), activated by the increase of free radical generation (di Bello, 2018). When members of Wnt family bind the receptor (Frizzled) on the target cellular surface, these induce the nuclear localization of β-catenin, which in turn activates the transcription of downstream genes (Funato, 2006). β-catenin expression is also regulated by Dv1-Nrx complex: when Nrx increases forms a complex with Dv1 and together, acting as free radical sensor, inhibit Wnt pathways inducing the β-catenin degradation; while, Nrx decrease leads to Wnt pathway expression (Kajla, 2012).

PGC1α is a transcription factor whose excessive activation is linked to chronic diseases. It is involved in mitochondrial biogenesis and respiration, fatty acid metabolism and oxygen species defence system, interacting with several transcription factors (St-Pierre, 2006; Zheqiong Tan, 2016).

1.2. Stem cells

Ernest A. McCulloch and James E. Till can be considered the starters of stem cell research, since in 1963 they demonstrated the existence of hematopoietic stem cells (HSCs) in murine bone marrow (Becker, 1963; Simonovitch, 1963). From that date, many different types of stem cells have been identified. Particularly, it is possible to divide them into three main groups: embryonic, fetal and adult stem cells (Latsinik, 1981; Thomson, 1998; Gucciardo, 2009). In 2007, Yamanaka discovered a fourth group of stem cells, called human induced pluripotent stem cells (hiPSCs), obtained by reprogramming human skin fibroblast into pluripotent stem cells. These cells behave similarly to embryonic stem cells, since these are able to originate all cells from the three embryo layers overcoming the ethical problems related to the embryonic stem cells (Takahashi, 2007).

Not all stem cells can differentiate into committed cell types; according to the differentiation potential, stem cells can be classified into four groups (Scheel and Weinberg, 2011):

- **Totipotent:** constituent of zygote, these stem cells can differentiate into all possible cell types;
- **Pluripotent:** part of the inner cell mass in the blastocyst, these stem cells can differentiate into all the cells deriving from the three germ layers, but not into the extra embryonic placental cells;
- **Multipotent:** present in both foetal and adult tissues, these stem cells can differentiate into a limited number of cells, typical of one embryonic layer;

- **Unipotent:** located in special microenvironments of adult tissues – the niches - these stem cells can differentiate into one mature cell type (Bernemann, 2011). In the niche, regulatory signals occur, such as cell-cell and cell-extracellular matrix interactions, driving stem cells to keep their quiescence or trigger the differentiation process (Dong, 2015).

In general, stem cells are quiescent, with the capability of self-renewal (Chen, 2006); in order to this, cells divide asymmetrically, originating two distinct daughter cells: one copy of the original stem cell, and the other one committed and differentiated into a non-stem cell. This mechanism helps to preserve the pool of stem cells in the niche (Okano, 2005; Gucciardo, 2009; Rashid, 2010; Soragni, 2014).

1.2.1. Stem cell sources

According to their source, it is possible to classify stem cells into four groups: embryonic stem cells, foetal stem cells, adult stem cells, and induced pluripotent stem cells.

1.2.1.1. Human embryonic stem cells (hESCs). Human embryonic stem cells were isolated for the first time in 1998 by Thomson et al., from the inner cell mass of a blastocyst (Thomson, 1998). Markers characterising the phenotype of these cells are the stage-specific embryonic antigen-3 (SSEA-3), stage-specific embryonic antigen-4 (SSEA-4), tumor rejection antigen-1-60 (TRA-1-60), tumor rejection antigen-1-81 (TRA-1-81), and alkaline phasphatase (Thomson, 1998). New markers (CD9, CD30, CD50, CD90 e CD200s, SSEA-5) have been recently discovered (Tang, 2011). Besides these, genes such as NANOG, OCT4 e SOX2, which encode transcription factors implicated in the maintenance of pluripotency, are also considered as markers of hESCs (Calloni, 2013; Skottman, 2005). hESCs are interesting for their therapeutic potential in regenerative medicine; nevertheless, their use faces several ethical problems, dependent on the destroy of embryos.

1.2.1.2. Foetal stem cells (fSCs). Foetal stem cells derive from foetal tissues (Campagnoli, 2001; Tsai, 2004), or foetal elements obtained after the birth, such as the placenta (In 't Anker, 2004). Furthermore, the foetal blood from umbilical cord and the amniotic fluid are considered sources of fSCs. From the umbilical cord blood, it is possible to isolate both hematopoietic (fHSCs) and mesenchymal stem cells (fMSCs) (Gucciardo, 2009). Other reservoirs of fMSCs are represented by fetal organs, such as bone marrow, liver, kidneys and lungs (Campagnoli, 2001; Almeida-Porada, 2002; In 't Anker, 2004). fHSCs express CD34 on their membrane surface, and they can differentiate into all the hematopoietic lineage cells. On the contrary, fMSCs express intracellular markers such as fibronectin, laminin and vimentin, and membrane markers such as CD105, CD733, CD45, CD34, CD14, and can differentiate into several cell types, from adipocytes to chondrocytes, but also from cardiomyocytes to neurons (Gucciardo, 2009).

1.2.1.3. Adult stem cells (aSCs). The best-known adult stem cell is the hematopoietic stem cell (HSC), isolated for the first time in 1961 by Ernest A. McCulloch and James E. Till from the murine bone marrow (BECKER, 1963; SIMINOVITCH, 1963). In 1982, Friedenshtein isolated and described another group of cells with staminal features from the murine bone marrow: even though showing a wider differentiation panel than the HSCs, these cells were initially called as colony forming unit-fibroblasts (CFU-F), better known nowadays as mesenchymal stem cells (MSCs) (Latsinik, 1981). Bone marrow and peripheral blood are both sources of HSCs. These cells can give rise to all cells in the hematopoietic line. The pattern of markers expressed by HSCs is represented by CD34, CD90 and CD133 (Hao, 1995; Nielsen and McNagny, 2009; Yin, 1997).

MSCs can be isolated not only from bone marrow (Mendez-Ferrer, 2010), but also from adipose tissue, (Shiratsuki, 2015), nervous tissue (Calzolari, 2015), the heart (Mayfield, 2014) and skin (Mehrabi, 2015). These cells express CD105, CD73 e CD90 as specific surface markers, while there is no evidence of CD45, CD34, CD14 o CD11b, CD79α o CD19. MSCs are able to differentiate into adipocytes, osteoblasts and chondroblasts (Dominici, 2006). Moreover, recent studies show

that these cells are also able to differentiate into non-mesenchymal cells such as pancreatic isles (Mehrabi, 2015), neuron-like cells (Johnstone, 2015), and hepatocytes (Stock, 2014).

1.2.1.4. Human induced pluripotent stem cells (hiPCSs). In 2007, Yamanaka et al. were able for the first time to reprogramme human dermal fibroblasts (HDFs) into pluripotent stem cells by viral transfection: these cells were then able to express markers such as OCT 3/4, SOX2, KLF4 e c-MYC (OSKM cocktail), typically expressed in pluripotent stem cells. Furthermore, they were able to reprogramme human fibroblast-like sinoviocytes (HFLS), obtaining same results (Takahashi, 2007).

These results were later confirmed by another lab, which obtained pluripotent stem cells with the OCT 3/4, SOX2, NANOG e LIN28 cocktail (Yu, 2007). Both these studies were based on invasive samples collection, while another study demonstrated that is possible to obtain hiPSC from urine samples, avoiding scars and bleeding and allowing the collection of cells also in subjects suffering of coagulopathies such as haemophilia (Jia, 2014). In theory, any type of cell can be reprogrammed, possibly generating hiPSCs (Singh, 2015).

hiPSCs express similar surface markers of hESCs, such as SSEA-3, SSEA-4, TRA-1-60, TRA-1-81, alkaline phosphatase, and Nanog protein. These cells also express OCT3/4, SOX2, NANOG, growth and differentiation factor 3 (GDF3), reduced expression protein 1 (REX1), fibroblast growth factor 4 (FGF4), embryonic cell-specific gene 1 (ESG1), developmental pluripotency-associated 2 and 4 (DPPA2 and DPPA4), and telomerase reverse transcriptase (hTERT) at similar levels to those seen in hESCs (Takahashi, 2007).

hiPSCs are pluripotent stem cells, with the ability to differentiate into cells derived from all three embryo layers. Indeed, several studies show that cardiomyocytes (Citro, 2014), neuronal cells (Wheeler, 2015), hepatocytes (Jia, 2014), pancreatic isles cells can be obtained from differentiation of hiPSCs (Kuise, 2014).

These cells exhibit an immense potential for research and therapy: first of all, their use does not face any ethical-religious problems; moreover, since these cells are taken directly from the patient and reprogrammed *in vitro*, the compatibility and rejection concerns are reduced. Unfortunately, the major problem, to date, remains the risk of carcinogenicity: indeed, 20% of rats with hiPSCs implants developed teratomas or teratocarcinomas.

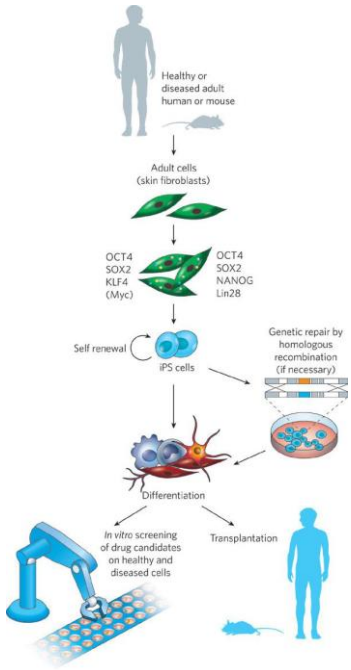


Figure 4: human induced Pluripotent Stem Cells. Cells can be infected to be reprogrammed and express genes normally expressed in pluripotent stem cells. In this way hiPSCs can be originated, able to differentiate into any cell type (Passier, 2008).

1.3. Reactive species drive stem cell fate

For a long time, reactive species have been considered as dangerous, but it is now known that, at low levels, these molecules are involved in the regulation of quiescence/self-renewal/differentiation axis (Blanchetot and Boonstra, 2008; Chiarugi and Fiaschi, 2007; Sart, 2014), together with metabolism (Takubo, 2013). On the contrary, high oxidant amounts cause cell damage and the alteration of homeostasis in the niche, particularly interfering with the molecule adhesion of multipotent stem cells by the focal adhesion kinase (FAK), Src and integrins downregulation. Moreover, oxidized DNA is associated with the reduced expression of telomeric repeat binding factor (TRF) 1 and 2, inducing cell senescence (Borodkina, 2014; Harbo, 2012). Furthermore,

oxidative stress causes cell cycle arrest via p38-MAPK and p16 in multipotent stem cells (Lee, 2009).

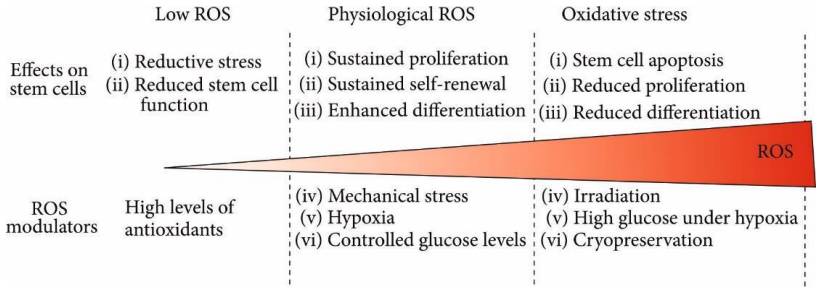


Figure 5: Reactive species as modulators of stem cell fate. Free radical levels are important to preserve the depletion of stem cell niches and to promote cell differentiation to replace old and damaged cells. Low ROS levels assure the maintenance of quiescence, while their physiological increase induces cell proliferation, self-renewal and differentiation. On the contrary, in case of ROS overproduction, premature senescence and apoptosis occur (Sart, 2015).

For instance, redox balance drives stem cell differentiation in ESCs: here, the increase of oxidants levels induces p38 activation and the expression of phosphatidylinositol-4,5-biphosphate 3-kinase (PI3K). These events drive the nuclear translocation of the Myocyte Enhancer Factor 2C (MEF2C), inducing ESCs differentiation toward cardiomyocytes. Free radicals produced during cardiac differentiation activate nuclear factor kappa-light-chain-enhancer of activated B cells (NF-kB) signalling, as well as phosphatidylinositol 3-kinase enhancer (PIKE) and PI3K activation. Also, the neural differentiation of ESCs is induced by the high amount of free radical generation and down-regulation of antioxidant defences (Sart, 2014).

Quiescent state is characterised by glycolysis and the inhibition of oxidative phosphorylation by HIF (hypoxia-inducible factor)-induced hypoxia (Mandal, 2011; Zhou, 2012). When HSCs activate to differentiate and replace mature blood cells, their metabolism shifts from glycolysis to oxidative phosphorylation (Takubo, 2013). Indeed, lower reactive species, mitochondrial mass and respiration, and higher glycolysis metabolites have been shown in HSCs rather than in downstream progenitors (Jang and Sharkis, 2007; Simsek, 2010; Le Belle, 2011; Norddahl, 2011); similar findings were described in neuronal stem cells (NSCs) and MSCs (Funes, 2007; Le Belle, 2011; Paik, 2009; Yeo, 2013). Furthermore, FoxO proteins seem to be involved in NSCs differentiation: particularly, FoxOs deletion in these cells promotes the decrease of antioxidant factors and induces

the increase of free radicals, with consequent proliferation of NSCs (Paik, 2009). On the contrary, the excess of free radical generation induces cell toxicity and premature senescence (Le Belle, 2011).

Further investigation is needed to focus on the impact of oxidants and antioxidants in determining progenitor cell fate, in order to modulate their capability to differentiate for therapeutic use.

1.4. Liver diseases and oxidative stress

Liver disorders, such as viral hepatitis, alcoholic liver disease (ALD), non-alcoholic fatty liver disease (NAFLD), and drug-induced liver injury (DILI) are widely spread all over the World. These conditions are characterised by a consistent alteration of tissue structure, inflammation, fibrosis, and the attempt by liver cells to regenerate the organ (Krahenbuhl, 2001; Arduini, 2011; Björnsson, 2017; di Bello 2018).

Both acute and chronic liver diseases are associated with oxidative stress, which leads to the alteration of structure and function of all cellular components (proteins, lipids, nucleic acids) (Roskams, 2003), causing metabolic and cell cycle failure (Muriel, 2009). For example, it is known that increased malondialdehyde (MDA) and decreased antioxidants characterise B and C viral hepatitis infection (Ames and Shigenaga, 1992; Emerit, 2000; Jain, 2002; Yadav, 2002; Demirdag, 2003; Bolukbas, 2005; Dikici, 2005; Venturini, 2010). ALD is due to the persistent alcohol abuse, whose prolonged consumption leads to cirrhosis (Cohen and Nagy, 2011) accompanied by free radical production (Cederbaum, 2009). NAFLD, caused by an alteration between the intake and the removal of lipids in the liver (Serviddio, 2008), is characterised by the decreased levels of reduced glutathione (GSH) (Leung and Nieto, 2013), which alters the balance between oxidants and antioxidants. This condition may also trigger the pro-inflammatory cascade of cytokines, with consequent activation of hepatic stellate cells to produce connective tissue; moreover, an excess of

oxidative stress causes hepatocytes death by receptor Fas-ligand activation and Kupffer cell stimulation (Koek, 2011).

Liver transplantation is, to date, the only effective treatment for end-stage liver diseases, though this procedure remains slightly used because of the paucity of organs, the high costs, and the long-term immunosuppressive therapy (Flechtenmacher, 2015). For these reasons, cell-based procedures have been recently proposed; in this context, the use of stem cells might be useful to overcome the limitation of liver donors (Fiegel, 2006).

1.5. Hepatic progenitor cells

Liver regeneration is a complex and unique process. When two-thirds of a mouse liver are removed, the remaining hepatocytes, normally in the quiescent phase, reactivate the cell cycle and restore the hepatic mass through their proliferation (Higgins and Anderson, 1931).

The regeneration process appears as a "compensatory" mechanism, and the proliferation of the hepatocytes ends once the original mass is restored (Russo and Parola, 2011; Fausto, 2012). This ability to regenerate is very important as it allows to perform liver transplants from living donors. However, if the liver damage is severe and protracted (for example in chronic viral hepatitis, ALD and NAFLD), and the loss of hepatic cell mass is associated with inflammatory and fibrotic processes, regeneration is not due to the proliferation of mature hepatocytes, but to the activation and differentiation of immature cells with an intermediate phenotype between hepatocytes and cholangiocytes, called hepatic progenitor cells (HPCs) (Roskams, 2003; Fausto, 2004; Roskams, 2004; Turanyi, 2010). This process is defined as ductular reaction, or oval cell hyperplasia, since in the beginning these cells were isolated from the murine liver and identified as oval cells.

HPCs are bipotent cells expressing epithelial cell adhesion molecule (EpCAM), neuronal cell adhesion molecule (NCAM), CD133, CXCR4, SOX9, SOX17, FOXA2, cytokeratins 8/18/19, while show low albumin expression, and no expression of α-fetoprotein, intracellular adhesion molecule

(ICAM-1), P450 enzymes or hematopoietic (CD34/38/45/90), endothelial (VEGFR, CD31, Von Willebrand factor) or mesenchymal (CD146, desmin, vitamin A, CD105) cell markers (Saxena and Theise, 2004; Schmelzer, 2006; Cerec, 2007; Schmelzer, 2007).

HPCs are located in the niches within the adult organ. Several HPCs stem cell niche sites have been proposed, although the most reliable are the canals of Hering (Theise, 1999; Eleazar, 2004), which are also the most suitable site from an anatomical point of view, as they represents the interface point between hepatocytes and cholangiocytes.

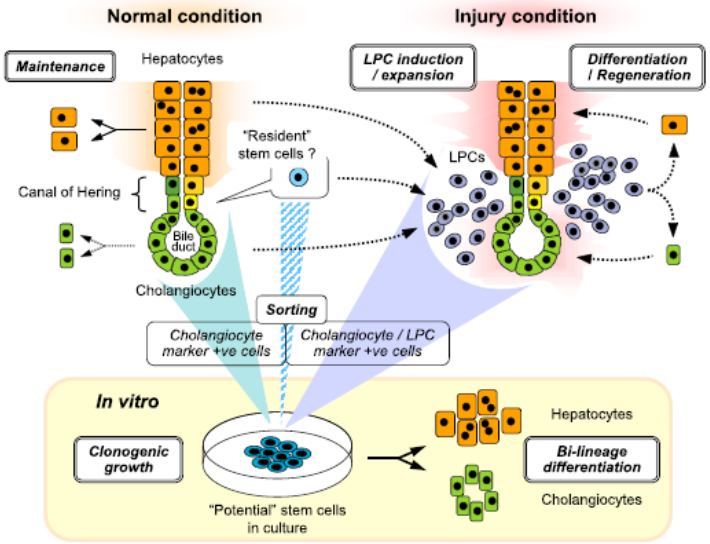


Figure 6: Liver progenitor cells (LPCs). LPCs are located in the canals of Hering and they are normally quiescent, and able to differentiate into both hepatocytes and cholangiocytes during chronic liver injury (Miyajima 2014).

1.6 Stem cell therapy in liver diseases

The current knowledge about molecular mechanisms, functions, and morphology of stem cells allows them to be widely used for therapies. Bone marrow stem cells were the first to be transplanted in patients affected by leukaemia; the transplant of umbilical cord stem cells in patients with haematological diseases has shown similar results to those induced by bone marrow transplant (Barker and Wagner, 2003).

To date, several clinical studies using neurons, cardiomyocytes and β pancreatic cells have been performed, in order to treat diseases for which pharmacological therapy is not successful, such as

neurodegenerative diseases, heart failure, and type 1 diabetes mellitus (Gilchrist and Plevris, 2010; Kattman, 2011; Konagaya and Iwata, 2015).

1.6.1. Therapeutic potential of stem cells in liver diseases

Liver diseases affect millions of patients worldwide. Liver transplant is nowadays the only end-stage treatment, but in the last years cell-based procedures are developing because of the low availability of donor organs, high costs and the subsequent need for long-life immunosuppressive therapy (Di, 2003; Fiegel, 2006).

Cell therapies in hepatology show many potential advantages when compared with transplantation, since cells can be:

- *in vitro* expanded, eliminating the limit of organ deficiency;
- genetically manipulated, to correct genetic errors;
- cryopreserved for future use;
- injected without the need of surgery and invasive techniques;
- obtained from the patient himself, avoiding the risk of rejection and the need for subsequent immunosuppressive therapy (Piscaglia, 2008).

A possible source for cell therapy is represented by primary adult hepatocytes, i.e. hepatocytes extracted directly from the hepatic tissue. Despite some encouraging results, studies with primary adult hepatocytes are hampered by several factors: firstly, these cells are difficult to be grown and *in vitro* expanded, and they can also be damaged during freezing/thawing procedures.

Several clinical trials have been performed using MSCs in the treatment of cirrhosis. The first clinical study, performed in 2007 by Mohamadnejad et al., involved four patients with decompensated cirrhosis, treated with MSCs infused via peripheral vein; the results showed a general improvement in the quality of life (Mohamadnejad, 2007). In 2009, a second clinical trial by Kharaziha et al. demonstrated an improvement in liver function in patients with cirrhosis who underwent autologous MSCs injection via peripheral or portal vein (Kharaziha, 2009); Jang et al.

obtained positive results in the transplantation of autologous MSCs in the treatment of alcoholic cirrhosis: of eleven patients treated, six showed histological improvement, and the levels of TGF-β1, collagen type 1 and α-SMA were significantly reduced (Jang, 2014). Unfortunately, problems regarding MSCs need to be considered: firstly, all these studies have been carried out on patients with advanced chronic liver disease, while similar promising results have not been found in patients with acute liver injury. Moreover, there was a greater differentiation in myofibroblast-like elements rather than hepatocyte-like, causing a pro-fibrotic risk (Bonzo, 2008; Baertschiger, 2009). Finally, but not the last, the oncogenic risk should not be underestimated (Zhu, 2006).

hiPSCs are also considered a new source to replace the damaged liver. Indeed, these cells can differentiate into hepatoblasts/hepatocytes, able to express liver markers (Rashid, 2010). They also revealed functional properties not only in *in vivo* mouse transplant, but also in humans (Dianat, 2013).

It is conceivable that the use of HPCs in the treatment of liver diseases may solve the problem of the oncogenic risk by MSCs transplantation, as well as the risk of infection and rejection due to xenogenic transplant (Khan, 2008).

Foetal liver hepatocytes showed high regenerative capacity, thanks to their resistance to cryopreservation, *in vitro* plasticity and their ability to repopulate tissue *in vivo* (Pietrosi, 2015). It has been recently described that the infusion of these cells via the hepatic artery in patients with liver cirrhosis did not cause any rejection; moreover, after six months of follow-up, the patients showed both biochemical and clinical improvement (Cardinale, 2014).

Nonetheless, further studies need to be performed to avoid ethical problems and teratomas formation, allergy and rejection. In this view, autologous transplant of HPCs could represent a solution, but to date little is known about the molecular pathways involved in their activation.

1.7 Redox balance and hepatic progenitor cells

Patients affected by NAFLD and ALD showed a correlation between fibrosis stage, inflammatory cytokines, and the activation of HPCs (Roskams, 2003). In these conditions, damaged hepatocytes were unable to regenerate the tissue because of cell cycle inhibition due to increased ROS generation and unbalanced GSH content (Roskams, 2003).

The activation of HPCs in chronic liver disease and its association with increased oxidative stress markers was also studied in paediatric patients affected by NAFLD and non-alcoholic steatohepatitis (NASH) (Nobili, 2012).

In the last decade, the role of β-catenin to promote cell survival during constant oxidative stress has been underlined (Essers, 2005; Hoogeboom, 2008), demonstrating to be protective in models of alcoholic and non-alcoholic steatohepatitis, as well as liver ischemia-reperfusion (IR) (Behari, 2010; Lehwald, 2011; Liu, 2012).

However, it has been observed that populations of extrahepatic adult stem cells can migrate into the liver and contribute to its repopulation and cell turnover (Piscaglia, 2008). Particularly, MSCs show high degree of plasticity, which can give rise to different cell phenotypes, including hepatocyte-like cells. Indeed, it has been demonstrated that, after grafting, these cells can both trans-differentiate into epithelial parenchymal cells and merge with the resident cells in the host tissue (Piscaglia, 2008).

In addition, MSCs express various growth factors and cytokines that stimulate resident cells and remodel the matrix by promoting the ductular reaction as previously mentioned, and factors with anti-fibrotic and down-regulating activity on myofibroblasts. Finally, MSCs show a cytoprotective activity inducing AREs in models of hepatic damage induced by carbon tetrachloride (CCl4) or thioacetamide (TAA) (Cho, 2012; Quintanilha, 2014).

Molecular mechanism involved in the activation of HPCs are not completely elucidated yet, but they could represent the resource to treat end-stage liver diseases. Understanding the pathways that

are involved in the maintenance, activation and differentiation of HPCs is a goal to reach now to better understand liver regeneration.

One of the first cytokines described to have a great impact on HPCs is the TNF-like weak inducer of apoptosis (TWEAK), produced by monocytes, T lymphocytes and macrophages, the expression of which increases during acute damage, inflammatory diseases and cancer. The expression of its Fn14 receptor, present in epithelial and mesenchymal cells, is normally low in healthy tissues, but significantly increases in damaged and diseased tissues, such as the murine liver following treatment with DDC (3,5-diethoxycarbonyl-1,4-dihydrocollidine), which induces cholangitis. The activation of HPCs is significantly reduced in knock-out mice for Fn14 or in wild-type mice following administration of antibodies against TWEAK, while the overexpression of TWEAK in hepatocytes (Jakubowski, 2005) or administration of exogenous TWEAK leads to oval cell hyperplasia (Bird, 2013). Indeed, the response of HPCs to hepatocyte damage was attenuated in mice with Fn14 deficiency, and stimulation of a Fn14-positive HPCs line with TWEAK led to regeneration levels similar to those of wild-type mice (Tirnitz-Parker, 2010).

Miyajima et al. identified the central role of FGF7 in the activation of HPCs during severe hepatic injury (Takase, 2013). To confirm this, it was observed that the expression of FGF7 was induced simultaneously with the proliferative response of HPCs in mouse models of liver injury (such as DDC), as high levels could be found in the serum of patients with acute liver failure (Steiling, 2004; Dezso, 2007; Murakami, 2011). In addition, the knock-out mice for the FGF7 gene exhibited markedly reduced HPCs and a higher mortality following liver injury. In addition, the *in vivo* transgenic expression of FGF7 leads to the induction of cells with features of HPCs and improves liver dysfunction (Takase, 2013).

In mice with FGF7 deficiency, the activation of HPCs was never induced, even long time after the damage induction (Takase, 2013).

Several different factors are believed to be involved in the activation and differentiation of HPCs, such as Hepatocyte Growth Factor (HGF)/c-MET and Epidermal Growth Factor (EGF)/EGF

receptor (EGFR). The HPCs response was significantly lower after DDC-induced damage in c-MET knock-out mice (Ishikawa, 2012), suggesting that HGF is an important factor in the activation of HPCs. On the other hand, EGFR appears to be necessary for the differentiation of HPCs, since stimulation of EGFR activates the Notch1 pathway, leading the cells to differentiation into colangiocytes and inhibiting hepatocytes differentiation (Kitade, 2013).

In experimental models of hepatocytes injury induced with choline-deficient, ethionine-supplemented (CDE) diet, macrophages express Wnt3 leading to Notch inhibition in HPCs and therefore to hepatocyte regeneration; on the contrary, the biliary damage activates Notch in HPCs, resulting in a colangiocytes differentiation. Of note, macrophage depletion during hepatocyte injury is accompanied by the activation of the Notch pathway - and not the Wnt pathway - favouring commitment toward cholangiocyte lineage (Boulter, 2012; Boulter, 2013). In addition, other cytokines such as TNFα, IFNγ, IL-6, and oncostatin may stimulate HPCs activation (Shiojiri, 1997; Knight and Yeoh, 2005; Znoyko, 2005; Yeoh, 2007).

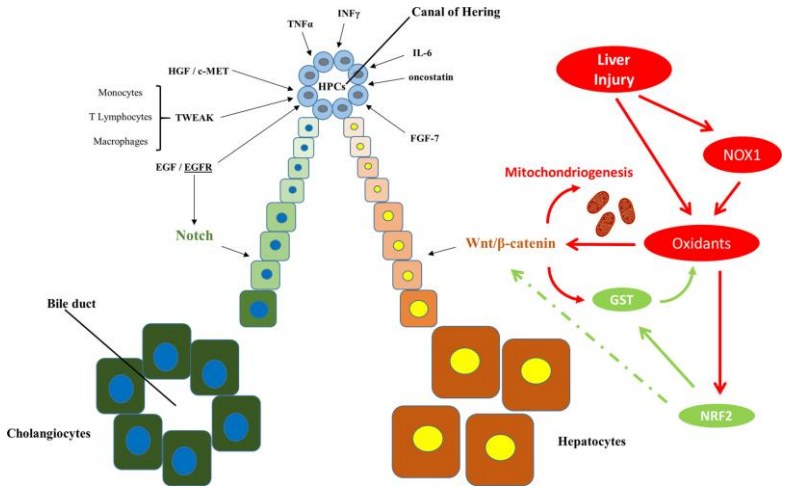


Figure 7: HPCs activation and differentiation after chronic liver injury. Oxidative stress, cytokines (TNFα, INFɣ, IL-6) and factors such as TWEAK, c-MET and EGF, released during persistent liver damage, induce the activation of HPCs located in the niches (canals of Hering). Progenitor cells can differentiate into cholangiocytes or hepatocytes, depending on the signalling pathway activated: particularly, Notch promotes the differentiation process toward cholangiocyte, inhibiting differentiation into hepatocytes; on the contrary, Wnt signalling pathway induces the differentiation of HPCs toward hepatocytes (di Bello, 2018).

Keap1-Nrf2-ARE is the main signalling pathway involved in the oxidative stress response (Kensler, 2007), since it is involved in the antioxidant response and phase II drug metabolism.

The role of Keap1-Nrf2 system in promoting stem cell differentiation is well known. For example, the Nrf2 homolog in Drosophila (CnC protein) is constitutively active in intestinal stem cells (ISCs), keeping these cells in their stemness; on the contrary, ISCs differentiation is induced by the increase of reactive species due to the inhibition by Keap1 (Hochmuth, 2011).

Interestingly, Nrf2 is also determinant in pluripotent stem cells reprogramming (Hawkins, 2016), neural stem cells fate (Pistollato, 2017; Robledinos-Anton, 2017), and the differentiation of mesenchymal stem cells into osteoblasts (Tao, 2016; Yuan, 2017). Moreover, Nrf2 seems to be involved in liver regeneration after partial resection (Wakabayashi, 2010), but there is no evidence about the role of Keap1-Nrf2-AREs signalling pathway in the modulation of activation/differentiation of HPCs.

2. AIMS

Chronic liver disease causes high morbidity and mortality worldwide. The only cure for end-stage liver disease is liver transplantation; however, donor organ availability cannot meet demand, and many patients die. Recently, cell-based procedures have been proposed; in this context, the use of stem cells might be very attractive to overcome the limitation of donor liver tissue (Fiegel, 2006).

In case of severe or protracted damage, the organ renewal is mediated by HPCs, capable of differentiating toward both the biliary and the hepatocyte lineages (Fausto, 2004). Even though HSCs transplantation for acute and chronic liver diseases has a promising therapeutic effect, the differentiation efficiency is too low to generate enough numbers of functional mature hepatocytes (Ichinohe N Cell Transplant 2012; Ichinohe N Hepatology 2013). Thus, it is conceivable that the identification and modulation of key cellular pathways that control and drive the activation/differentiation of HPCs would improve their transplantation efficiency.

Redox cell signalling is crucial to determine stemness/proliferation of several cell types. Nrf2, a master regulator of reactive species production, may drive cell fate (Kensler, 2007; Bigarella, 2014, di Bello 2018). To date, the role of Nrf2 in the modulation of HPCs proliferation and differentiation has not been demonstrated yet. Thus, the aim of this project is to study the involvement of Nrf2 in HPCs activation and/or differentiation *in vitro*, and to explore the effects of Nrf2 modulation on HPCs transplantation *in vivo*.

3. MATERIALS AND METHODS

3.1. Cell Cultures

First, we studied the impact of redox balance, the involvement of Nrf2, and the effect of Nrf2 modulation in HPCs activation/differentiation. Preliminary experiments were performed on primary HPCs extracted by rodent models of HPC activation (male wild-type C57BL/6 fed on a choline-deficient diet for up to 2 weeks, and their water supplemented with 0.15% (wt/vol) DL-ethionine), kindly provided by prof. Stuart Forbes (Edinburgh, UK).

mHPC culture. After isolation by medium containing collagenase B and DNAse I, cells were centrifuged through a discontinuous Percoll gradient and cultured as previously described (Tirnitz-Parker JE, Int Biochem Cell Biol, 2007). FACS separation of mHPCs was performed using EpCAM+/CD24+/CD133+/CD31-/CD45-/Ter119- sorting optimised in the Forbes Laboratory (see below for examples of sorted and plated HPCs).

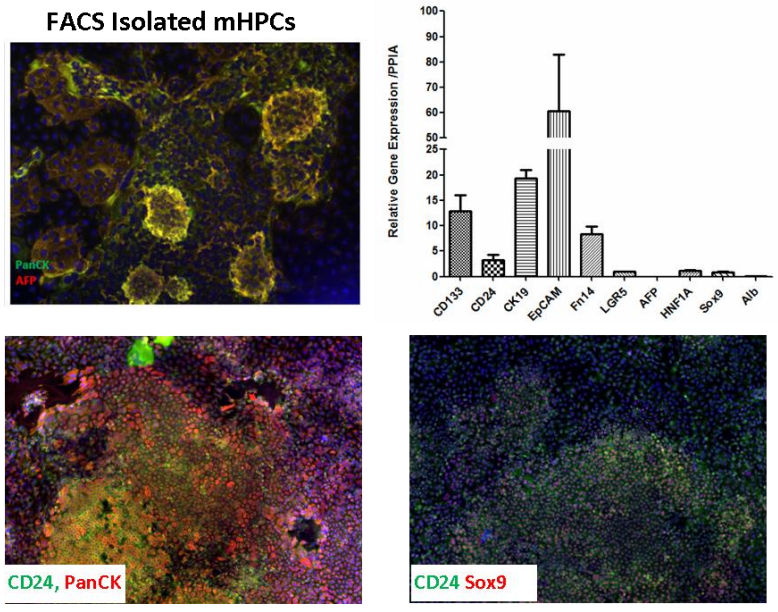


Figure 8: Primary HPCs. Mouse primary HPCs were isolated by FACS. EpCAM+/CD24+/CD133+/CD31-/CD45-/Ter119- cells were *in vitro* cultured (images courtesy of prof. S. Forbes, Scottish Centre of Regenerative Medicine, Edinburgh, UK).

HepaRG culture. To further study and track the impact of Nrf2 modulation on *in vivo* transplantation efficiency, the human cell line HepaRG (purchased by Millipore) was used. These cells were isolated for the first time from differentiated grade 1 Edmonson hepatoma associated with chronic C hepatitis (Parent 2004; Cerec, 2006). Even though undifferentiated HepaRG cells exhibit a fibroblast-like morphology, the differentiation process induces both hepatocyte-like and biliary-like epithelial phenotypes at confluence, indicating bipotent progenitor features. HepaRG cells express a wide panel of liver genes, including drug metabolizing enzymes (e.g. cytochrome P450), similarly to human primary hepatocytes (Cerec, 2006; Aninat, 2006; Aninat, 2008).

HepaRG cells were seeded at 27000 cell/cm^2 confluence in a base medium composed by William's E Medium + GlutaMAX (Gibco, 3255-020) supplemented with 10% FBS (Sigma, F7524), 100U/ml penicillin (Sigma), and 100ug/ml streptomycin (Sigma, P4333). To induce the trans-differentiation, cells were shifted to a Growth Medium composed by Base medium + 5ug/ml insulin (Actrapid®, Novo Nordisk), and 50uM hydrocortisone hemisuccinate (Pfizer) for two weeks, and then to the same medium supplemented with 2% DMSO (Differentiation Medium) (Sigma, 67-68-5). The medium was renewed every 2 or 3 days (Aninat, 2006; Cerec, 2006).

When seeded, HepaRG cells (control cells, CTRL) show an elongated morphology characterized by a clear cytoplasm. Then, they progressively form typical colonies of smaller epithelial cells with a granular and dark cytosol, and prominent nuclei, like hepatocytes surrounded by more flattened and clearer epithelial cells. Addition of DMSO induces the appearance of clusters of hepatocyte-like cells surrounded by biliary-like cells (Differentiated cells, DIFF) (Fig. 9).

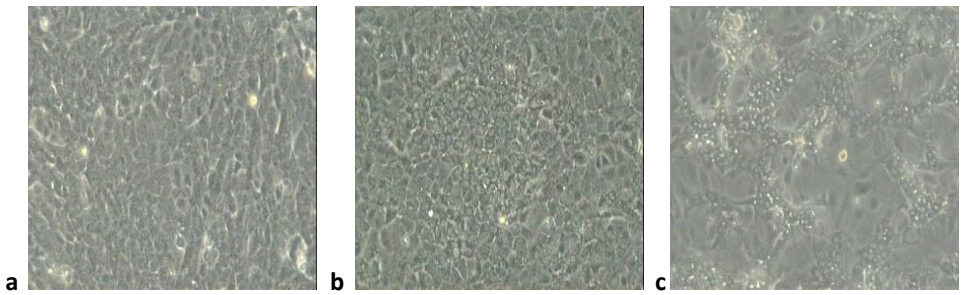


Figure 9: HepaRG phenotype changes. a) Undifferentiated cells' morphology; **b)** HepaRG after two weeks differentiation process; **c)** Phenotype of HepaRG cells at the end of the differentiation treatment. 10X magnification.

3.2. Oxidative stress induction and redox manipulation

Oxidative stress was induced by exposing HPCs to hydrogen peroxide (H_2O_2) added to the medium. Two formulas of treatments were used: HPCs were treated with (1) different concentrations of H_2O_2 (20, 50, 100, 300µM) for the entire course of experiment to determine their tolerance to H_2O_2 toxicity (long-term treatment) and (2) with 150µM H_2O_2 for 2h (short-term treatment). This treatment is sublethal and is designed to assess the effects of H_2O_2 on different cellular activities. To render cells more reduced, medium was supplemented with N-AcetylCysteine (NAC).

3.3. Transfection of NRF2 siRNA

Primary HPCs were seeded 0.4–1.6 x 10^5 cells per well in a 24-well plate, transiently transfected with 37.5ng of a pre-designed siRNA directed against mouse Nrf2, using the HiPerFect Transfection Reagent (Qiagen). According to the manufacturer procedure, siRNA was diluted in 100µl culture medium; subsequently, 3µl of HiPerFect Transfection Reagent were added to the diluted siRNA, to form transfection complexes which were loaded in the wells for 24h. The following day, cells were used for gene analysis by Real Time PCR.

3.4. AEM1 treatment

Are **E**xpression **M**odulator **1** (**AEM1**) was used to inhibit Nrf2 binding to AREs. AEM1 does not induce modifications in the expression of both Nrf2 and Keap1 protein (Bollong, 2015). AEM1 (Sigma, SML-1556) was used at the final concentration of 1uM dissolved in Growth Medium for 2 weeks (see paragraph 3.1 for medium composition). The medium was renewed every 2 or 3 days.

3.5. RNA Isolation, Reverse Transcription PCR (RT-PCR), and Real Time PCR (qPCR)

To study the levels of genes expressed in both HPCs and HepaRG cells, RNA was extracted and converted into cDNA which was used as template in the following Real Time PCR.

3.5.1. RNA extraction

To isolate RNA from frozen livers, 30mg/sample were used. The tissue was included in Lysis buffer (+1% β-mercapthoethanol) and homogenized by potter. The RNA isolation was then performed as well as for cells, as described below.

RNA extraction was performed by the "Pure Link RNA Mini kit" (Thermo Fisher, 12183025). 1.0 * 10^6 cells/sample were processed as follows: cells were washed twice with PBS and lysed with Lysis Buffer; a volume of 70% EtOH was added. After being vortexed, the samples were transferred into the columns furnished by the kit and centrifuged at max speed for 15 sec. A first wash was made with Wash Buffer I: samples were centrifuged at max speed for 15 sec, and the eluate discarded. Two following washes with Wash Buffer II (+EtOH) were made: in both cases, the samples were centrifuged at max speed for 15 sec, and the eluate was discarded. To completely dry the membranes, samples were centrifuged for 2 min, and RNA of each sample was eluted with RNase-free water. RNA concentration was determined by spectrophotometer method at Nanodrop, measuring absorbance at $\lambda = 260nm$. $A_{260}/A_{280} > 2$ was evaluated to guarantee protein-free samples.

3.5.2. Reverse-transcriptase PCR (RT-PCR)

RT-PCR was performed by High-capacity cDNA Reverse Transcription Kit (Applied Biosystems, 4368814). Mix for each sample was composed of:

- 2 ul 10X RT Buffer;
- 0.8ul 25X dNTPs Mix (100mM);

- 2ul 10X RT Random Primers;
- 1ul Multi Scribe Reverse Transcriptase;
- 1ul RNase Inhibitor;
- 1ug RNA
- Nuclease-free water up to 20ul final volume.

The RT-PCR reaction was performed as follows:

	STEP 1	**STEP 2**	**STEP 3**	**STEP 4**
Temperature	25°C	37°C	85°C	4°C
Time	10 min	120 min	5 min	∞

3.5.3. Real Time RT-PC

To detect new synthesized cDNA molecules by Real time RT-PCR, SYBR Green (Biorad, 172-5271) was used as fluorescent probe. Murine and Human *actin* was chosen as housekeeping gene. The genes analysed by Real Time are typically expressed in either stem or liver differentiated cells, as explained below:

- Albumin: it encodes a protein produced in the liver and released into the plasma. It is expressed in differentiated hepatocytes;
- CYP3A4: it encodes an enzyme involved in drug metabolism in differentiated hepatocytes;
- CEA (carcinoembryonic antigen): it encodes a glycoprotein involved in cell adhesion and it is typically expressed in foetal, undifferentiated or tumour cells;
- Cytokeratin-19 (CK-19): it is the marker of liver progenitor cells;
- Gamma-glutamyl transferase-1 (GGT-1): it encodes an enzyme expressed on the cell membrane surface of mature hepatocytes.
- Hes1 and Hes5: Notch downstream genes, expressed in cholangiocytes;

- Hnf1a, Hnf4a, Sox9, Myc, Axin2: genes expressed in hepatic cell lines whose differentiation process, starting from progenitor cells, is driven by Wnt.

The sequences of forward and reverse primers of all the human genes studied are listed as follows:

Gene name	Specie	Forward/Reverse	Sequences
ACTIN	Mouse	FOR	5'-GGCGGGACTGTTACTGAGCTGCG-3'
		REV	5'-GCTGTCGCCTTCACCGTTCCA-3'
ACTIN	Human	FOR	5'-TGGACATCCGCAAAGACCTG-3'
		REV	5'-GCCGATCCACACGGAGTACTT-3'
ALBUMIN	Human	FOR	5'-CCTGTTGCCAAAGCTCGATG-3'
		REV	5'-GAAATCTCTGGCTCAGGCGA-3'
CYP3A4	Human	FOR	5'-CTTCATCCAATGGACTGCATAAAT-3'
		REV	5'-TCCCAAGTATAACACTCTACACAG-3'
CEA	Human	FOR	5'-GGTCTTCAACCCAATCAGTAAGAAC-3'
		REV	5'-ATGGCCCCAGGTGAGAGG-3'
GGT-1	Human	FOR	5'-TTTGGTGTGCTGCTGGATGAC-3'
		REV	5'-ACCTGAGCTTCCCCACCTATG-3'
CK	Human	FOR	5'-TCCGAACCAAGTTTGAGACG-3'
		REV	5'-GCCCCTCAGCGTACTGATTT-3'
SOD1	Human	FOR	5'-TGTGGGGAAGCATTAAAGG-3'
		REV	5'-CCGTGTTTTCTGGATAGAGG-3'

The FOR/REV primers used to amplify mouse-specific NRF2, HES1, HES5, HNF1a, HNF4a, SOX9, MYC and AXIS2 genes were purchased by Qiagen (QuantiTect Primers Assay).

For each gene, mix was prepared as follows:

- 10ul Sybr green Supermix (Biorad SSoAdvanced Universal Sybr Green);
- 0.5ul FOR primer 10uM;
- 0.5ul REV primer 10uM;
- 8ul RNase-free water.

Mix (19ul) was loaded in the well of 96-wells plate and 1ul of cDNA was added. Each condition was prepared in triplicates and cDNA was amplified for 40 cycles. The amplification protocol is shown below:

	STEP 1	STEP 2	STEP 3
Temperature	95°C	95°C	60°C
Time	30 sec	15 sec	1 min

At the end of differentiation treatment, gene analysis was done to check the differentiation state of HepaRG. After the isolation of RNA from CTRL and DMSO-treated (DIFF) cells, and its retro-transcription via RT-PCR, cDNAs were amplified and evaluated via Real Time PCR.

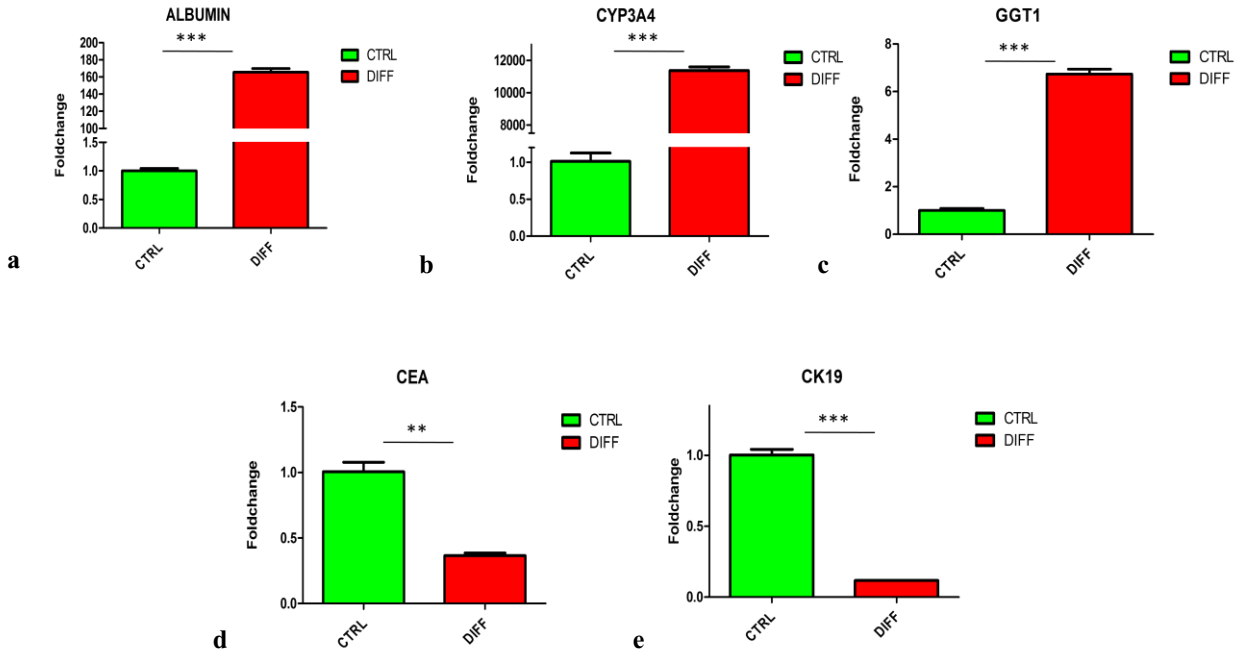


Figure 10: Gene expression levels in HepaRG cells. Gene expression levels of target genes, before and after four weeks treatment. DMSO-treated cells show an increased expression of ALBUMIN (a), CYP3A4 (b) and GGT1 (c), while a reduced expression of CEA (d) and CK19 (e). * = $p<0.05$, ** = $p<0.01$, *** = $p<0.001$.

It is possible to appreciate a high foldchange in ALBUMIN, CYP3A4 and GGT1 expression levels (Fig. 10 a-b-c) in DIFF cells compared to CTRL cells; on the other hand, the expression of CEA

and CK19 is decreased in DIFF cells (Fig. 10 d-e). This means that the differentiation process induced the commitment of progenitor cells toward hepatocyte and cholangiocyte-like cells, correctly.

3.6. Identification of Nrf2 cellular localization by confocal microscopy

1.5 * 10^5 cells/well were seeded on the glass coverslip in a 24-multi wells plate. The day after, cells were washed three times with PBS, fixed with 4% paraformaldehyde for 10 min at RT, and washed again two times with PBS. Cells were firstly permeabilised with PBS + 0.1% X-100 Triton (Fluka, 93418) for 10 min, then blocking buffer (3% BSA (Sigma, A7906) + 0.3M Glycine (Sigma, G-7126-500-50)) was added for 30 min at RT. Subsequently, cells were treated for 1 h and a half with anti-Nrf2 primary antibody (AbI, Abcam, ab31163) at RT, and then washed 3 times with PBS. Cells were then labelled with AlexaFluor 488-conjugated secondary antibody (AbII, Abcam, ab150073) in the dark for 1h at RT. Nuclei were counterstained with DAPI included in the mounting medium (Abcam, ab104139). Cells were analysed by a Nikon Eclipse Ti-E confocal microscope.

3.7. Flow cytometry

Phenotype changes, cell cycle and mitochondrial membrane potential were investigated in HepaRG cells by flow cytometry analysis, using FlowSight Cytometer (Amnis, Merck Millipore) and IDEAS Software.

3.7.1. Cell markers

The markers investigated are intracellular or expressed on the surface of both undifferentiated and differentiated cells (hepatocytes/cholangiocytes), as detailed below:

- CD34 (130-081-002) - single-chain transmembrane glycoprotein expressed in human stem and progenitor cells, but absent in differentiated cells;
- CD49a (α1 integrin) (130-101-397) - transmembrane glycoprotein which forms VLA-1 heterodimer complex, the collagen and laminin-1 cell receptor, with CD29 (β1 integrin). It is expressed typically in hepatocytes lineage;
- CD49f (integrin α6) (130-097-246) - transmembrane glycoprotein which forms VLA-6 heterodimer complex with CD29, while α6-β4 complex with CD104 (β4 integrin), both necessary as laminin cell receptors. CD49f expression is observed in biliary cells;
- CD184 (CXCR4 or fusin) (130-098-354) - G protein-coupled 7 transmembrane helixes receptor expressed in progenitor cells;
- EpCAM (CD326) (130-091-253) - epithelial adhesion molecule expressed on the basolateral surface in epithelial cells. Its expression is tipically observed in differentiated epithelial cells;
- Cytokeratins (CK) (130-080-101) - proteins constituting intermediate filaments of the cytoskeleton. Particularly, the antibody used recognizes CK7, considered as marker of liver progenitors.

To study the phenotype of HepaRG cells, antibodies against CD34/CD184(CXCR4)/ CD49a/CD49f/CD326(EpCam) (PE-labelled) and CK (FITC-labelled) antigens were purchased by Miltenyi Biotec.

Briefly, 1.0 * 10^6 cells/sample were resuspended in PBS and left 10 min at 4°C in the dark; then, cells were centrifuged at 300g for 10 min, and washed twice with PBS. Finally, samples were resuspended in PBS and analysed by flow cytometry.

Cells labelled with anti-CK AbI were permeabilised with PBS + 0.1% X-100 Triton before staining.

The expression of differentiation markers in HepaRG cells was evaluated before and after the treatment with DMSO.

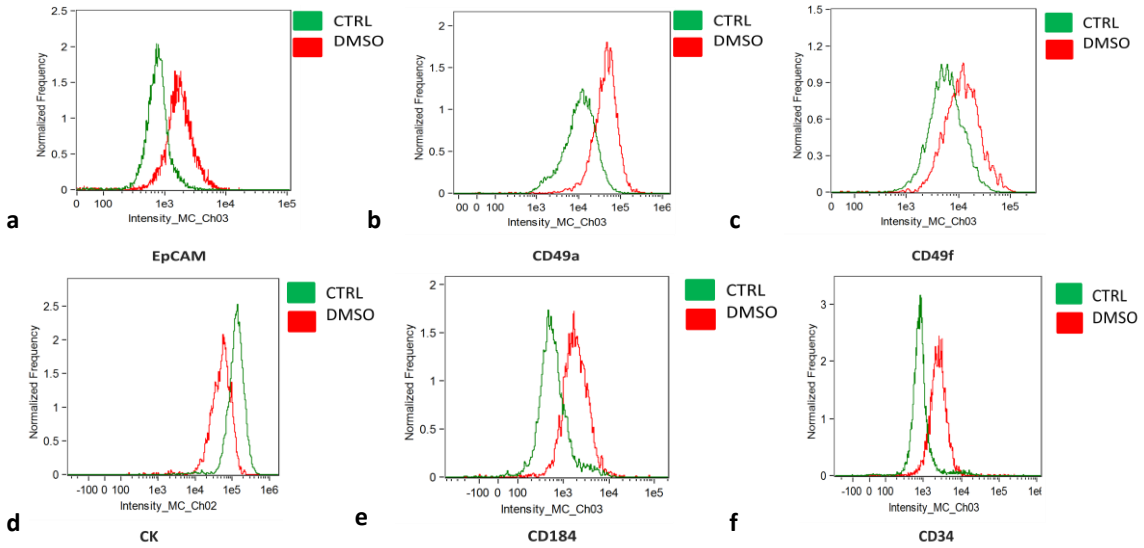


Figure 11: Study of HepaRG phenotype by flow cytometry. Histograms show the expression of markers to study phenotype of both CTRL (green) and DIFF cells (red).

As shown in Fig. 11 a-b-c, the expression of EpCAM, CD49a and CD49f increases in DIFF cells, indicating a trans-differentiation toward hepatic lineages; nevertheless, the differentiation process is not complete, as demonstrated by the expression of CD184 and CD34 (Fig. 11 e-f).

3.7.2 Cell cycle analysis

Cells were washed with PBS and centrifuged at 300g for 3 min; pellet was resuspended in the medium and cells counted. The supernatant was discarded and cold EtOH was added to the pellet; after this, the samples were vortexed and soon preserved in -20°C. The day after, cells were centrifuged at 300g for 3 min and stained with DRAQ5 (5uM, BioLegend, 424101) for 15min, at RT. When the incubation time ended, cells were washed and resuspended in PBS to be analysed by flow cytometry.

3.7.3 Mitochondrial membrane potential ($\Delta\Psi$) measurement

JC-10 (Abcam, ab112133) was used as a probe to measure mitochondrial membrane potential. It enters mitochondria and changes its colour from green to orange, as $\Delta\Psi$ increases. This property is due to the JC-10 aggregation upon membrane polarization that causes shifts in emitted light from 520 nm 570 nm.

5.0 * 10^5 cells were incubated with JC-10 for 15min at 37°C in the dark before flow cytometry analysis. In the resulting graph, it is possible to appreciate two cellular populations: the upper one consists of cells with higher ΔΨ, while cells with lower ΔΨ are represented in the bottom panel.

DIFF (Fig. 12 b) cells showed a higher ΔΨ than CTRL cells (Fig. 12 a).

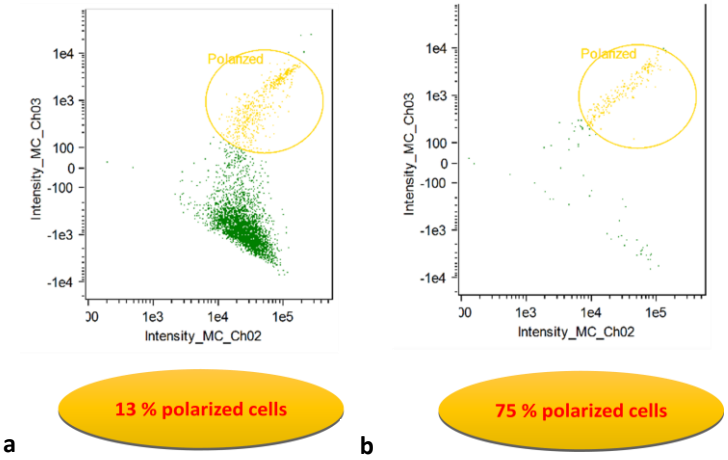


Figure 12: Mitochondrial membrane potential. Comparison between CTRL (a) and DIFF (b) cells in comparing mitochondrial membrane potential measurements.

3.8. Mitochondrial Respirometry

5,0 * 10^6 HepaRG cells were washed with PBS and resuspended in 10mM KH_2PO_4, 27mM KCl, 1mM $MgCl_2$, 40mM HEPES, 0.5mM EGTA buffer (pH 7.1), and assayed for O_2 consumption by oxymetry at 37°C under continuous stirring. Oligomycin (8µg/ml) was added followed by the addition of valinomycin (2µg/ml) after 5 min. The rates of oxygen consumption (OCR) were corrected for 3mM KCN-insensitive respiration and normalized to the cell number (Scrima, 2014).

The analysis showed higher mitochondrial respiration in DIFF rather than CTRL cells (Fig. 13). This condition is typical of cells that are differentiating or are already differentiated, while undifferentiated cells are characterized by lower mitochondrial respiration (di Bello, 2018) (Fig. 13 b).

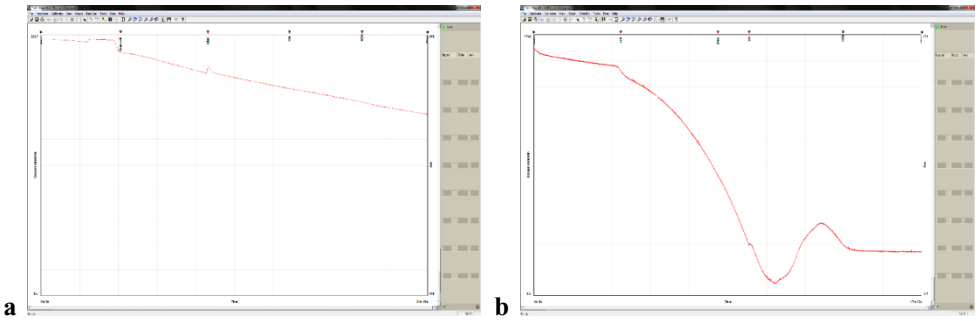


Figure 13: Measurements of oxygen consumption. Cellular respiration in CTRL (a) and DIFF (b) cells.

These data are in line with ΔΨ data, meaning that DIFF cells are more committed than CTRL cells.

3.9. DIR labelling

1, 1' - Dioctadecyl - 3, 3, 3', 3'- Tetramethylindotricarbocyanine Iodide (DIR, Thermo Fisher, D12731) is a near IR fluorescent, lipophilic dye, quite photostable when incorporated into membranes: once applied to cells, the dye diffuses within the plasma membrane. This dye is useful for *in vivo* cell tracking; however, we first tested its toxicity *in vitro*.

To evaluate the highest non-toxic concentration, cells were labelled with DIR. At two different final concentrations (5 and 2.5uM). 1.0 * 10^5 cells/sample were incubated for 20 min with DIR at 37°C in the dark. When the incubation time ended, cells were washed with PBS and counted with Trypan Blue (Sigma, T8154) to measure cell viability. As shown in figure 14, the final concentration of 2.5uM was not toxic for HepaRG cells.

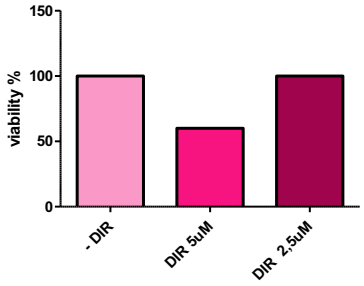


Figure 14: DIR staining. To test DIR toxicity, cells were labelled with two different concentration of DIR, 5 and 2.5uM, respectively. After 20min incubation, cell viability was evaluated.

3.10 HepaRG cell transplantation

SCID Beige mice (Charles River) were chosen as animal model. These mice are characterized by severe immunodeficiency affecting B and T lymphocytes, and they are also defective in natural killer (NK) cells.

To evaluate the intrahepatic diffusion of cells after transplant, 1.0 * 10^6 CTRL or AEM1-treated cells were labelled with DIR immediately before intrasplenic injection (Ezzat, 2012). Migration of transplanted cells from the spleen to the liver was detected after 30 min and 24h by In vivo F-PRO (Bruker).

Fig. 15a shows in red the site of transplantation (spleen), while no signal was detectable in the liver (Fig. 15 b); cellular homing was again evaluated after 24h from transplant, showing the migration from spleen (Fig. 15 c) to liver (Fig. 15 b).

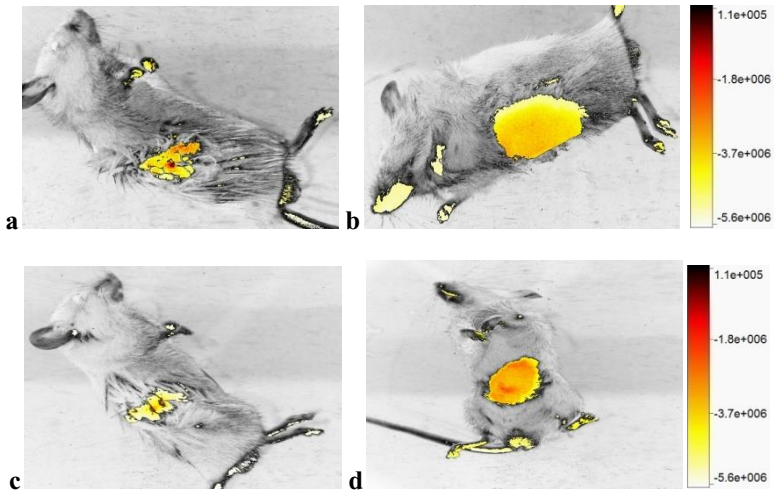


Figure 15: Cell transplant and DIR tracking. 30 min after transplant cellular tracking was evaluated in both spleen (a), the site of transplant, and liver (b); the same was repeated after 24h (c-d).

When livers were isolated (4 weeks after the treatment), the presence of transplanted HepaRG was evaluated in the spleen and the liver. As shown in fig. 16, a high fluorescence signal was detected in the liver but not in the spleen.

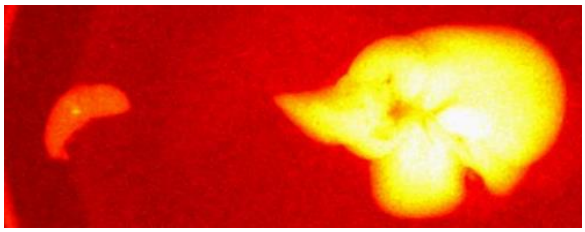


Figure 16: Evaluation of cellular migration. When the treatment to induce mice injury ended, the spleen and the liver were isolated and evaluated to verify whether transplanted cells migrated from spleen to liver.

3.10.1 Induction of liver injury

The day after HepaRG cell transplantation, liver injury was induced by administration of an anti-Fas monoclonal Ab (Jo2/CD95, BD Bioscences), once weekly for four weeks. Mice were divided in the following groups:

- untransplanted (n = 4);
- transplanted with CTRL HepaRG cells (n = 4);
- transplanted with AEM1-treated HepaRG cells (n = 4).

At the end of the fourth week, animals were sacrificed, the blood drawn, and the livers excised to be frozen at -80°C; some liver samples were fixed in 4% formalin for immunohistochemistry.

Isolated liver samples were frozen at -80°C.

3.11 Immunohistochemistry

Immunohistochemical analysis on 4-µm serial sections was performed by using Ventana Benchmark® XT autostainer and standard linked streptavidin-biotin horseradish peroxidase technique (LSAB-HRP), according to the best protocol for each antibody used in our laboratory: primary mouse polyclonal antibody anti-human Ck19 diluted 1:100 in PBS (Ventana, 760-4281); primary mouse monoclonal antibody anti human Glypcan3 diluted 1:300 in PBS (Ventana, 790-4564) and incubated overnight. Negative control slides without primary antibodies were included

for each staining. Same protocol was used for HNE (Abcam, ab46545) and Nrf2 (Abcam) analysis. The results of the immunohistochemical staining were evaluated separately by two observers. In each tissue section 10 representative high-power fields (HPFs) were analyzed at optical microscope (Zeiss Axioscope).

3.12 Serum transaminases

Aspartate aminotransferase (AST) and alanine aminotransferase (ALT) are used as markers of liver disease associated to hepatocellular necrosis. Their serum levels are measured by UV kinetic assay.

AST catalyses transamination of L-aspartate (240 mmol/L) and 2-oxyglutarate (12 mmol/L) leading to the conversion into L-glutamate and oxaloacetate. The last is converted into L-malate by dehydrogenase malate (MDH) (≥ 0,9 mmol/kU/L), while NADH is converted into NAD^+. The decrease of the absorbance (A) is due to the reduction of NADH (λ = 340nm) and its decrease is proportional to AST amount in the sample.

ALT is an amino transferase which catalyses the reversible transformation of α-chetoacids into aminoacids by transferring amines groups. It transfers the alanine (500mmol/L) aminic group to 2-oxyglutarate (12 mmol/L) originating pyruvate and glutamate. The adding of pyridoxal phosphate guarantees maximum catalytic action of AST. Pyruvate is then reduced to L-lactase by LDH (≥ 1.8 kU/L) in the presence of NADH (0.20 mmol/L), which is instead reduced to NAD^+.

4. RESULTS

4.1. Models of HPCs activation are characterized by hepatic oxidative stress

Chronic cholestatic or hepatocellular liver injury was induced by feeding C57Bl-6 mice a Dipin 3,5-diethoxycarbonyl-1,4-dihydrocollidine (DDC) or a methionine-choline deficient (MCD) diet, respectively. Immunohistochemical analysis showed that oxidative damage, analysed via detection of hydroxynonenal in the liver, was higher in chronic hepatitis rather than controls (Figure 17).

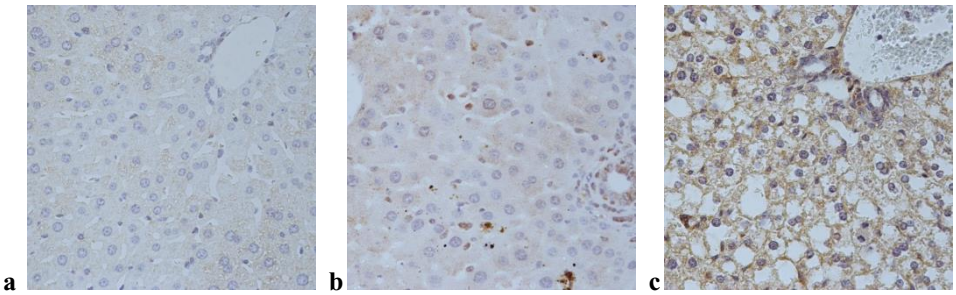


Figure 17: Chronic liver injury is characterised by oxidative damage. Liver samples from healthy mice (a) or animals fed Dipin 3,5-diethoxycarbonyl-1,4-dihydrocollidine (DDC) or a methionine-choline deficient (MCD) diet to induce cholangitis (b) or steatohepatits (c), were incubated with anti-hydroxynonenal primary antibody. 40X magnification.

4.2. Oxidative stress activates HPCs

To reproduce *in vitro* the oxidative stress condition which occurs in case of chronic liver injury, primary murine hepatic progenitor cells were exposed to different concentrations of H_2O_2. This exposure revealed that sublethal amounts of H_2O_2 induced the activation of cell cycle and promoted replication. Indeed, hepatic progenitor cells treated with H_2O_2 exhibited higher percentage of G2 phase as compared to CTRL (35.2% vs 26.1%, Fig. 18 a-b); the addition of a free radical scavenger such as N-acetylcysteine (NAC) resulted in unaltered G2 phase (Fig. 18 c-d). Taken together, these results suggest that altering redox balance by the induction of a pro-oxidative environment may activate HPCs.

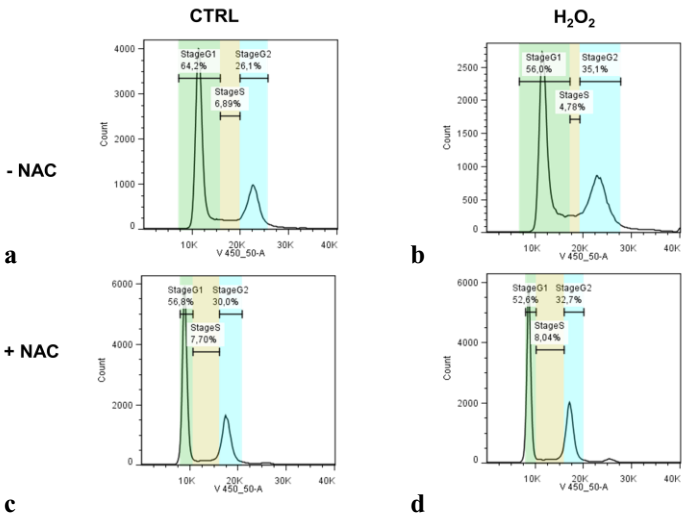


Figure 18: H_2O_2 activates HPCs. Effects of H_2O_2 on cell cycle: H_2O_2-treated cells (b) are characterized by higher percentage of G2 phase compared to the CTRL (a); the effects are cancelled after NAC addition (d), indeed in this case there are no differences compared with CTRL (c).

4.3. Nrf2 is constitutively activated in HPCs to maintain stemness, while it is inhibited in case of HPCs activation

The link between H_2O_2 exposure, Nrf2 activation and primary HPCs quiescence was demonstrated by immunocytochemistry. Particularly, H_2O_2 treatment was associated to the presence of Nrf2 into the nucleus and HPCs proliferation, as shown in figure 19 c; on the contrary, co-treatment with H_2O_2+NAC resulted in Nrf2 cytoplasmic localization and no proliferation (Fig. 19 f).

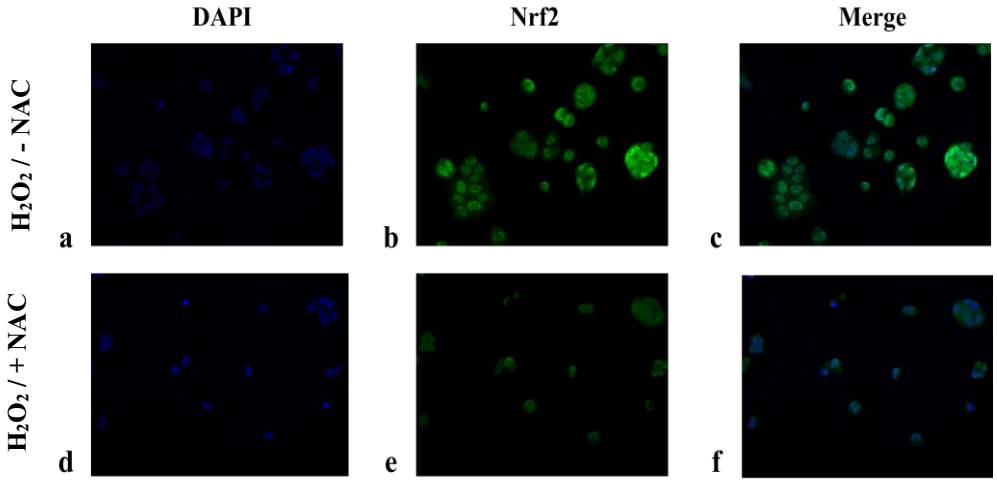


Figure 19: H_2O_2 exposure induces Nrf2 nuclear localization in HPCs. When HPCs are treated with H_2O_2, Nrf2 is located into the nucleus (b-c), while it is cytoplasmic during H_2O_2 + NAC co-treatment. (a) and (d) = DAPI staining for nuclei; (b) and (e) = anti-Nrf2 AbI + 488 AlexaFuor AbII; (c) and (f) = merge. 200X magnification.

The activation/deactivation state of Nrf2 in the hepatic stem cell niches was demonstrated by immunohistochemistry. Particularly, in healthy liver, Nrf2 reveals to be activated only in the niches (Fig. 20 a, red arrows). On the contrary, in case of both cholestatic (Fig. 20 b) and hepatocellular (Fig. 20 c) injury, Nrf2 is activated in the parenchyma but not in the niches.

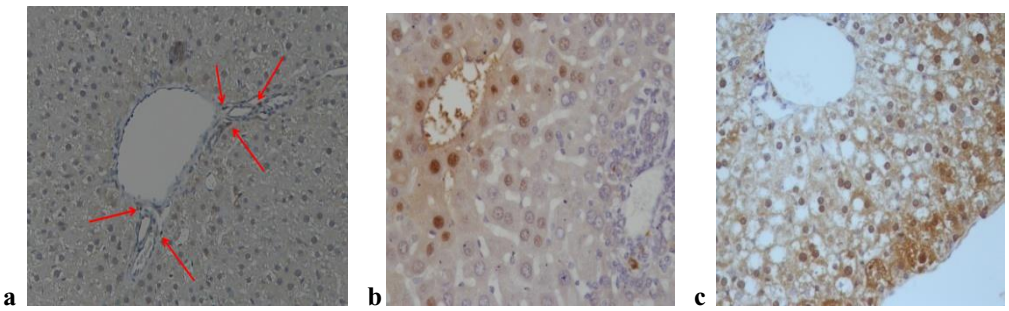


Figure 20: Nrf2 activation in the liver. In the healthy liver, Nrf2 signal is activated in the niches but not in the parenchyma (a); on the contrary, Nrf2 is active in the parenchyma but not in the niches during both cholestatic (b) and hepatocellular injury (c).

4.4. Nrf2 silencing promotes HPCs differentiation

To underline the key role of Nrf2 in determining quiescence and/or proliferation/differentiation of liver stem cells, we used a specific siRNA for gene silencing in HPCs (Fig. 21).

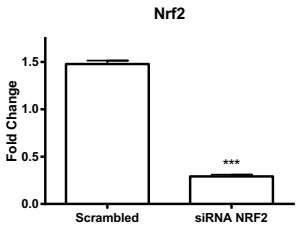


Figure 21: Nrf2 silencing. Real Time PCR evaluation of Nrf2 silencing by siRNA.

Notch and Wnt pathways modulate the differentiation process of HPCs, determining the commitment toward cholangiocytes or hepatocytes, respectively (Boulter, 2012; Boulter, 2013). Thus, to evaluate the effects of Nrf2 inhibition in the activation and/or differentiation process of HPCs, the expression of Notch and Wnt downstream genes was studied. Particularly, Hes1 and Hes5 were considered as Notch-downstream genes, while Sox9, Myc, Axin2, Hnf1a and Hnf4 were studied as Wnt-downstream genes.

Fig. 22 shows that the expression of all the Notch and Wnt-downstream genes evaluated was increased in HPCs subjected to Nrf2 silencing, suggesting the importance of this transcription factor in the activation of HPCs.

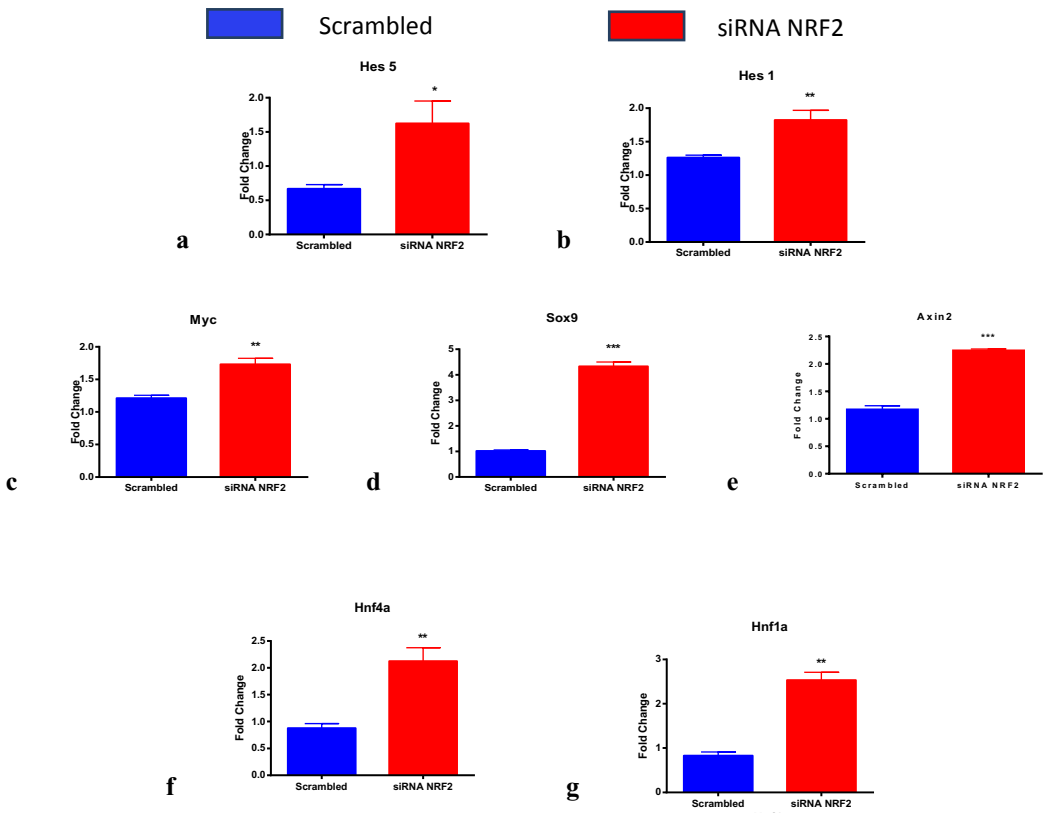


Figure 22: Gene expression analysis. Nrf2 was silenced and the expression of Notch and Wnt-downstream genes was evaluated by Real Time. Hes1 (a) and Hes5 (b) are Notch-downstream genes, while Sox9 (c), Myc (d), Axin2 (e), Hnf1a (f) and Hnf4 (g) are Wnt-downstream genes. * = $p<0.05$, ** = $p<0.01$, *** = $p< 0.0001$.

4.5. Nrf2 nuclear internalization is reduced in trans-differentiated HepaRG cells

We aimed to verify whether the inhibition of Nrf2 *in vitro* would improve the transplant efficiency of HPCs *in vivo*. To track HPCs in rodent models, we moved to HepaRG cells, a human immortalized cell line able to trans-differentiate toward bipotent progenitor cells when stimulated with DMSO.

Nrf2 is highly active in progenitor cells, to keep low levels of intracellular ROS, since it migrates from cytoplasm into the nucleus to promote the expression of antioxidant factors.

To verify the impact of Nrf2 on the trans-differentiation process, its intracellular localization was evaluated by flow cytometry and confocal microscopy after DMSO treatment. In CTRL cells (Fig. 23 a), Nrf2 shows a perinuclear localization; indeed, during the trans-differentiation process induced by DMSO, the pattern of Nrf2 localization is cytoplasmatic (Fig. 23).

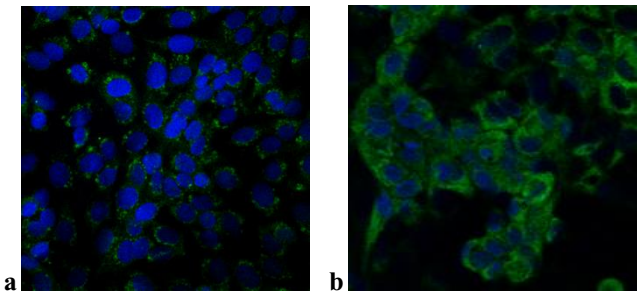


Figura 23: Intracellular localization of Nrf2. In CTRL cells (a), Nrf2 (green) has a perinuclear localization, while it is cytoplasmic in DMSO-treated cells (b). 60X magnification.

4.6. Pharmacological inhibition of Nrf2 promotes trans-differentiation of HepaRG cells

Since the inhibition of Nrf2 expression by siRNA lasted after 48h, we blocked Nrf2 by the chemical compound AEM1, which does not alter either Nrf2 or Keap1 expression (Bollong, 2015).

To study the effects of AEM1 treatment in HepaRG cells, gene expression levels in CTRL and AEM1-treated cells were evaluated.

The expression of ALBUMIN, CYP3A4 and GGT1, which are characteristic of liver mature cells, was increased, while CEA and CK19 was reduced in AEM1-treated cells with respect to CTRL (Fig. 24).

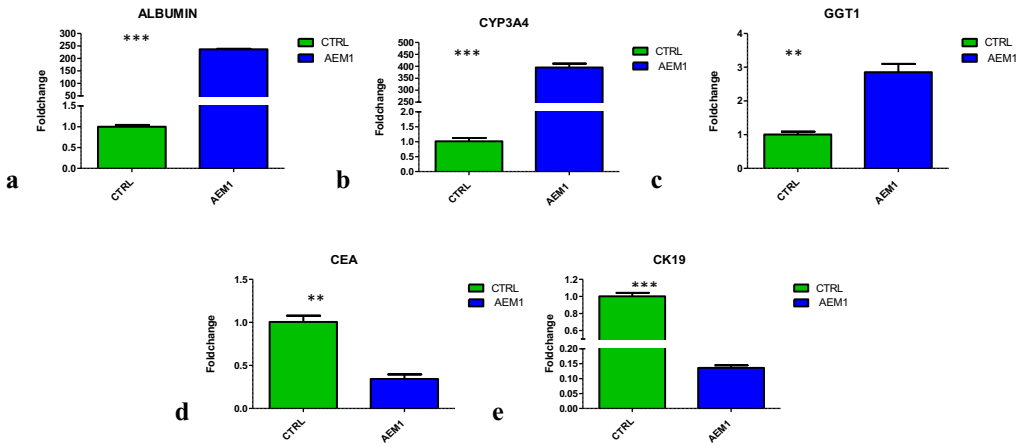


Figure 24: Gene expression analysis after AEM1 treatment. Gene expression levels of target genes, tipically expressed in hepatic progenitors or mature cells, after inhibiting Nrf2 activity by AEM1 treatment. * = $p<0.05$, ** = $p<0.01$, *** = $p<0.001$.

AEM1-treated cells were further characterized by flow cytometry in order to evaluate their phenotype.

The expression of EpCAM (a target of differentiated epithelial cells) was increased in AEM1-treated with respect to control HepaRG cells (Fig 25 e); on the contrary, the expression of CK was reduced (Fig. 25 f), as already observed by qPCR (Fig. 24 f).

CD49a and CD49f are markers of hepatocytes and cholangiocytes, respectively: their expression was increased in AEM1-treated rather than control HepaRG cells (Fig. 25 b-c).

Moreover, the expression of CD34 and CD184, both markers of stem and progenitor cells, was increased in AEM1-treated with respect to control HepaRG cells (Fig. 25).

Taken together, these results suggest that the inhibition of Nrf2 by AEM1 treatment induces the trans-differentiation of HepaRG cells.

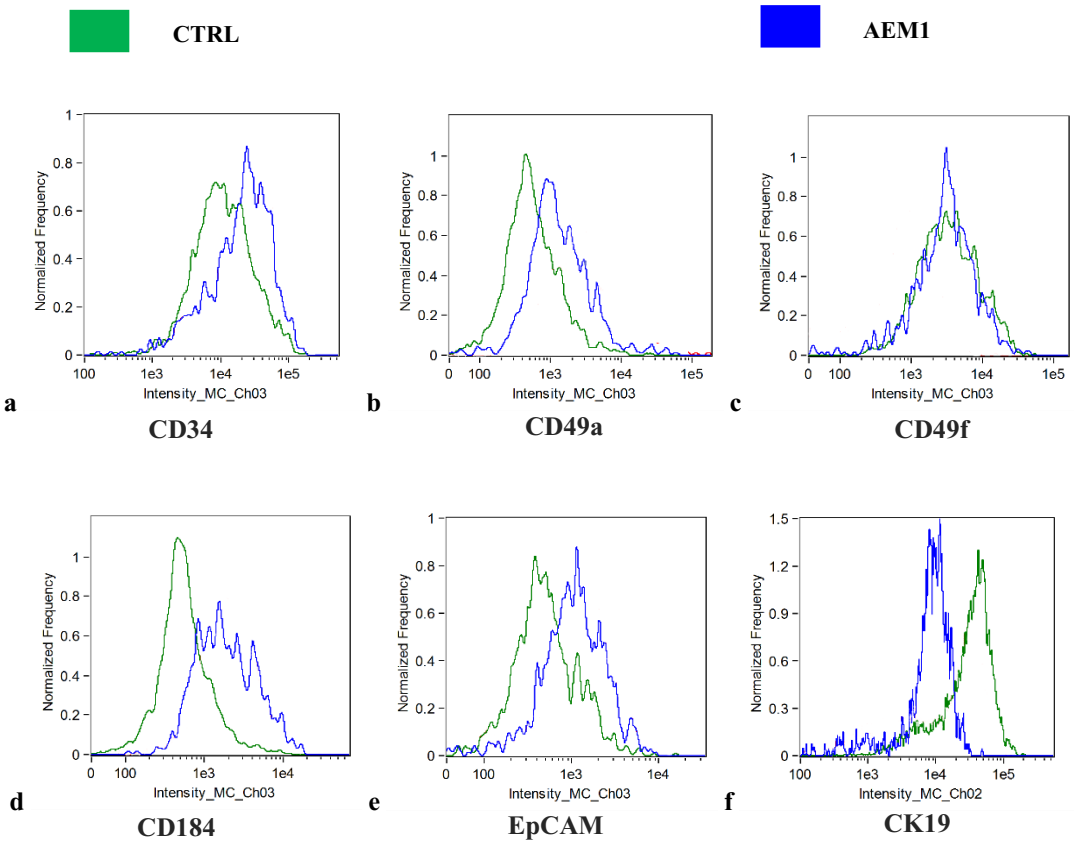


Figure 25: Study of phenotype by flow cytometry. a-b-c-d-e-f) Expression of phenotype markers observed in control cells (green) and AEM1(blue) treated cells.

4.7. AEM1 treatment increases mitochondrial respiration and oxygen consumption in HepaRG cells

Since the differentiation process of stem/progenitor cells is characterized by the increase of mitochondrial metabolism, to further confirm the commitment of HepaRG cells following AEM1 treatment we evaluated both mitochondrial respiration (oxygen consumption) and membrane potential.

The graphs below show a considerable higher mitochondrial respiration in AEM1-treated cells compared to the CTRL HepaRG cells (Fig. 26).

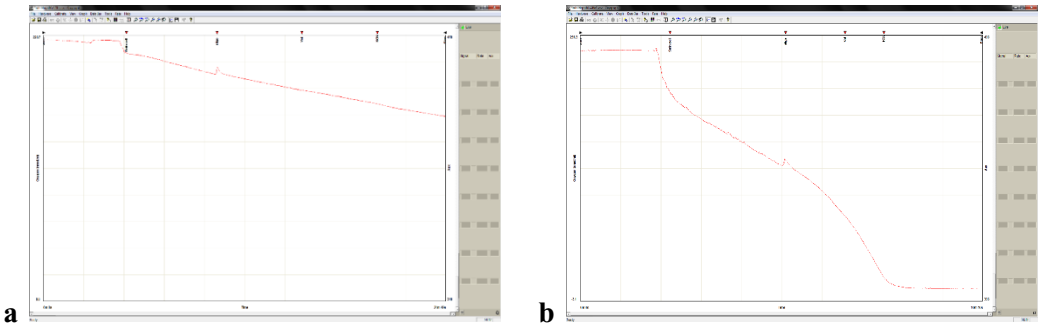


Figure 26: Measurements of oxygen consumption. Curves represent cellular respiration in CTRL (a), and AEM1-treated cells (b).

These data indicate that CTRL HepaRG cells (Fig. 26 a) are characterized by low oxygen consumption with respect to AEM1-treated cells, which show higher mitochondrial respiration (Fig. 26 b).

Mitochondrial transmembrane potential was measured by using a fluorescent probe (JC10). Fig. 27 shows that the percentage of HepaRG cells with higher mitochondrial ΔΨ is increased in the AEM-1 treated group when compared to CTRL.

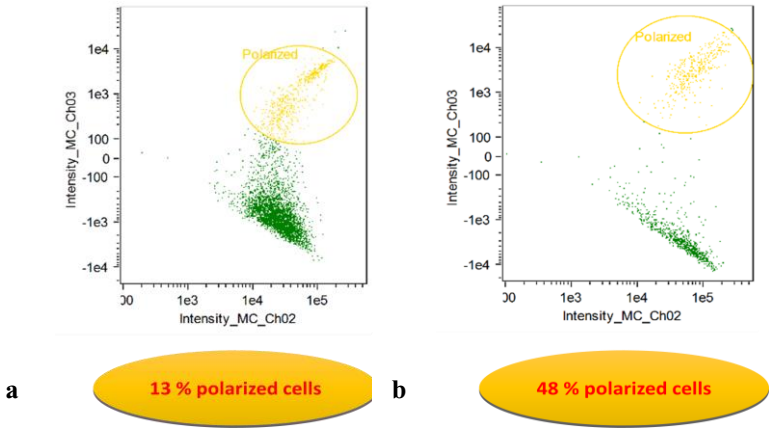


Figure 27: Mitochondrial membrane potential. The graphs represent two different cell populations: the upper one (yellow colour) is characterized by polarized cells, with higher ΔΨ, while the lower one (green colour) shows cells with low ΔΨ. (a) CTRL cells showed lower percentage of polarized cells than (b) AEM1-treated cells.

4.8. Transplant of AEM1-treated HepaRG cells into SCID-beige mice improves the efficiency of differentiation toward functional hepatocytes

SCID-beige mice were used as animal models for HepaRG transplant recipient. Both CTRL and AEM-1-treated HepaRG cells were injected intrasplenically in mice; starting from the following day, anti-Fas mAb (Jo2) was intraperitoneally injected once weekly for 4 weeks, to induce chronic liver injury (Jiang, 2010).

To evaluate hepatocellular necrosis, serum levels of both alanine (ALT/GPT) and aspartate (AST/GOT) aminotransferase were measured. Liver injury caused by Jo2 injection was characterized by increased GOT and GPT levels in untransplanted mice; no changes in the serum level of both transaminases were observed in mice transplanted with CTRL HepaRG cells, while they were both significantly reduced in those transplanted with AEM1-treated HepaRG cells (Fig. 28).

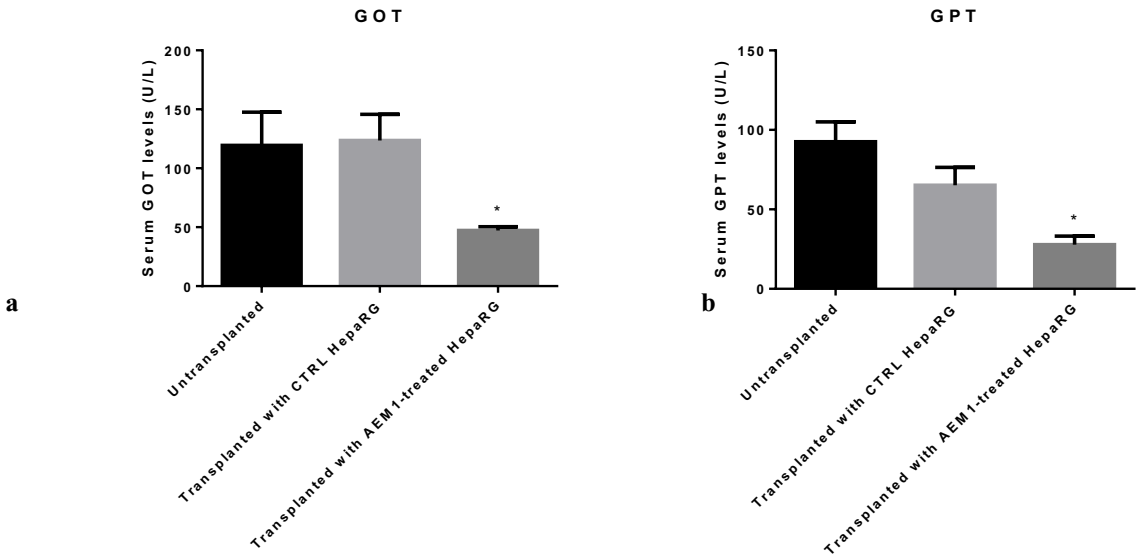


Figure 28: Transaminanases assay. After the induction of liver injury, mice were divided in three groups: one group was no transplanted, while the other two groups were transplanted with CTRL or AEM1-treated HepaRG cells. From the following day of transplant, injury was induced for 4 weeks and, when treatment ended, serum levels of transaminases (GOT and GPT) were evaluated.

To evaluate the functional effects of different cell transplantation on liver injury, gene expression analysis was performed to study the expression of human ALBUMIN and GGT1. The activation of

progenitors and consequent liver regeneration after injury was underlined by the increased expression of both human ALBUMIN and GGT1 in mice transplanted with AEM1-treated HepaRG cells, compared with those transplanted with control HepaRG cells (Fig. 29).

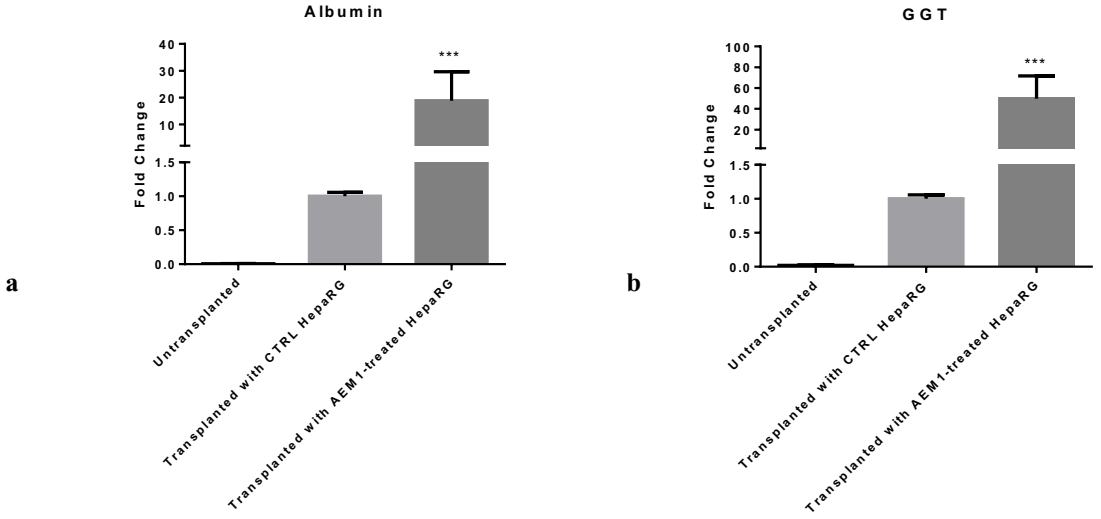


Figure 29: Gene expression analysis after HepaRG transplant. The expression of human ALBUMIN and GGT1 were evaluated when Jo2 treatment ended. The expression of both genes was higher in AEM1. * = $p<0.05$, ** = $p<0.01$, *** = $p<0.001$.

Immunohistochemical staining with anti-human CK19 and Glypican 3 showed that HepaRG cells engrafted the mouse liver but only those treated with AEM-1 differentiated and replaced damaged tissue (Figure 30).

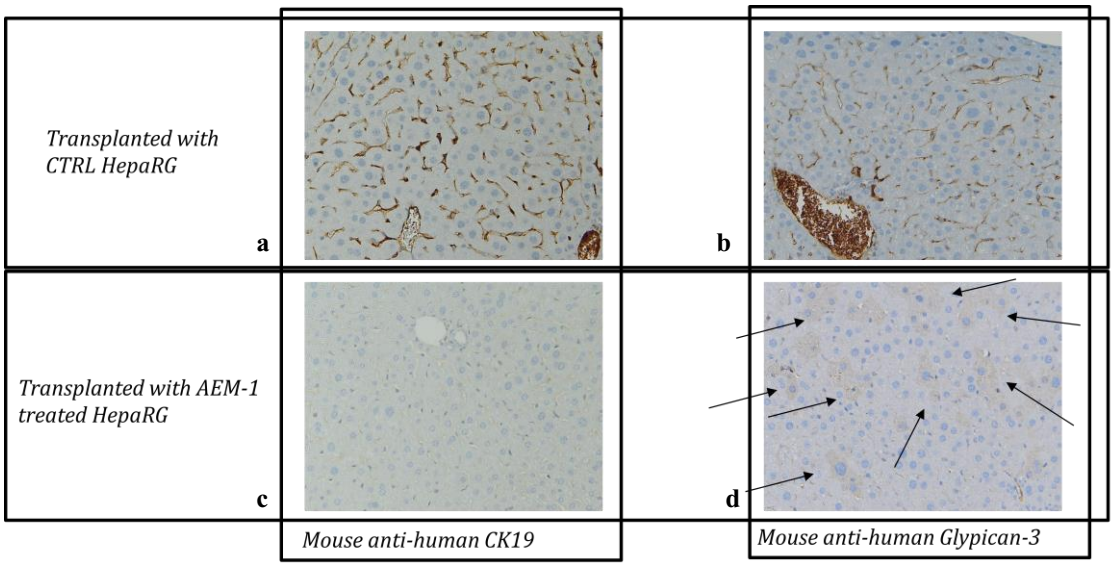


Figure 30: Immunohistochemistry analysis. Sections of transplanted livers were analysed to evaluate the engraftment of CTRL (a-b) and AEM1-treated HepaRG transplanted cells. The presence of HepaRG cells was studied by using Abs against human markers, such as Ck19 (a-c) and Glypcan3 (b-d). 200X magnification.

5. DISCUSSION

This study demonstrates the role played by the transcription factor Nrf2 in modulating the activation/differentiation process of hepatic progenitor cells (HPCs).

HPCs activation occurs in rodent models of both cholestatic and hepatocellular liver injury, replacing the damaged tissue (Boulter, 2013). The present data evidence that oxidative stress is associated with the activation and proliferation of HPCs in such models of chronic liver injury.

Nrf2 is a redox-sensitive transcription factor that activates cytoprotective signalling pathways against oxidative, inflammatory and pro-apoptotic damage through the induction of several genes encoding phase II detoxifying enzymes and antioxidants (Alam, 1999, Motohashi and Yamamoto, 2004). The activation of Nrf2 occurs in viral, alcoholic, non-alcoholic and toxic hepatitis, playing a crucial role in the protection of hepatocellular elements from oxidative damage (Tang, 2014). Our data show that Nrf2 is activated in the hepatic parenchyma of chronic disease. On the contrary, Nrf2 is activated in staminal niches from healthy liver, but de-activates in chronic liver disease models.

For instance, the main role played by the Keap1-Nrf2 system in intestinal stem cells (ISCs) has been well-characterized; indeed, differentiation of ISCs in Drosophila is triggered by Keap1 inhibition, while Nrf2 pathway is constitutively active to maintain stemness (Hochmuth, 2011). Nrf2 signalling pathway is relevant in determining pluripotent stem cells reprogramming (Hawkins, 2016), neural stem cells fate (Pistollato, 2017; Robledinos-Anton, 2017), and the differentiation of mesenchymal stem cells into osteoblasts (Tao, 2016; Yuan, 2017). Similarly, the present study suggests that Nrf2 is determinant in the modulation of activation/differentiation process of stem/progenitor liver cells.

Exposure of HPCs to reactive species induces specific changes in the cell cycle typical of replicative events, and triggers the expression of genes downstream the Notch and the Wnt pathways, typical of committed cells. It is then conceivable that, in the quiescent state, Nrf2 activation contributes to the maintenance of low levels of oxidants in the hepatic niche; on the

contrary, during chronic liver disease Nrf2 de-activates to allow a pro-oxidant environment in the niche and to promote the activation of progenitors.

Our experiments demonstrate that both the genetic and the pharmacological inhibition of Nrf2 promotes the proliferation and differentiation of HPCs. In the present study, Nrf2 was pharmacologically inhibited by AEM1. Together with its structurally related compound, named AEM2, AEM1 is also referred as selective inhibitor of sirtuin 2 (SIRT2), a p53 deacetylase (Hoffmann, 2013). SIRT2 inhibition by AEM1 or AEM2 increases SIRT2-dependent deacetylation and induces the expression of p53 targets, promoting apoptosis and cell cycle arrest. We excluded the effects of AEM1 on SIRT2 in our experiments because of the following reasons:

- AEM1 has a weaker inhibitory effect on SIRT2 than AEM2;
- AEM1 is *per se* able to induce apoptosis only in less than 5% of the entire cellular population considered, when treated at the final concentration of 1uM (the same we used); more significant results, in terms of cell cycle arrest and apoptosis induction in *in vitro* studies described by Hoffman et al. (Hoffmann, 2013), are observed when 20uM final concentration of AEM1 is used.
- Furthermore, really high percentage of apoptosis (about 20%) is reported when AEM1 and AEM2 are associated with etoposide, an important DNA-damaging agent.

Nrf2 inhibition is associated with the high expression of Notch and Wnt-downstream genes, the two responsible factors in committing liver progenitors toward cholangiocytes and hepatocytes, respectively (Boulter, 2012; Boulter, 2013). Further, Nrf2 inhibition is able to drive the trans-differentiation of progenitor cells, which show phenotypical markers of hepatocyte-like and cholangiocyte-like cells.

Quiescent stem cells in the niches are characterized by low levels of reactive species and a reduced rate of oxidative phosphorylation, while both increase in differentiated cells. Several studies demonstrate the different metabolic phenotype between stem and differentiated cells, underlining

the shift from anaerobic to aerobic metabolism and the increased production of reactive species during the differentiation process. Particularly, HSCs residing in the bone marrow (BM) niches are surrounded by a hypoxic microenvironment, producing energy by high glycolysis rate rather than via oxidative phosphorylation (Suda, 2011). To respond to low O_2 levels, HIF1α reveals to be essential as transcription factor. Indeed, it plays an important role in keeping low levels of reactive species in NSCs, to maintain the quiescence under hypoxic conditions, promoting Wnt-β catenin system activation (Mazumdar, 2010; Suda, 2011). The present data demonstrate that Nrf2 inhibition in HPCs is associated with an increase in mitochondrial oxygen consumption and membrane potential.

HPCs may be used as an important source for liver cell transplantation. However, HPCs need to undergo maturation to become functional liver cells. Several investigations so far have described that the transplantation efficiency of HPCs is too low to produce sufficient numbers of functional mature hepatocytes (Sharma, 2008, Ichinohe, 2013). *In vitro* models of HPCs maturation, other than being useful for elucidating the differentiation mechanism of progenitors into mature liver cells, might improve the efficiency and biosafety profile of possible clinical applications for liver stem cell transplantation (Gridelli, 2012). We then verified whether HPCs maturation induced by Nrf2 inhibition would effectively enhance the effects of stem cell transplantation in terms of structural and functional recovery during chronic liver damage. To this, the human HepaRG cell line was transplanted in SCID-beige mice, characterised by a defective immune system to avoid the rejection of transplanted cells. Our data demonstrate that Nrf2 inhibition in HepaRG cells favours engraftment and re-population of damaged cells by transplanted elements. Furthermore, Nrf2 inhibition is associated with an *in vivo* functional outcome – production of albumin and GGT – in transplanted HepaRG cells.

In conclusion, this study demonstrates that the *in vitro* inhibition of Nrf2 at a pre-transplant stage in HPCs, other than proving the crucial role of this transcription factor for the activation/differentiation of progenitors, is promising in terms of cell engraftment and

differentiation improvement following transplantation. Even though it is not always possible to translate strategies used in animal models to human disease, this study paves the way for further investigation aimed at achieving clinical outcomes.

ABBREVIATIONS

ALD = alcoholic liver disease

ALT = alanine aminotransferase

AREs = antioxidant response elements

aSCs = Adult stem cells

AST = Aspartate aminotransferase

BM = bone marrow

CAT = catalase

CCl4 = carbon tetrachloride

CDE = choline-deficient, ethionine-supplemented

CFU-F = colony forming unit-fibroblasts

CoQ = coenzyme Q

DDC = 3,5-diethoxycarbonyl-1,4-dihydrocollidine

DILI = drug-induced liver injury

DPPA2 = developmental pluripotency-associated 2

DPPA4 = developmental pluripotency-associated 4

EGF = Epidermal Growth Factor

EGFR = EGF receptor

EpCAM = epithelial cell adhesion molecule

ESG1 = embryonic cell-specific gene 1

ETC = electron transport chain

FAK = focal adhesion kinase

FBS = Fetal Bovine Serum

FGF4 = fibroblast growth factor 4

fHSCs = foetal hematopoietic stem cells

fMSCs = foetal mesenchymal stem cells

FoxO = Forkhead box O

fSCs = Foetal stem cells

GDF3 = growth and differentiation factor 3

GPxs = glutathione peroxidases

GSH = reduced glutathione

GSK-3β = Glycogen Synthase kinase

GSSG = disulphure glutathione

H_2O_2 = hydrogen peroxide

HDFs = human dermal fibroblasts

hESCs = Human embryonic stem cells

HFLS = human fibroblast-like sinoviocyets

HGF = Hepatocyte Growth Factor

HIF = hypoxia-inducible factor

hiPSCs = human induced pluripotent stem cells

$HO^{\cdot}$ = hydroxyl radicals

HPCs = hepatic progenitor cells

HPFs = high power fields

HSCs = hematopoietic stem cells

HSCs = hematopoietic stem cell

HSCs = hematopoietic stem cells

hTERT = telomerase reverse transcriptase

ICAM-1 = intracellular adhesion molecule

IMM = inner mitochondrial membrane.

IMS = intermembrane space

IR = ischemia-reperfusion

ISCs = intestinal stem cells

JNK1 = c-Jun N-terminal kinase 1

Keap1 = Kelch-like ECH-associated protein 1

LPCs = Liver progenitor cells

MDA = malondialdehyde

MDH = dehydrogenase malate

MEF2C = Myocyte Enhancer Factor 2C

MSCs = mesenchymal stem cells

N_2O_3 = dinitrogen trioxide

NAFLD = non-alcoholic fatty liver disease

NASH = non-alcoholic steatohepatitis

NCAM = neuronal cell adhesion molecule

NF-kB = nuclear factor kappa-light-chain-enhancer of activated B cells

$NO^{\cdot}$ = nitric oxide

$NO_2^{\cdot}$ = nitrogen dioxide

Nrf2 = nuclear factor (erythroid-derived 2)-like 2

Nrx = Nucleoredoxin

NSCs = neuronal stem cells

O_2 = oxygen

$O_2^{\cdot -}$ = superoxide anions

PGC1α = Peroxisome proliferator-activated receptor gamma coactivator 1-alpha

PI3K = phosphatidylinositol-4,5-biphosphate 3-kinase

PIKE = phosphatidylinositol 3-kinase enhancer

PRDM16 = PR domain containing 16

REX1 = reduced expression protein 1

RNS = reactive nitrogen species

ROS = reactive oxygen species

SOD = superoxide dismutases

SSEA-3 = stage-specific embryonic antigen-3

SSEA-4 = stage-specific embryonic antigen-4

TAA = thioacetamide

TRA-1-60 = tumor rejection antigen-1-60

TRA-1-81 = tumor rejection antigen-1-81

TRF 1= telomeric repeat binding factor 1

TRF 2= telomeric repeat binding factor 2,

TRP53 = p53tumour suppressor

Trx = thioredoxin

TWEAK = TNF-like weak inducer

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