

***In vivo* reprogramming of hepatic myofibroblasts into hepatocytes attenuates liver fibrosis: back to the future?**

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Orthotopic liver transplantation (OLT) represents the standard curative treatment for human patients affected by an end-stage form of life-threatening liver disease involving, whatever the etiology (acquired or inborn) and development (acute or chronic), significant loss of hepatocytes and organ failure (1,2). The need for reliable therapeutic approaches alternative to OLT, in particular involving cell therapy, has become progressively more urgent because of the increasing worldwide incidence of liver diseases, particularly end-stage chronic liver diseases (CLDs), which is unfortunately associated to a significant shortage in organ availability (1,2). In the last two decades, several laboratories have performed studies designed to establish cell therapy procedures able to provide stable and reliable sources of functional hepatocytes or of hepatocyte-like cells (HLCs) to be used for repopulation of damaged liver parenchyma. To this purpose intra- or extrahepatic cells have been used in pre-clinical studies as a potential source of functional HLCs to be transplanted, including primary hepatocytes, liver sinusoidal-endothelial cells, mesenchymal stem cells, endothelial progenitor cells, liver progenitor cells and even macrophages. However, due to several limitations [reviewed in (3-6)], these procedures offered sometimes positive results in animal models but, with few limited exceptions, were not always translated to successful clinical therapeutic approaches.

A potentially more promising strategy, based on the development of the original studies of Yamanaka's group on the generation of induced pluripotent stem cells (iPSC) from adult cells (7,8), has led, using protocols involving either integrative or non-integrative strategies, to successful

reprogramming of either murine or human fibroblasts into HLCs (9-12). Whatever the protocol, HLCs derived from iPSC usually has the ability to: (I) express hepatocyte-related markers (albumin, α 1-anti-trypsin and CYP3A4); (II) secrete albumin; (III) express CYP3A4 activity; (IV) store glycogen; and (V) take up cholesterol and indocyanine green. Moreover, in most cases HLCs have been shown to have the potential to engraft murine liver and to be used as tools for disease modelling (9-12). Human induced, PSC-derived hepatocytes (hiHeps) can indeed represent a multi-purpose and potentially inexhaustible source of viable and functional HLCs. hiHeps from a healthy donor can be used for drug screening and toxicology, for allogenic therapy as well as a source of cells to be used in bioengineered livers. hiHeps from a patient donor can be used in specific disease modelling (i.e., modelling of HCV infection or malaria to investigate disease progression) or drug screening/toxicology but reprogrammed and corrected iPSC, than differentiated into iPSC-derived hepatocytes, may be in principle also used for autologous cell therapy (9-11). However, for any of these applications limitations have been described, ranging from the general problems still represented by the incomplete efficiency of either reprogramming or differentiation step to limitations related to the more specific application of choice, including cell function and viability of these iPSC-derived hepatocytes as well as homogeneity of the cell population obtained [see ref. (9)]. In addition, for allogenic or autologous cell therapy involving *in vivo* transplantation of iPSC-derived hepatocytes one should still consider the concerns associated with safety issues as well as the usually low

engraftment potential of transplanted iPSC-derived hepatocytes and their functionality at both short and long term (9-12). Whether cell therapy approach is specifically concerned, iPSC-derived hepatocytes have been used in a number of experimental models, but best results in terms of engraftment and repopulation have been reported in studies employing a protocol of acute liver failure performed in rather specific mouse models (for example fumarylacetoacetate hydrolase *Fah*^{-/-}/*Rag2*^{-/-}/*IL2r γ* ^{-/-} and urokinase (alb-uPA) severe combined immunodeficient transgenic mice), in which transplanted HLCs efficiently repopulated damaged livers because of the proliferative advantage over resident hepatocytes granted by the model itself (3,9-11). This need for a procedure offering a growth advantage/proliferation to transplanted HLCs, already outlined also by previous studies employing cells obtained from non-iPSC sources (3-6), is still a relevant problem for translation to humans.

More recently, some laboratories obtained HLCs by means of direct reprogramming of adult fibroblasts through overexpression of specific hepatocyte-related transcription factors in order to circumvent the induced pluripotent state (13-16). Murine fibroblasts have been reprogrammed to HLCs by either combining transduction of *Hnf1 α* , *Gata4*, and *Foxa3* with *p19(Arf)* inactivation (13) or through ectopic expression of *HNF4*, *Foxa1*, *Foxa2* or *Foxa3* (14). Similarly, reprogramming of human fibroblasts into HLCs was obtained by either forced ectopic expression of *HNF1A*, *FOXA3* and *HNF4A* (15) or by combining forced ectopic expression of *HNF1A*, *HNF4A* and *HNF6* with the maturation factors *ATF5*, *PROX1*, and *CEBPA* (16). The transplanted HLCs so obtained exhibited typical hepatocyte-like characters and properties and repopulated the livers of either *Fah*(-/-) mice (13-15) or Tet-uPA/*Rag2*(-/-)/ γ c(-/-) mice (16), significantly ameliorating liver functions and/or prolonging survival of recipients following an experimental acute liver failure protocol. However, most of limitations intrinsically related to the employment of transplanted HLCs for cell therapy, either for treatment of acute or, even more relevant, of CLDs, still remain and direct reprogramming until now has only been shown in culture settings.

In a recent outstanding experimental study Song and coworkers (17) have offered what can be really envisaged as a novel and very promising approach to counteract the progression of CLDs: the establishment of a procedure designed to obtain *in vivo* direct reprogramming of pro-fibrogenic hepatic myofibroblasts (MFs) into HLCs in

fibrotic murine livers (i.e., to concur to repopulate liver parenchyma), that also resulted in a significant attenuation of liver fibrosis. Two nice ideas were at the basis of this study. The first was of course to investigate the feasibility of *in vivo* reprogramming of adult cells into HLCs, as a rationale development of procedures found to be efficient in culture. However the most relevant idea was without any doubt the “choice” of pro-fibrogenic hepatic MFs as the cellular target for direct reprogramming in experimental conditions reproducing ongoing CLDs. Indeed, experimental and clinical literature data from the last two decades indicate that liver fibrogenesis is a dynamic and highly integrated process that, irrespective of the aetiology (viral, toxic, metabolic, autoimmune or genetically-related) drives the progression of CLDs resulting in a progressive accumulation of extracellular matrix components (i.e., liver fibrosis) (18-20). In this scenario, hepatic MFs, which mainly originate from a process of activation/trans-differentiation of hepatic stellate cells (HSCs), are widely accepted to exert a major pro-fibrogenic role. Persistent activation of hepatic MFs, unequivocally detected practically in any clinical condition of CLDs, irrespective of etiology, is believed to represent the result of a complex interaction between growth factors, cytokines, chemokines, reactive oxygen species (ROS) and other mediators (18-20). In the pro-fibrogenic environment these factors are released by, and interact with, any liver cell population, including damaged hepatocytes, activated inflammatory cells and MFs. This means that direct reprogramming of hepatic MFs does not simply originate HLCs contributing to repopulate damaged parenchyma and improve liver functions but at the same time can diminish the number of MFs, an event fundamental in ameliorating liver fibrosis (21), then realizing in a futable and even improved perspective the old dream of generations of hepatologists that devoted efforts to find a way to specifically block and/or revert *in vivo* activated hepatic MFs.

In their study, Song and coworkers (17) first successfully obtained HLCs, defined as iHep, by direct reprogramming murine MFs derived from activation of primary culture of HSCs by means of simultaneous overexpression of *FOXA3*, *GATA4*, *HNF1A* and *HNF4A* (i.e., by cloning cDNAs into a polycistronic lentiviral vector). The hepatocyte-like phenotype was obtained within 14 days and confirmed by several analyses, including the classical assays for Cyp3A and Cyp1A1 activity, glycogen storage, uptake of LDL and albumin secretion. In particular, Authors transduced cells at day 10 with a reporter lentiviral vector expressing dTomato under the transcriptional control of albumin promoter

and found that approx. 12% of cells expressed dTomato to confirm generation of HLCs. Following fluorescence-activated cell sorting, iHep showed increased transcription of typical markers of primary hepatocytes in parallel with disappearance of critical MFs markers. Of interest, iHep maintained genomic integrity and, although acquiring a hepatocyte-like gene expression profile, remained a distinct cell type when compared to primary murine hepatocytes.

Authors then established a complex lineage-tracing murine model suitable to detect *in vivo* reprogramming of non-parenchymal cells into iHep with the four transcription factors (4TFs). Authors used Gt(ROSA)26Sortm4(ACTB-tdTomato, -EGFP) mice (mT/mG mice), with all liver cells expressing tdTomato before Cre-mediated recombination; endogenous hepatocytes in mT/mG mice were then labeled following administration of adenoviral articles expressing Cre-recombinase under the control of the liver specific transthyretin (Ttr) promoter. Endogenous hepatocytes showed then positivity for EGFP membrane fluorescence and non-parenchymal cells for tdTomato membrane fluorescence. MFs were generated following a protocol for experimental fibrosis based on chronic administration of carbon tetrachloride (CCl₄) and, in order to overexpress the four TFs, a procedure requiring the administration of a p75NTRp-tagged recombinant adenoviral vector expressing the 4TFs from a polycistronic transgene cassette. *In vivo* targeting of MFs was obtained through portal vein administration of Ad.GFP-S11-NGFp, a vector modified to couple adenoviral fiber knobs with a peptide fragment of NGF (NGFp) allowing specific binding to the p75 neurotrophin receptor (p75NTR) expressed on HSCs and MFs (22). Administration of Ad.GFP-S11-NGFp carrying the 4TFs (Ad4TF) led to overexpression of the 4TFs in sorted MFs without any sign of morphological or liver function alteration.

Authors, having established the model to identify iHeps reprogrammed from MFs, performed a number of experiments to evaluate iHep formation *in vivo* and to characterize them using different murine protocol of fibrosis induction. In first experiments using chronic CCl₄ protocol (involving for mT/mG mice treatment with AAV-Ttr-Cre at time zero, CCl₄ treatment twice a week from day 30 to 90, Ad4TF administration at day 97 and detection point at day 127) the following major data were obtained: (I) iHep were not detected when Ad4TF was administered to control animals (no chronic injury); (II) the percentage of iHep derived from MFs (i.e., positive for dTomato and hepatocyte markers) in chronically injured liver receiving Ad4TF, was estimated as 0.2–1.2% among the total hepatocyte

population, corresponding to an estimated reprogramming efficiency of less than 4%; (III) iHep isolated from *in vivo* conditions and sorted as single-tdTomato positive cells (i.e., cells unequivocally converted from MFs) were functionally behaving as HLCs by secreting albumin and urea, taking up indocyanine green, storing glycogen and exhibiting activity of CYP3A and other Cytochrome-p450 isoforms; (IV) sorted dt-tomato-positive cells showed no chromosomal alterations and absence of expression of 4TFs, indicating stable reprogramming of MFs into iHep; (V) administration of Ad4TF resulted in a significant amelioration of CCl₄-induced liver fibrosis and injury as compared to animals treated with CCl₄ but receiving the empty vector (as properly evaluated by assessing L-OH-proline hepatic levels, fibrosis score, Sirius red staining, immunohistochemical staining for desmin and p75NTR and aminotransferases levels). It is interesting to underline that similar results (with a percentage of iHep derived from MFs of 0.9–2.5%) were also obtained by employing a different lineage-tracing model like the mice expressing Cre under the transcriptional control of lecithin-retinol acetyltransferase (Lrat) promoter also containing mT/mG transgenes previously used to trace MFs deriving *in vivo* from activated HSCs (23). Moreover, the previously described major results, including the attenuation of liver fibrosis, were confirmed also in a model of cholestasis-induced liver fibrosis obtained in mice fed a 3,5-diethoxycarbonyl-1,4-dihydrocollidine (DDC) diet.

Of relevance, when the protocol was modified (i.e., by administering Ad4TF at day 60 during chronic CCl₄ administration), the procedure was still resulting in the *in vivo* generation of iHep and reduction of liver fibrosis during ongoing chronic liver injury. However, reduction of fibrosis and improvement of liver functions was not detected in mice treated with CCl₄ for 12 weeks, mice that develop a murine histopathologic equivalent of cirrhosis, and then receiving Ad4TF. This lack of effect may depend on the fact that although the number of iHep reprogrammed was similar at 8 and 12 weeks, the number of MFs at 12 weeks was massively increased. Finally, overall *in vivo* and *in vitro* evidence provided in this study also outlines three additional relevant concepts: (I) the anti-fibrotic effect is due to the reduction of MFs rather than to suppression of MFs properties, fully in agreement with previous results (21–23); (II) following Ad4TF administration, iHep were detectable only in the liver (no evidence in other p75NTR-expressing tissues like brain, heart, lung and kidney), a result indicating that the procedure is then very selective for liver; (III) iHep generated *in vivo* were not the result of cell fusion, which is

an “old” but significant concern here correctly investigated and excluded.

The outstanding study by Song *et al.* (17) is of extreme interest because it outlines a reliable novel therapeutic strategy designed to counteract and/or ameliorate fibrogenic progression of CLDs which are, at present, in relation to their increased worldwide incidence, the most relevant concern for hepatologists. Of course, since this can be considered as a pioneer study, there are still limitations as well as unanswered questions, some of these correctly acknowledged by authors (17). A first relevant obvious point relies on the fact that the efficiency of *in vivo* reprogramming of MFs is still relatively low, and then future studies should pursue the goal to enhance the overall efficiency of iHep generation, possibly by improving targeted transduction (as Authors themselves suggest), in order to maximize positive effects. This is something that some of the Authors involved in the study are trying to finalize, as already show by data published in an accompanying paper in the same issue of *Cell Stem Cell* in which they used adeno-associated virus 6 (AAV6) vector expressing hepatic transcription factors (24). Another question is relying on the precise origin of *in vivo* iHep: although the models used in this study and literature data support a predominant origin from HSCs-derived MFs, one can not exclude that some iHep may originate from other cellular sources, including portal fibroblasts, smooth muscle cells around portal vein, bone marrow-derived mesenchymal stem cells or fibroblasts around central vein (18-23). Along these lines, and because data from this study indicate that iHep are detected only near to the portal vein or near central vein, it would be of interest to know what happens (i.e., *in vivo* procedures to reprogram MFs into iHep) in a murine model of the human non-alcoholic fatty liver disease (NAFLD, a major CLD worldwide), in which fibrosis starts with a peculiar perisinusoidal/pericellular pattern mainly involving activated HSCs. Similarly, since Authors state that they have detected limited, but significant, genetic memory of MFs, it would be nice to have information related to longer studies, in mice at this stage, to know whether the procedure could be considered overall safe. Finally, as recognized by Authors, one can not exclude that overexpression of TFs in endogenous hepatocytes may contribute to liver fibrosis amelioration, as already suggested for *HN4A* overexpression by a study performed on rat exposed chronically to CCl_4 (25).

However, all the limitations and unanswered questions seem really to disappear in front of an outstanding study

that open a novel and very promising way to counteract *in vivo* fibrogenic progression of CLDs: it needs just to be refined and improved.

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Footnote

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