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Abstract

In the present study rat liver acellular scaffolds were used as biological support to guide the differentiation of human liver stem cells (HLSC) to hepatocytes. Once recellularized, the scaffolds were maintained for 21 days in different culture conditions to evaluate hepatocytic differentiation. HLSC lost the embryonic markers (alpha fetoprotein, nestin, nanog, sox2, Musashi1, Oct 3/4 and pax2), increased the expression of albumin and acquired the expression of lactate dehydrogenase and three subtypes of cytochrome P450. The presence of urea nitrogen in the culture medium confirmed their metabolic activity. In addition, cells attached to tubular remnant matrix structures expressed cytokeratin-19, CD31 and vimentin. Rat extracellular matrix provides not only a favorable environment for differentiation of HLSC in functional hepatocytes (hepatocyte-like) but also promoted the generation of some epithelial-like and endothelial-like cells. When FGF-EGF or HLSC-derived conditioned medium were added to the perfusate an improvement of survival rate was observed. Conditioned medium from HLSC potentiated also the metabolic activity of hepatocyte-like cells repopulating the acellular liver. In conclusion, HLSC have the potential, in association with natural extracellular matrix, to generate *in vitro* a functional “humanized liver-like tissue”.

Keywords

Stem cells, adult human liver stem cells, functional liver regeneration, acellular bioscaffolds, natural extracellular matrix

Introduction

About 170 million of people worldwide are affected by chronic liver diseases eventually progressing to fibrosis and in several cases culminating in cirrhosis (1). Liver transplantation is the only efficient treatment that radically improves the outcome of liver failure. However, the accessibility of whole livers for transplantation is limited by the number of donors. Furthermore, the transplants of mature hepatocytes or hepatocytes obtained by neonatal livers are considered potential candidates for transplantation as an alternative therapy. Nevertheless, availability of organs for isolation of mature hepatocytes as well as the difficulty to expand them *in vitro* are the main limitation to their use (2).

Recently, researchers focused on stem/progenitor cells as a potential strategy for treatment of acute or chronic liver diseases. Stem cells (SC) are characterized by a self-renewal capacity and possess a high potentiality to differentiate in diverse cell progeny. The generation of mature hepatocytes from SC could offer an alternative for treatment of liver diseases and for correction of genetic disorders of liver metabolism. Embryonic stem cells (ESC) have been extensively studied for their potential to differentiate into different hepatic cell phenotypes (3, 4). However, the formation of teratoma has been observed in the liver and other organs after ESC transplantation in mice (5, 6).

Therefore, alternative sources of human stem cells have been explored. At present, bone marrow mesenchymal stem cells (BM-MSC) are preferred for potential clinical applications as they have some advantages related to their commitment to hepatic lineage (7, 8, 9, 10, 11, 12, 13). Adult human liver stem-like cells (HLSC) isolated by our group may represent an alternative for regenerative medicine because easily expandable (14, 15). HLSC have multiple differentiating capabilities distinct from those of oval stem cells. They express several mesenchymal but not hematopoietic stem cell markers and express embryonic

markers such as alpha fetoprotein (AFP), nestin, nanog, sox2, Musashi1, Oct 3/4 and pax2 (14, 16). Moreover HLSC express albumin, alpha fetoprotein and cytokeratin 18 (CK18) supporting their partial hepatic commitment (14). The effectiveness in restoring of the hepatic mass and function has been also described (16). Indeed, HLSC are able to enhance survival and to improve the tissue recovery in SCID mice with fulminant liver failure (FLF). These characteristics make the HLSC potential candidates for generation of functional hepatocytes to be used in regenerative medicine.

The dream in regenerative medicine is to develop strategies to reconstitute whole organ morphology and to re-establish its function. To promote a regeneration of a functional organ, it is not only necessary to generate tissue-specific cells, but it is also important to recreate the micro and macro environments critical for cell structural organization and function. Currently efforts of researchers are directed to design synthetic scaffolds in order to mimic the macro and microstructure of tissue that favor vascular network formation (17, 18, 19, 20). Alternative strategies such as including the co-seeding with endothelial cells to promote the spontaneous formation of capillary-like networks have been used (21). Incorporation of angiogenic peptides and growth factors into synthetic scaffolds has been also attempted to promote angiogenesis within engineered tissues (22, 23, 24, 25). Nevertheless, in these synthetic scaffolds, the vessel connectivity to host circulatory system is incomplete and restricted to the scaffold edges when they are transplanted (26).

To solve these difficulties, natural scaffolds with intact tridimensional anatomical architecture have been recently successfully used for different organs including liver (27). The natural extracellular matrices (ECMs) provide some advantages over the synthetic scaffolds. ECMs have the complex composition of bioactive molecules and lacks of

immunoreactivity (28), offer the type-specific niches necessaries for cell engraftment and are also able to regulate the cellular behavior and functionality (29).

In this respect, generation of natural liver bioscaffolds may offer the tridimensional mechanical support for a favorable cell engraftment and commitment. Moreover, natural liver bioscaffolds may allow optimal delivery of nutrients and offer an appropriate environment for regeneration of a fully functional organ.

In this study, we explored the potential of rat acellular liver bioscaffolds to promote differentiation of HLSC into mature hepatocytes and into other non-hepatocyte cells. We also explored the contribution of different culture conditions in improving the maturation of Hepatocyte-like cells. Finally, we analyzed the capacity of Hepatocyte-like cells to modify their micro and macro environment, substituting the native rat extracellular matrix with the human counterpart.

Material and methods

Animals

Young male Wistar rats (250 to 300 g) were obtained from the local animal facility. Animal studies were approved by the local Ethic Committee and conducted in accordance with the National Institute of Health Guide for the Care and Use of Laboratory Animals. Animals were sacrificed by cervical dislocation.

Isolation, characterization and culture of Human Liver Stem-like Cells (HLSC)

HLSC were isolated from human cryopreserved normal hepatocytes obtained from Lonza (Basel, Switzerland) and were cultured and characterized as previously described (14). Specifically, HLSC were cultured in a standard culture medium containing a 3 to 1 proportion of α-minimum essential medium and endothelial cell basal medium-1, supplemented with L-glutamine 2 mM, penicillin 100 UI/ml/streptomycin 100 µg/ml and 10% Fetal Calf Serum (α-MEM/EBM/FCS; Gibco/Cambrex) and maintained in a humidified 5% CO2 incubator at 37°C. Cells up to a ≈ 80% confluence are trypsinized and harvested by centrifugation at 1200 rpm for 5 minutes. HLSC at passages 3 to 7 and ≈ 80 % of confluence were used in all the experiments. By confocal microscopy and FACS analysis HLSC expressed several embryonic (alpha fetoprotein (AFP), Nestin, Nanog, Sox2, Musashi1, Oct 3/4 and Pax2), mesenchymal stem cells (CD29, CD73, CD44, and CD90) and the hepatic marker albumin as previously described (14).

Preparation of HLSC-derived conditioned medium

The HLSC-derived conditioned medium (HLSC-CM) was obtained as described previously by our group (30). Briefly, supernatants of HLSC cultured in α-MEM/EBM/FBS were collected after 24 h. After centrifugation at 3000g for 10 min to remove cell debris, cell-free

supernatants were concentrated ~25 fold by centrifugation at 2700g for 75 min, using Ultra-PL 3 ultrafiltration units (Amicon-Millipore Concord Road, Billerica, MA, USA) with a 3-kDa molecular weight cutoff and stored at -20°C until its use.

Generation of acellular liver scaffolds

After cervical dislocation, a longitudinal abdominal incision through the midline was performed, and all three inferior vena cava (IVC), portal vena (PV) and the cystic duct (CD) were cannulated using 22G or 24G cannulas (as appropriate) and ligated proximally with a silk non-absorbable surgical suture 6-0 (Ethicon. Johnson-Johnson Intl. Belgium). Connecting a 10 ml syringe to the cannulas, 1 ml of PBS containing 1U.I/µl of heparin (EPSOCLAR 25.000 U.I/5ml; HOSPIRA, Italy) was perfused trough each cannula to prevent coagulation. Posteriorly, a midline sternotomy was done; the superior vena cava (SVC) was cannulated and fixed as described about, then perfused with PBS-heparin solution. Liver was dissected and removed from the abdominal cavity (with its capsule intact), transferred to the perfusion camera and connected to the Langendorff system (Hugo Sachs Elektronik-Harvard Apparatus, Germany). Then, the decellularization was proceeding according with the protocol of Shupe and collaborators (27), with some modifications. Briefly, liver was perfused with: a) PBS 1X to clear the blood (300 ml), b) PBS solution containing different concentrations of Triton X-100 (1, 2 and 3%, 300 ml each one) to disrupt the lipids of the membranes and clear most of the cellular components and c) 1000 ml of PBS solution containing SDS (0.1%) to remove the remnant nuclear components containing DNA. The cannula connected to the perfusion system was changed time to time to permit a best perfusion of the entire liver. Then, the remnant detergent was cleared from the scaffolds by perfusion with 1000 ml of PBS and sterilized with a 50 ml of peracetic acid solution (PA, 0.1 % ethanol 4%). Finally, scaffolds were

washed with 300 ml of sterile PBS to eliminate the PA. All solutions were perfused by peristaltic pump at 75 mmHg at a constant flow rate of 5 ml/minute.

Recellularization and culture of bioscaffolds

A total of 10 ml α-MEM/EBM/FCS was perfused into the scaffolds as pre-treatment, 30 minutes previously to seed the cells in order to promote a best engraftment. Then, a quarter of the cell suspension was injected into the scaffolds through the cannula inserted in the vena porta, whereas the other three cannulas (IVC, SVC and CD) were clamped to avoid the efflux of the cells outside the scaffold. The same procedure was repeated to the IVC, SVC and then by the CD.

Three different protocols were designed in order to explore the best one promoting cell engraftment, differentiation and maturation: a) only HLSC cells in standard medium (Hepatocyte-like; 80 to 100x10^6 cells), b) HLSC + 10 ng/ml EGF-10 ng/ml FGF4 (Hepatocyte-like+GF) and c) Hepatocyte-like + HLSC-derived conditioned medium (HLSC-CM; Hepatocyte like+HLSC-CM). Once cells were seeded, the bioscaffolds were cultured in static condition in humid incubator at 37°C, air and 5% CO2 for 21 days, medium was changed with fresh one each other day during the entire experiment.

Assessment of urea production

Samples of the culture medium were collected at 2, 4, 6, 14 and 21 days during the culture. The samples were centrifuged 5 minutes at 1200 rpm to eliminate cellular detritus and then the supernatant was frozen at -20°C until their analysis. The presence of urea nitrogen in the medium was assessed by colorimetry using a specific Human BUN colorimetric detection kit (Arbor Assays. Michigan, USA. Reference K024-H1) following the

instruction of the provider. Standard medium (α-MEM/EBM/FCS) was used as blank for the normalization and considered as the BUN concentration at time 0.

Histology

At the end of the experiments, a part of each bioscaffold was formalin-fixed and paraffin-embedded and 3-5 µm sections were used for histological analysis. Hematoxylin and eosin (H&E) staining was conducted by the standard method to evaluate the tissue architecture, morphology, the cell engraftment and the organization of the Hepatocyte-like in the bioscaffold. Further, immunocytochemistry was performed to analyze the human nature of the cells by their positivity to MHC-I (HLA). Briefly, endogenous peroxidase activity was blocked with 6% H_2O_2 for 10 minutes at room temperature. Rabbit anti-human polyclonal antibody 1:100 (HLA-1, Santa Cruz Biotechnology) were applied to slides and incubated overnight at 4°C. The next day, samples were washed three times with PBS-Tween 0.1 %, 5 minutes, incubated for 30 minutes at room temperature with the horseradish peroxidase-labeled anti-rabbit (Dako Denmark A/S). The reaction product was developed using 3,3-diaminobenzidine. Omission of the primary antibody or substitution with unrelated rabbit antibody served as negative control.

Immunofluorescence

Immunofluorescence was performed to characterize the ECM composition and the cell phenotype. Briefly, at the end of each experiment, parts of each bioscaffold were included in Tissue-Tek-II and frozen at -80°C. Serial slides were cut longitudinally (3-5 µm) in a cryostat and fixed in acetone then, the expression of human matrix proteins and typical markers of mature hepatocytes, epithelial and endothelial cells were analyzed by immunofluorescence using specific antibodies (table 2). Briefly, tissue sections were first

washed with PBS 1X, incubated during 30 minutes at room temperature with a permeable solution containing PBS 1X and 0.25 % Triton X-100, washed three times with PBS 1X for 5 minutes each one, incubated for 20 minutes with a blocking solution containing PBS 1X, 0.1% tween and 0.1% bovine serum albumin (wt/vol) during 30 minutes at room temperature, then incubated overnight with specific primary antibodies. For the characterization of EM in rat acellular bioscaffolds, performed by immunofluorescence using primary antibodies recognizing anti-rat proteins (table 1). The expression of human markers in the HLSC-recellularized bioscaffolds were analyzed by immunofluorescence using specific antibodies anti-human proteins: HLA-1, CD31, oct3/4, nestin; albumin, alpha fetoprotein (AFP), collagen IV, vimentin, cytochrome P450 1a1, cytochrome P450 3a4, cytochrome P450 0 7a1, LDH, fibronectin, CD31, laminin, vimentin, cytokeratin 8-18, cytokeratin 19, nanog, sox2, Musashi1, and pax2 (table 2). Non immune isotypic control IgG from mouse, rabbit or sheep were used where appropriated as negative controls. After washing with PBS-tween solution, sections were incubated with the corresponding secondary antibodies: Alexa Fluor 488 or Texas Red, as appropriate (table 3), washed with PBS-tween solution and incubated 10 minutes with DAPI (DAKO), after a final washing step with PBS, slides were mounted with Fluoromount (SIGMA). Specificity of primary antibodies recognizing human markers was tested a) in acellular rat liver scaffolds or using as primary antibody non immune isotypic control IgG from mouse, rabbit or sheep where appropriated (supplementary figure 1). Microscopy analysis was performed using a Cell Observer SD-ApoTome laser scanning systems (Carl Zeiss, Germany).

Analysis of differentiated cell population derived from HLSC

Ten micrographs (x10 magnification) were taken for each experimental condition. Total number of cells and number of positive cells were counted for each marker: cytokeratin-19 (CK-19), vimentin (VIM) or CD31 and the percentage of positive cells was calculated.

Cell proliferation and apoptosis

Specimens of paraffin fixed scaffolds were routinely processed and sectioned at 3 to 5 µm. Then immunohistochemistry for detection of proliferation and apoptosis was performed as previously described (31) using the anti-PCNA monoclonal antibody (Santa Cruz) for proliferation or by terminal deoxynucleotidyl transferase dUTP nick end-labeling (TUNEL) for apoptosis. The percentage of positive cells was calculated as described above. Percentage of positive cells = (positive cells/total cells)*100.

Statistical Analysis

Values are presented as the mean of three independent experiments in each condition ± the standard error (SE). Data were analyzed using a t-test, analysis of variance (ANOVA) and Pearson. A probability (P) < 0.05 or 0.01 was considered significant.

Results

The macroscopic architecture was preserved in the acellular liver structures after decellularization (figure 1A-D). The translucent appearance of these bioscaffolds permitted to observe the presence of tubular arrangements pertaining to vessel and biliary duct remnants (figure 1D). The hematoxylin and eosin staining demonstrated the absence of cells in the decellularized liver bioscaffolds (figure 1F) when compared with normal liver tissue (figure 1E). A fine fibrous network was preserved in the decellularized liver bioscaffolds. The immunohistochemical analysis showed that the remnant native ECMs contained collagen IV, cytokeratin 8-18, fibronectin, actin, laminin and vimentin (figure 2 A-F).

Once the acellular bioscaffolds were obtained and pre-treated with culture medium (3A), they were recellularized with HLSC, maintained in culture for 21 days in three different conditions (see material and methods). The initial transparency of the acellular bioscaffolds allowed to observe cellular distribution after infusion. By conventional microscopy, the presence of the cells inside the structures was observed (figure 3B). The efficiency of HLSC to repopulate the rat acellular liver bioscaffolds was assessed after 21 days by hematoxylin and eosin staining of histological preparations. We observed that cells were distributed throughout the matrix of the acellular liver parenchyma but also around and inside of tubular structures that seemed to belong to remnants of vessel and/or biliary duct systems (figure 3C). These cells were positive to the human marker HLA-1 (figure 3D), demonstrating their human nature and excluding their origin from rat remaining cells. No differences were observed in the different culture conditions (data not shown).

The viability of Hepatocyte-like cells was maintained until the end of the experiment. The TUNEL analysis (figure 4), revealed that after 21 days in culture, 13 % of the cells were

apoptotic in the bioscaffolds cultured in basal conditions (Hepatocyte-like). The viability rate was significantly improved when the HLSC-derived conditioned medium (HLSC-CM) was added (2.8 % of apoptotic cells). Similar effect was observed when recellularized bioscaffolds were stimulated with EGF and FGF factors (Hepatocyte-like+GF; 2.5 % of apoptotic cells).

Considering that the HLSC have a high degree of proliferation, we explored at the end of the experiment, if this capability was modified by the direct interaction with the extracellular matrix. PCNA assay was performed in order to identify the cells in proliferation. The histological analysis demonstrated that in all the bioscaffolds, independently from the condition, the great majority of Hepatocyte-like cells were negative to the PCNA indicating that cells lost their self-renewal capability (data not shown).

The histological analysis showed a major cell population characterized macroscopically, by large cells with a central nucleus and granular cytoplasm that were attached to each other and dispersed through the entire bioscaffold (figure 3). This phenotype suggested the apparent differentiation of the HLSC to Hepatocyte-like cells. Differentiated Hepatocyte-like cells lost positivity the embryonic cell markers, alpha fetoprotein (AFP), nestin, nanog, sox2, Musashi1, Oct 3/4 and pax2 (not shown) and acquired markers typical of mature cells. The immunocytochemistry analysis showed that the great majority of the cells were positive for human albumin and co-expressed the enzyme lactate dehydrogenase (LDH) and three different subclasses of the cytochrome P450 (Cyp450) (figure 5). These cells were also able to synthesize *de novo*, diverse human proteins of the extracellular matrix such as collagen IV, laminin, fibronectin (figure 6). These were specific for human proteins and did not react with the rat extracellular matrix (figure 6; Neg Crtl). The expression of these proteins suggested that Hepatocyte-like cells were able to “humanize” their

environment, substituting the rat native matrix with the human homologous. This deposit of human matrix proteins was observed in all the recellularized bioscaffolds independently of the treatment (data not shown).

In addition to the major population (Hepatocyte-like), we identified a second population forming, small groups of cells dispersed through the entire bioscaffold. This population was constituted by two different subpopulations differing for the expression of specific cell markers (figure 7A). The first subpopulation was formed by cytokeratin-19-expressing cells (14.5%), whereas the other subpopulation expressed vimentin (16.1%) or CD31 (10.5%). The presence of cells expressing these markers suggests the generation of epithelial and endothelial lineages from the HLSC. The quantitative analysis showed that the percentage of the vimentin positive cells had a tendency to be reduced in the scaffolds cultivated in presence of FGF-EGF (Hepatocyte-like+GF, 9.2 %) or in a medium enriched with HLSC-CM (Hepatocyte-like+HLSC-CM, 13.2 %) whereas the other two subpopulations were not affected (figure 7).

The presence of vimentin+ and CD31+ cells suggested a possible neo-angiogenesis derived from this cell subpopulation. As it is shown in the figure 8, we observed elongated and square-shaped cells forming a continuous monolayer contiguous or close to some tubular structures. Co-expression of albumin with the endothelial markers vimentin and CD31 indicated an immature phenotype of these cells. Cells non-adherent to ECMs were negative for vimentin and CD31. The presence of these double positive cells attached to the matrix of remnant tubular structures suggested the importance of a direct modulation exerted of ECMs in guiding the HLSC specific cell commitment.

To evaluate metabolic activity of the Hepatocyte-like population, culture medium was collected at different experimental times (2, 4, 6, 14 and 21 days) and the urea nitrogen

synthesis was assessed. Hepatocyte-like cells produced urea detectable already after 2 days from seeding HLSC in the acellular bioscaffolds (figure 9). In standard conditions (Hepatocyte-like) cells were able to produce ~1 mg/dl and this concentration was maintained during the entire experiment. The production of urea was significantly increased up to 2-2.5 mg/dl when recellularized bioscaffolds were cultured in presence of the HLSC-CM (Hepatocyte-like+HLSC-CM), whereas not major differences were observed in Hepatocyte-like+GF bioscaffolds.

Discussion

The liver is able to regenerate itself maintaining an adequate volume and function. When liver is undergoing up to 70% of resection, the restoration of the liver mass is carried out by the proliferation of hepatocytes and/or progenitors (32, 33). After an acute injury, the regenerative capacity of the liver is enough to maintain, temporally, the function of the organ. However, this effect is limited, especially in chronic or fulminant diseases, where the regenerative capacity of remnant resident cells is not enough to restore the normal anatomy and function of the organ, making orthotopic liver transplantation mandatory.

In this work, we were able to generate functional “humanized livers” using HLSC in combination with rat acellular liver bioscaffolds. The tridimensional structure of the native matrix and the preservation of bioactive molecules promoted differentiation and maturation of HLSC to functional hepatocytes. In addition, subgroups of cells were differentiated to endothelial-like or epithelial-like cells, suggesting that HLSC could participate also in the regeneration of both vascular and biliary systems when seeded in acellular natural bioscaffolds. Pluripotency of HLSC, including the ability to differentiate in endothelial cells as was previously shown (14).

Expression of the drug metabolizing enzymes such as cytochrome P450 (Cyp450) are commonly used as markers of hepatocyte differentiation (14, 34). The histological analysis made evident the expression of three types of CytP450 (1a1, 3a4 and 7a1) by the HLSC at the end of the experiments (21 days).

The hepatocytes control the homeostasis of fuel molecules such as glucose, glycogen, triglycerides, cholesterol, bile acids and vitamins; metabolize amino acids and endogenous compounds such as heme and bilirubin, and are also responsible of the systemic ammonia

detoxification in connection with urea synthesis, maintaining the ammonia and bicarbonate homeostasis under physiologic and pathologic conditions (35). In our experiments, we observed production of urea nitrogen in the medium of the recellularized bioscaffolds detectable after 2 days in culture and maintained up to day 21 (figure 6). A significant increase in the metabolic activity was observed in the Hepatocyte-like+HLSC-CM bioscaffolds, indicating that factors secreted by the cells, have important trophic effects on the differentiation of HLSC.

Even if ECM constitutes less than 3 % of the liver tissue, it is formed by diverse proteins which are organized in a tissue-specific arrangement, composed by collagens, laminins, proteoglycans, glycosaminoglycans, elastins and other proteins. Physiologically, the ECM not only provides the architecture of tissues, but also participates in the cellular adhesion, migration, patterning and phenotype (36). Thus content of the diverse proteins forming the ECM seems to be important to maintain the differentiated functions in each individual cellular compartment of the liver (29). Due to the high complexity of the ECM *in vivo*, it is difficult to construct synthetic scaffolds mimicking their natural composition as well as its microarchitecture that are able to support the hepatocyte survival and function when transplanted in rats. Baptista and collaborators demonstrated that decellularized liver matrix favor colonization of human fetal liver cells and human umbilical vein endothelial cells (37) confirming the potentiality of bioscaffolds to be used in regenerative medicine to clinical applications. The natural matrices, due to the lack of immunogenicity, represent a potential alternative in tissue regeneration (38). Barakat and collaborators described the capacity of acellular liver bioscaffolds to support and induce phenotypic maturation of human fetal hepatocytes, demonstrating the possibility to use recellularized natural bioscaffolds in xenotransplantation therapies to replace or supplement allotransplantation

(39). The use of embryonic or fetal tissues is limited by the number of donors, but also by the ethical and legal reasons.

Uygun and collaborators demonstrated that acellular bioscaffolds obtained from ischemic rat livers, supported the efficient engraftment and function of adult primary hepatocytes (40). This study served as proof of concept for generation of recellularized liver, but the use of xenogenic matrix would be limited by immunogeneicity. Based on this observation the authors suggested that organs derived from deceased donors, unsuitable for transplantation, could be used for fabrication of acellular human liver bioscaffolds. However a limitation of this approach is the need of a large amount of normal human hepatocytes. The novelty of our approach is the use of HLSC that can be easy expanded in culture at variance of normal adult hepatocytes and that could be used to recellularize liver from deceased donors. Moreover, this experiments add new information on the differentiation capabilities of HLSC which have been recently designed as “Orphan Drug” by the European Medical Agency for urea cycle disorders (EU/3/12/971; EU/3/11/904) and acute liver failure (EU/3/12/983). HLSC are easily obtainable from small surgical sample or from biopsy of human adult liver (14, 16). In addition, these cells exhibit a great proliferation potential, remain stable for over thirtieth culture passages, without chromosomal aberrations and good manufacturing protocol.

In conclusion, this work demonstrated that HLSC can be expanded satisfactorily and differentiated to functional Hepatocyte-like cells, epithelial and endothelial cells when seeded in liver acellular scaffolds.

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Table 1.

Rat marker	Antibody	Reference	Dilution
Collagen IV	Rabbit polyclonal IgG	ABCAM (ab19808)	1:500
Fibronectin	Rabbit polyclonal IgG	ABCAM (ab23751)	1:200
Laminin	Rabbit polyclonal IgG	ABCAM (ab11575)	1:200
Vimentin	Mouse monoclonal IgG1	ABCAM (ab8978)	1:200
Actin	Mouse monoclonal IgG2A	ABCAM (ab11003)	1:200
Cytokeratin 8-18-19	Mouse monoclonal IgG1	ABCAM (ab41825)	1:100

Table 2.

Human marker	Antibody	Reference	Dilution
MHC class 1 (HLA-1)	Rabbit polyclonal IgG	Santa Cruz (SC-25619)	1:100
Albumin	Mouse monoclonal IgG2A	R&B Systems (MAB1455)	1:50
CYTP450 1a1	Rabbit polyclonal IgG	Abcam (ab79819)	1:100
CYTP450 3a4	Sheep polyclonal IgG	Abcam (ab22702)	1:100
CYTP450 7a1	Rabbit polyclonal IgG	Abcam (ab65596)	1:100
LDH	Rabbit monoclonal IgG	Abcam (ab52488)	1:100
Fibronectin	Rabbit polyclonal IgG	Abcam (ab23750)	1:100
Laminin	Mouse monoclonal IgG1	Abcam (ab49726)	1:100
Vimentin	Rabbit monoclonal IgG	Abcam (ab16700)	1:100
Collagen IV	Mouse monoclonal IgG1	Abcam (ab6311)	1:100
ACTIN	Rabbit monoclonal IgG	Abcam (ab115777)	1:100
Cytokeratin 19	Mouse monoclonal IgG1K	Dako (M0888)	1:100
Oct 3/4	Goat polyclonal IgG	Abcam (ab14520)	1:50
AFP	Mouse monoclonal IgG1	R&D Syatem (MAB1368)	1:20
Nanog	Rabbit polyclonal IgG	Abcam (ab21603)	1:100
Sox2	Rabbit polyclonal IgG	Abcam (ab15830)	1:100
Musashi 1	Rabbit polyclonal IgG	Abcam (ab33251)	1:100
CD31	Rabbit polyclonal IgG	Abcam (ab28364)	1:100
Pax2	Rabbit polyclonal IgG	Covance, Princeton	1:100

Table 3.

Fluorophore	Secondary antibody	Reference	Dilution
ALEXA FLUOR 488	Goat anti rabbit IgG (H+L)	ABCAM (ab11008)	1:200
ALEXA FLUOR 488	Goat anti mouse IgG (H+L)	ABCAM (ab11029)	1:200
ALEXA FLUOR 488	Donkey anti sheep IgG (H+L)	ABCAM (ab11015)	1:200
TEXAS RED	Goat anti rabbit IgG (H+L)	ABCAM (T2767)	1:200
TEXAS RED	Goat anti mouse IgG (H+L)	ABCAM (T6390)	1:200

Legends

FIG. 1. *Ex vivo* decellularization of whole rat livers. Macroscopic appearance of the liver during different phases of decellularization (A-C). Translucent structures were obtained at the end of the process (D) and there is possible to identify a network of tubular structures derived from the remnants of vessel and biliary duct systems. Representative micrographs of hematoxylin and eosin staining (H&E) of normal (E) and decellularized liver tissues (F) showing that after the decellularization there was conserved a fine ECM network with the absence of cellular components. Scale bar = 100 µm.

FIG. 2. Representative micrographs of immunofluorescence analysis of ECM proteins in acellular rat liver bioscaffolds. After the decellularization, the ECM matrix proteins such as collagen IV (A), fibronectin (B), actin (C), laminin (D), and vimentin (E), cytokeratin 8-18 (F) were conserved after the decellularization. Scale bar = 100 µm.

FIG. 3. Recellularization of acellular rat liver bioscaffolds with HLSC. The acellular bioscaffolds was perfused with α-MEM/EBM/FCS as a pre-treatment 30 minutes before of their recellularization (A) and subsequently recellularized with HLSC ($80X10^6$ cells). The presence of cells into the bioscaffold was immediately confirmed by conventional microscopy (B). Representative micrographs of H&E staining (C) showing the preservation of HLSC at the end of the experiment (21 days), whereas the human origin of the cells was demonstrated by their positivity to HLA (D; brown cells). Scale bar = 100 µm.

FIG. 4. Cell viability of bioscaffolds cultured for 21 days in different conditions. The TUNEL analysis demonstrated the presence of apoptotic cells independently of the culture condition. However, the quantitative analysis demonstrated that 13.7% of the cells fall in apoptosis when the bioscaffolds were cultured in standard conditions (Hepatocyte-like).

The apoptotic rate was reduced when the HLSC-CM (Hepatocyte-like+HLSC-CM) or the factors EFG-FGF (Hepatocyte-like+GF) were added to the medium (2.8 % and 2.5%, respectively). Data are presented as the media of three independent experiments ± the SE. * P < 0.01. Scale bar = 100 µm.

FIG. 5. Expression of human typical markers of mature hepatocytes in recellularized bioscaffolds. The immunofluorescence analysis demonstrated that after 21 days in culture, the Hepatocyte-like cells (HLA+) co-expressed albumin, LDH and three different subtypes of cytochrome P450 (1a1, 7a1 and 3a4), all of them typical markers of mature hepatocytes. Scale bar = 100 µm.

FIG. 6. Immunohistochemical characterization of human ECM proteins in recellularized bioscaffolds. After 21 days in culture, the HLSC (albumin+ cells) were able to synthesize *di novo*, typical human ECM proteins such as collagen IV, fibronectin and laminin (A). Only background staining was present in the negative controls (Neg Ctrls), confirming the specificity of anti-human primary antibodies (B). Scale bar = 100 µm.

FIG. 7. Presence of epithelial- and endothelial-like cells in recellularized bioscaffolds. A) Representative micrographs of immunofluorescence analysis demonstrating the presence of cytokeratin-19, vimentin and CD31 positive cells. B) The quantitative analysis demonstrated that cytokeratin-19+, vimentin+ and CD31+ cells represent respectively, 14.5%, 16.1% and 10.5% of the total cells. The addition of FGF-EGF (Hepatocyte-like+GF) reduced the percentage of vimentin+ cells (9.2%), whereas not changes were observed when the HLSC-CM was added to the culture medium (Hepatocyte-like+HLSC-CM). The other subpopulations (cytokeratin-19+ and CD31+) did not differ between treatments. Data are presented as the media of 3 different experiments ± STDEV. * P < 0.05. Scale bar = 100 µm.

FIG. 8. Representative micrographs of endothelial-like cells forming tubular structures. Hematoxylin and eosin staining showing more square-shaped Hepatocyte-like (HLA; brown cells) were observed lining each to other in a continuous monolayer forming tubular structures. Some cells expressing albumin were negative to vimentin.

FIG. 9. Hepatocyte-like cells are metabolically active. The urea nitrogen is detected just than 2 days after the recellularization. In most conditions, the levels were maintained during the entire experiment (~1 mg/dl). The addition of HLSC-CM (Hepatocyte-like+HLSC-CM) improved the metabolic activity of the cells, because the levels of urea nitrogen were ~ 2-fold higher (2-2.5 mg/dl) when compared with the other conditions. Standard culture medium (see methods) was used as blank in the normalization and also represents the levels of BUN at time 0. Data are presented as the media of three independent experiments ± the SE. *P < 0.01 respect to the other three conditions.

Supplementary figure 1. Supplementary figure 1. Representative micrographs of negative controls for human markers. Rat acellular bioscaffolds were incubated with anti-human primary antibodies. The absence of reactivity confirmed the specificity of antibodies. Only background signal was observed. Scale bar = 100 µm.

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Disclosure statement

No competing financial interests exist.