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Blood-Derived Stem Cells (BDSCs) Plasticity: In Vitro Hepatic Differentiation

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The limited availability of hepatic tissue suitable for the treatment of liver disease and drug research encourages the generation of hepatic-like cells from alternative sources as support for the regenerative medicine. Human blood derived stem cells (BDSCs) express surface markers and genes characteristic of pluripotent stem cells and have the ability to differentiate into different cell types, including tissues of endodermal origin (i.e., liver). Therefore they can represent a valuable source of hepatocytes for medicine. In this investigation, we exploited a fast hepatic differentiation protocol to generate hepatocyte-like cells from human BDSCs using only hepatocyte growth factor (HGF) and fibroblast growth factor-4 (FGF-4) as growth factors. The resulting cell population exhibited hepatic cell-like morphology and it was characterized with a variety of biological endpoint analyses. Here, we demonstrate how human BDSCs can be reprogrammed in hepatocyte-like cells by morphological, functional analysis, reverse transcriptase (RT)-PCR, and Western Blot assay. This study defines a fast and easy reprogramming strategy that facilitates the differentiation of human BDSCs along a hepatic lineage and provides a framework for a helpful source in the stem cells therapy and liver disorders.

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Despite the remarkable regenerative potential and tissue turnover of liver, its diseases are becoming one of the most common causes of mortality in developing countries (Fox and Roy-Chowdhury, 2004). The World Health Organization estimates that 20 million people worldwide are affected by cirrhosis and/or liver cancer, predominantly affecting among the estimated 500 million people (nearly 10% of the world population) bearing persistent viral infections like hepatitis B (HBV) or hepatitis C (HCV) (Dietreich, 2005; Rozga, 2006). Although some people with end stage liver disease can be effectively treated with orthotopic liver transplantation (OLT), considerable morbidity and mortality are associated with this treatment. In addition, a shortage of available donors and the cost of surgery have made this treatment unavailable to many patients suffering from liver diseases in most countries. As a result, thousands of patients die each year while on a waiting list for transplantation, and many more are never put on the list. In view of these shortfalls, cell-based therapy would offer a safer and readily available alternative source of treatment for patients with chronic liver diseases (Selden and Hodgson, 2004; Nussler et al., 2006; Zhou et al., 2012). However, some major limitations of this therapy are the availability of human hepatocytes (besides the storage of donor hepatocytes), the difficult large scale hepatocytes amplification and function maintenance (Ankrum and Karp, 2010).

The normal source of cells for hepatocyte transplants are livers with >50% steatosis, vascular plaques or other factors which render the tissue unsuitable for whole organ transplantation (Strom et al., 1997; Fisher et al., 2000; Muraca et al., 2002; Horslen et al., 2003; Strom and Fisher, 2003). The isolation of viable and useful cells from discarded organs has made possible the small proof of concept studies in humans (Fox et al., 1998; Fisher et al., 2000; Horslen et al., 2003; Parasassi et al., 2005). A wider use of hepatocyte transplants will require alternative and more reliable sources of cells. Xenotransplants (Nagata et al., 2003), immortalized human hepatocytes (Cai et al., 2002; Wege et al., 2003; Cai et al., 2007) and stem cells or induced pluripotent stem cells (Petersen et al., 1999; Alison et al., 2000; Theise et al., 2000; Campard et al., 2008; Basma et al., 2009; Miki et al., 2009; Iwamuro et al., 2010; Ayatollahi et al., 2011; Marongiu et al., 2011; Lee et al., 2012) have been proposed as alternative sources of cells for clinical transplants, research and toxicology studies (Parolini et al., 2010).

The peripheral blood derived stem cells (BDSCs) represents a promising source of cells for regenerative medicine because their ability to differentiate into several cell types (Giovani et al., 2008). Our recent in vivo studies have shown how BDSCs are able to repopulate different organs such as muscle, bone, liver,

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or heart and generate differentiated cells in response to acute or chronic organ damage.

The peripheral blood is a readily available, non-controversial source of adult stem cells. Moreover, BDSCs are not tumorigenic upon injection and recent reports indicate that they do not induce immune reaction when injected (Spaas et al., 2011; Marfe et al., 2012a,b). These are evident advantages for the potential clinical use of this stem cell source.

In the last decade, many authors have described the differentiation, to different extents, of various stem cell types towards a hepatocyte-like phenotype (Dua et al., 2004; Lee et al., 2012). However, differentiation of human BDSCs into functional hepatocytes has not been reported so far.

The aim of this study is to investigate the ability of human BDSCs to proliferate and differentiate in vitro into mature and functional hepatocytes. The cell population, derived by culturing human BDSCs for 7 days under a well-defined differentiation protocol into endodermal lineage, displayed hepatic cell-like morphology, loss of expression of marker of pluripotency or "stemness," such as the transcription factor Nanog homeobox (*NANOG*), increased expression of α-fetoprotein (AFP), an important hepatic marker. In conclusion, our results outline a unique and feasible approach that allows human BDSCs differentiation into hepatocyte-like cell lineage.

Materials and Methods

Stem cells expansion

Human BDSCs were obtained by few milliliters of a blood sample as previously described (Marfe et al., 2012a,b). The nucleated blood cell fraction was isolated by ammonium chloride incubation (dilution 1:3 in NH_4Cl 1 M), centrifuged at 400*g* and washed several times with phosphate-buffered saline, pH 7.2 (PBS) (Oxoid, Hampshire, England) to remove the majority of erythrocytes. Cells were then resuspended in 5 ml PBS and incubated for 72 h at 37°C in the presence of 50 nM Macrophage Colony-Stimulating Factor (M-CSF, Sigma–Aldrich, St. Louis, MO) and 5 μM gentamicin sulfate (Sigma–Aldrich).

Hepatocyte differentiation

To promote hepatic differentiation in vitro the human BDSCs were plated on collagenated (0.1 mg/ml Collagen type I, BD Biosciences, Bedford, MA) 24 wells.

The cells were grown in a maintenance medium composed by DMEM-F12 (Biowest, Nuaillé, France) supplemented with 2 mM stable L-Glutamine (Gibco-Invitrogen, NY), 2.4 mg/ml sodium carbonate (NaHCO3) (Merck, Frankfurter, Germany), 1% Glucose, 10% fetal bovine serum (FBS) (Biowest), penicillin/streptomycin (100 U/ml) (Biowest), 1% non-essential amino acids (Euroclone, Milano, Italy), 10 ng/ml epidermal growth factor (EGF) (BD Biosciences), 10 ng/ml IGF-1 (Insulin Growth Factor) (Provitro, Berlin, Germany). After 2 days, the cells were grown for other 5 days in a differentiation medium (Hu et al., 2003) composed by a basal medium DMEM-F12 (Biowest) supplemented with 2 mM stable L-Glutamine (Gibco-Invitrogen), 2.4 mg/ml sodium carbonate ($NaHCO_3$) (Merk, Germany), 1% Glucose, 10% FBS, Penicillin/Streptomycin (100 U/ml), 1% non-essential amino acids (Euroclone), 20 ng/ml hepatocyte growth factor (HGF) (Provitro) and 10 ng/ml fibroblast growth factor-4 (FGF-4) (Provitro).

Reverse transcriptase-polymerase chain reaction

Total RNA was extracted from differentiated cells using the *RNAeasy Mini Kit* (QIAGEN, Hilden, Germany). PCR was performed using One-Step RT-PCR Kit (QIAGEN) according to the manufacturer's instructions. Human-specific primers were designed to detect the expression of human α-fetoprotein AFP mRNA in differentiated cells. Glyceraldehydes 3-phosphate dehydrogenase (GAPDH) was used as the internal standard. The primer sequences are listed below.

AFP primers:
Forward: 5′-GGAGCGGCTGACATTATTATCG-3′;
Reverse: 5′-TGGCCAACACCAGGGTTTA3′
GAPDH primers:
Forward: 5′-CCTGCACCACCAACTGCTTA-3′;
Reverse: 5′-CATGAGTCCTTCCACGATACCA-3′

PCR was performed in a thermal cycler (Gene-amp PCR System; Perkin-Elmer, MA).

Protein extraction and Western blot analysis

Cells were homogenized directly into following buffer: 50 mM HEPES, 150 mM NaCl, 1 mM EDTA, 1 mM EGTA, 10% glycerol, 1% NP-40, 1 mM phenylmethylsulfonyl fluoride, 1 μg/ml aprotinin, 0.5 mM sodium orthovanadate, and 20 mM sodium pyrophosphate. The lysates were centrifugated at 15,000 *g* for 10 min. Protein concentrations were determined by the Bradford assay. Equivalent amounts of proteins were loaded and electrophoresed on sodium dodecyl sulfate (SDS)-polyacrylamide gels. Subsequently, proteins were transferred to nitrocellulose membranes. After blocking with Tris-buffered saline-BSA, the membrane was incubated with the following primary antibodies: anti-Nanog (1:1,000) (Cell Signaling, MA), anti-AFP (1:1,000) (Cell Signaling) and anti-β-actin MAb (dilution 1:7,500) (Sigma, St. Louis, MO). Membranes were then incubated with the appropriate horseradish peroxidase-conjugated secondary antibody and the reaction was detected with an enhanced chemiluminescence system. The relative amount of protein expression was quantified using a Gel-Doc phosphorimager and Quantity One software and normalized by the intensity of β-actin.

Immunocytochemistry analyses

After 7 days of growth, the cultured cells were washed twice with PBS and fixed with 10% formalin (ScyTek Laboratories, Logan, UT) for 30 min at room temperature (RT) and permeabilized with 0.3% Triton X-100 for 45 min at 4°C. After blocking solution with bovine serum albumin, the washed cells were incubated with the primary antibody mouse anti-AFP (Abcam, Cambridge, UK) overnight at 4°C. Subsequently, the cells were incubated with secondary anti-mouse Alexafluor 488 (Invitrogen, NY) and 10 μg/ml 4′,6-diamidino-2-phenylindole (DAPI) (Sigma).

Glycogen storage

Intracellular glycogen was analyzed by periodic acid-schiff (PAS) staining. The cells were fixed with 10% formalin (ScyTek Laboratories) for 45 min. The samples were then oxidized in 1% periodic acid for 5 min, rinsed 3 times in deionized water, incubated with Schiff's reagent for 15 min in the dark at RT and washed three times with H_2O for 10 min. The nuclei were stained with hematoxylin for 1 min, and rinsed with H_2O.

Urea production

Since the urea cycle is active only in hepatocytes, we determined urea concentration in the cultured cells. The cells, collected at Days 5 and 7 of the differentiation protocol, were analyzed by the Urea Assay Kit (Abcam) according to the manufacturer's instructions. Human BDSCs were used as negative control.

Results

Isolation of human BDSCs from peripheral blood

Stem cells from human peripheral blood were obtained as described in other papers of ours (Marfe et al., 2012a,b). After erythrocytes removal, the nucleated blood cells were incubated with M-CSF for 72 h. Within this period, the stem receptors were expressed on the surface of the nucleated cells. Because these cells were quickly dividing, after 72 h there were

a sufficient number of stem cells for sorting. In addition, such sorting resulted in the elimination of differentiated granulocytes and monocytes in order to obtain pure stem cell population. After sorting, the cells were plated. As shown in Figure 1 they exhibited morphological changes assuming a typical conformation called "string of pearls."

Morphological observation of human BDSCs derived hepatocytes

To evaluate the differentiation of human BDSCs into hepatocyte-like cells, morphological features, cytological staining, molecular assays to detect specific biological markers, and hepatic functions were made.

After the first 2 days in a maintenance medium as described in Materials and Methods section, the cells organized themselves into a spheroid structure known as embryoid bodies. These structures resembled the three embryonic sheets, characterized by a darker and compact nucleus surrounded by lighter and shining boards (Itskovitz-Eldor et al., 2000; Zhou et al., 2010). These bodies budded (day 2) and the cells started to differentiate (Fig. 2a–e).

After the treatment with FGF-4 and HGF, the cells transformed and within 5 days gradually progressed toward the typical polygonal hepatocyte-like morphology with several cytoplasmic granules, losing the typical spherical and regular shape of stem cells (Fig. 3).

Molecular characterization of human BDSCs cell-derived hepatocytes

The hepatic character is ultimately defined by acquisition of hepatic function. Thus, we performed RT-PCR analyses to assess expression of hepatic functional marker gene. Indeed, compared to human BDSCs, the hepatocytes-like cells exhibited increased mRNA expression of the liver-generated plasma proteins AFP following 7 days in culture (Fig. 4a).

Differentiation of human BDSCs is accompanied by the loss of expression of markers of pluripotency or "stemness," such as Nanog. Western blot analyses on the hepatocytes-like cell population revealed a disappearance in Nanog expression during differentiation protocol (Fig. 4c), while the AFP protein was visible after 7 day (Fig. 4d)

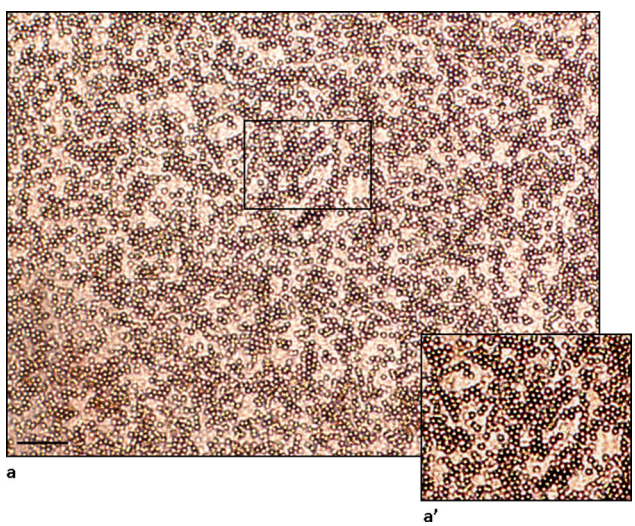

Fig. 1. Human BDSCs after sorting. a: The human BDSCs show a round-shining shape and they are organized in a "string of pearls." a′: Enlargement of a detail. (Magnification 10×, Bar = 100 μm).

Immunocytochemistry

For further characterization, the immunocytochemical staining against AFP, the marker for hepatocyte differentiation, was applied on the differentiated hepatocyte-like cells. AFP was detected in treated cells on 5 days of culture in differentiation medium and then were directly fixed and stained on 24-well plates as shown in Figure 5c and f. Immunocytochemical analysis with an anti-human hepatocyte antibody strongly stained differentiated cells (green; Fig. 5a and d), while the nuclei have been highlighted by DAPI staining (blue; Fig. 5b and e).

Functional assay of human BDSCs cell-derived hepatocytes

In order to assess whether these hepatocyte-like cells derived from human BDSCs also acquired typical liver metabolic features, glycogen storage, and urea production were evaluated.

Intracellular glycogen was analyzed by PAS staining. Upon treatment with hepatogenic media, glycogen storage was first seen after only 7 days (Fig. 6a–d). We further used a haematoxylin stain that spotlighted polynucleated mature hepatocyte cells characterized by peripheral nuclei (Fig. 6e).

In addition, we measured the urea synthesis by the differentiated cells. The urea production was significantly increased during the culture time compared to undifferentiated cells. Urea was not significantly present in human BDSC control cells (days 0), but it was exponentially increasing (from 5 to 7 days; Fig. 7).

Taken together these findings demonstrated that our differentiated cells showed hepatocyte-specific functions.

Discussion

Liver development occurs by an ordered sequence of biological events. Each step of cell growth and differentiation is tightly regulated by cell autonomous mechanisms and extracellular signals, including cytokines and growth factors (Kinoshita and Miyajima, 2002). Previously, Schwartz et al. (2002) have described a population of cells in postnatal rat bone marrow which were able of differentiating into endodermal-type cells (hepatocytes) upon exposure to hepatogenic factors. Lee et al. (2004) have reported hepatic differentiation of human mesenchymal stem cells upon treatment with HGF, FGF, and basic fibroblast growth factor (bFGF) while Snykers et al. (2006) have found hepatogenic differentiation of rat MSCs by using HGF, FGF, oncostatin M (OSM) and a cocktail of insulin–transferrin–selenium. Although other studies have used such liver-specific cytokines and growth factors for in vitro differentiation of stem cells into hepatocyte-like cells from adipose tissue (Seo et al., 2005) and human bone marrow (Lavon and Benvenisty, 2005), attempts at using various approaches to enhance the effectiveness of cell therapy is still a challenge.

Our recent studies (Marfe et al., 2012a,b) reported that BDSCs represent a novel source of stem cells for clinical application. In this article, we demonstrate the human BDSCs ability to differentiate into hepatocyte-like cells using only HGF and FGF-4 in combination. HGF is a multifunctional factor, which can promote cell survival and regeneration, inhibits the apoptosis of stem cells and increases the survival rate of the transplanted cells (Schmidt et al., 1995). FGF-4 was considered to be one of the most important FGF family members that can trigger the proliferation of mesodermal and endodermal cells and improve the development of fetal liver (Rappolee et al., 1994; Dong et al., 2010).

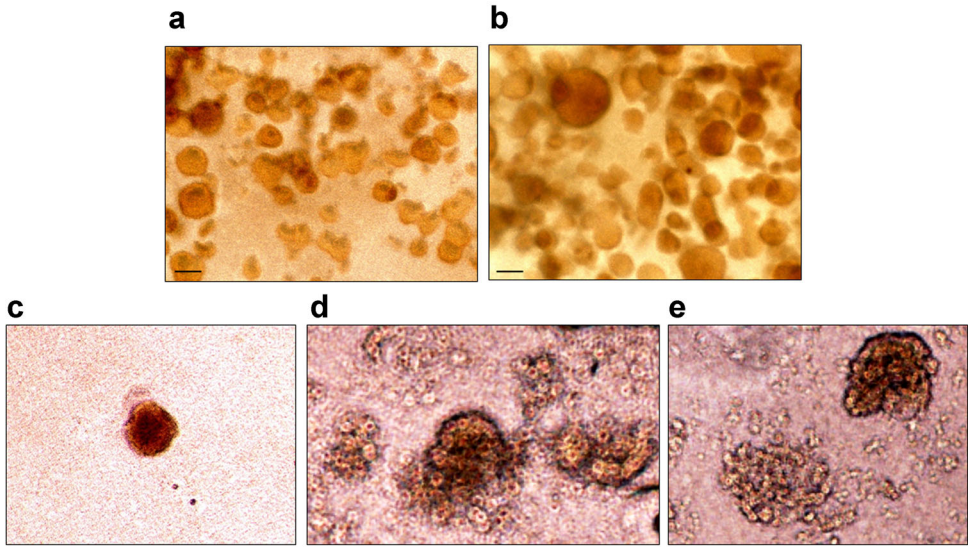

Fig. 2. Rapid organization of human BDSCs into embryoid bodies. a,b: Human BDSC samples organize themselves into a spheroid structure. c: Embryoid bodies appear with dark center and translucent boards. d,e: Embryoid bodies open and bud. (Magnification 10×, Bar = 100 μm).

Several laboratories have reported the differentiation of human stem cells into hepatocyte-like cells using different methods (Zhang et al., 2012). However, these protocols are lengthy (15–20 days) and need to be fine-tuned to obtain hepatocyte-like cells in sufficient numbers for autologous cell therapy. Here, we describe a quick and easy differentiation strategy that allows the transformation of human BDSCs into liver cells in 7 days only.

To reach this objective, we have adopted different steps in the differentiation protocol: (1) growth of human BDSCs cells for 2 days in a normal medium; (2) growth in a medium supplemented with HGF and FGF-4 for 5 additional days to obtain hepatocyte-like cells; (3) human BDSCs differentiation in hepatocyte-like cells using only collagen, although previous studies (Kazemnejad et al., 2008) reported that specific scaffolds were required to induce the formation of functional hepatocytes.

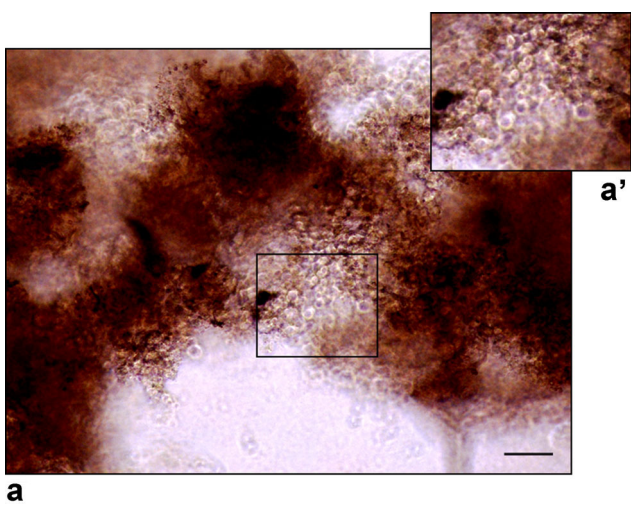

Fig. 3. Morphological features of the hepatic differentiated cells. a: The differentiated cells under hepatogenic conditions on day 7. (Magnification 10×, Bar = 100 μm). a′: Enlargement of a detail that shows the polygonal shape typical of mature hepatocytes.

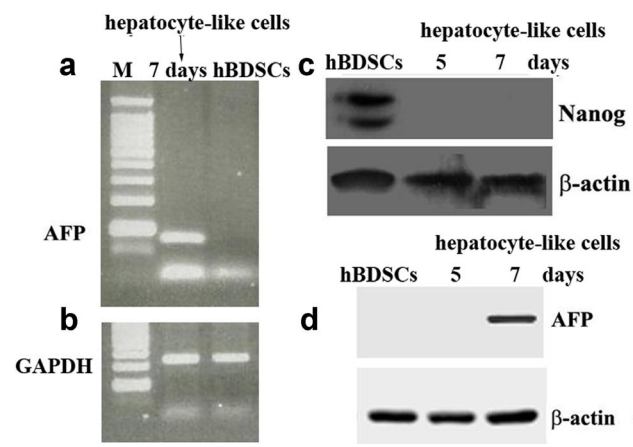

Fig. 4. AFP mRNA transcript by RT-PCR, AFP, and Nanog expression protein during hepatic differentiation. a: AFP mRNA transcript: M, molecular weight marker (100 bp); Hepatocytes-cells like (7 days); Human BDSCs (hBDSCs) sample as negative control cells. b: GADPH transcript (as an internal loading control). The gel is representative of three separate experiments. c: Whole-cell lysates were prepared from human BDSCs (hBDSCs) as positive control cells Hepatocytes-cells like (5 and 7 days). Western Blot analysis was performed with antibody specifically recognizing Nanog. β-actin was used as an internal loading control. Blot is representative of three separate experiments. d: Whole-cell lysates were prepared from human BDSCs (hBDSCs) as negative control cells and Hepatocytes-cells like (5 and 7 days). Western Blot analysis was performed with antibody specifically recognizing AFP. β-actin was used as an internal loading control. Blot is representative of three separate experiments.

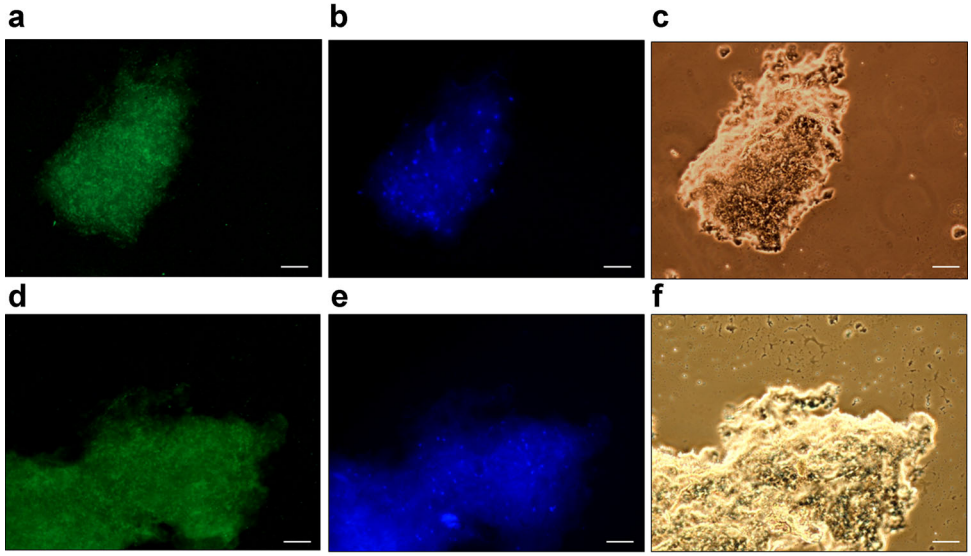

Fig. 5. The immunocytochemistry of the hepatic differentiated cells (7 days). a,d: Fluorescent staining for AFP protein (green). b,e: Nuclear staining using DAPI (blu) for AFP protein. c,f: Visible. (Magnification 10×, Bar = 100 μm).

In the final step >90% of the cells displayed hepatocyte-specific features and morphologic changes such as a large number of bi-nucleated cells and the mature cuboidal morphology (polygonal-shaped similar to those of primary hepatocytes). In addition, we demonstrated that the human BDSCs, reprogrammed in liver cells, expressed AFP by RT-PCR, Western blot, and immunohistochemistry assay. Moreover, we observed, for the first time, that the Nanog protein present in the deprogrammed BDSCs was gradually disappearing during the cellular reprogramming. We show that the differentiated cells acquired typical hepatocyte functions such as urea production which was practically absent in undifferentiated cells. The production of urea is performed by hepatocytes only and not by undifferentiated cells like those of yolk sac (Lavon and Benvenisty, 2005). In the same time, we also demonstrate the presence of another hepatocyte feature, that is, the cytoplasmic storage of glycogen.

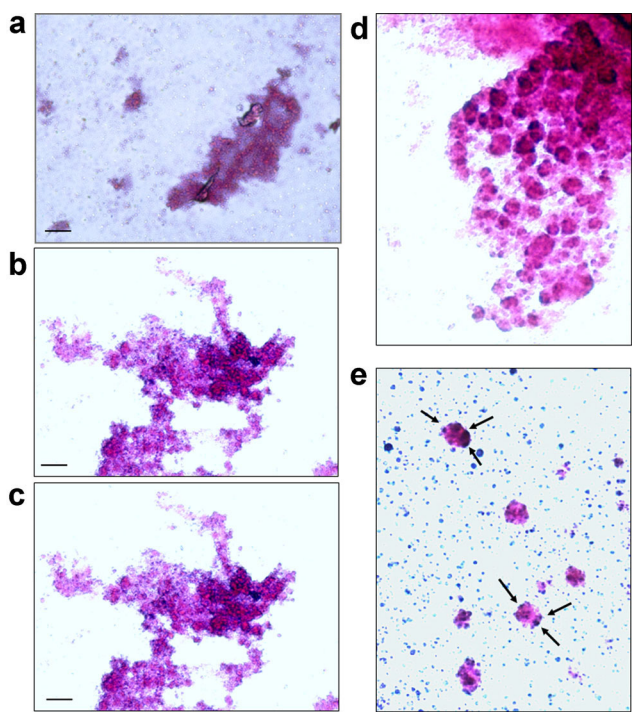

Fig. 6. Periodic Acid-Schiff staining assay in hepatic differentiated cells (7 days). a–d: Differentiated cells show the glycogen storage represented by the typical magenta staining; (e) polynucleated differentiated cells stained by hematoxylin. (Magnification 10X, Bar = 100 μm).

In summary, the hepatic differentiation approach defined in this study—culture in hepatocyte media on collagen type I substrate for 7 days—provides a simple, straight-forward methodology by which human BDSCs may be directed to differentiate along a hepatic lineage. Extensive characterization of the human BDSCs—derived hepatic-like cell population revealed disappeared protein expression of marker of pluripotency such as *NANOG* as well as marked increase in AFP expression levels. Urea production and glycogen storage capacity by the differentiated cells confirmed acquisition of hepatic function, properties that were obtained after only 7 days of the treatment.

This model opens new perspectives: it may be applicable not only to the study of cell fate and differentiate uncommitted cells toward endodermal lineages, but also indicates that the growth factors may have a great potential in cell therapy. As shown here, differentiated human BDSCs displayed both qualitative and quantitative traits of hepatocytes.

In conclusion, the BDSCs appear to have great potential for clinical applications in regenerative medicine and tissue/organ replacement. First, they can be easily isolated and expanded in amounts relevant for clinical application. Second, being autologous BDSCs can be used without rejection risks for clinical application in liver diseases.

Altogether, our findings point to BDSCs as potential tools in regenerative medicine and an alternative therapeutic strategy for different human pathologies.

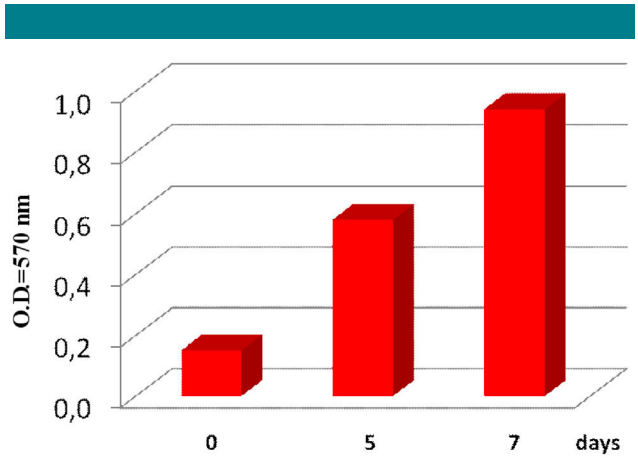

Fig. 7. Urea production in hepatic differentiated cells. The histogram shows the urea production at 0, 5 and 7 days during hepatic differentiation. This graphic is representative of three separate experiments.

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