Available online at www.sciencedirect.com
SCIENCE DIRECT®
Biochemical and Biophysical Research Communications 326 (2005) 66–73
BBRC
www.elsevier.com/locate/ybbrc

Human hepatocytes in mice receiving pre-immune injection with human cord blood cells

Paolo Turrini[a], Giovanni Monego[b], Josè Gonzalez[d], Sandra Cicuzza[a], Giuseppina Bonanno[c], Giovanni Zelano[b], Nadia Rosenthal[d], Giacomo Paonessa[a], Ralph Laufer[a], Julio Padron[a,*]

[a] *Pharmacology Department, Istituto di Ricerca di Biologia Molecolare "P.Angeletti" (IRBM), Merck Sharp and Dohme Research Laboratories, Rome, Italy*
[b] *Department of Anatomy, Catholic University of Sacred Heart, Rome, Italy*
[c] *Department of Obstetrics and Gynecology, Catholic University of Sacred Heart, Rome, Italy*
[d] *EMBL Monterotondo, Mouse Biology Programme, via Ramarini 32, 00016 Monterotondo—Scalo (RM), Italy*

Received 22 October 2004
Available online 11 November 2004

Abstract

It is well established that certain subpopulations of human adult stem cells can generate hepatocyte-like cells when transplanted into adult immunosuppressed mice. In the present study, we wanted to explore whether xeno-transplantation of human cord blood $CD34^+$ ($hCBCD34^+$) cells during pre-immune stages of development in immunocompetent mice might also lead to human–mouse liver chimerism. Freshly isolated $hCBCD34^+$ cells were xeno-transplanted into non-immunosuppressed mice by both intra-blastocyst and intra-fetal injections. One and four weeks after birth, immunostaining for different human-specific hepatocyte markers: human hepatocyte-specific antigen, human serum albumin, and human α-1-antitrypsin indicated the presence of human hepatocyte-like cells in the livers of transplanted animals. Detection of human albumin mRNA further corroborated the development of pre-immune human-mouse chimeras. The current report, besides providing new evidence of the potential of $hCBCD34^+$ cells to generate human hepatocyte-like cells, suggests novel strategies for generating immunocompetent mice harboring humanized liver.

Keywords: Xeno-transplantation; Stem cells; Intra-fetal; Intra-blastocyst; Liver; Hepatocytes

Mouse liver repopulation with donor cells of human origin has significant potential for cell and gene therapy applications and for the study of biological mechanisms underlying viral and metabolic diseases of the liver. Xeno-transplantation of freshly isolated human hepatocytes into the livers of immunosuppressed mice carrying genetically defective hepatocytes has shown a valuable potential for hepatitis B virus (HBV) and hepatitis C virus (HCV) research [1,2]. However, two important issues limit this approach. First, healthy and freshly isolated human hepatocytes are frequently not available on a regular basis. Second, the need to use immunosuppressed animals hampers studies on the immuno-ethiopathogenesis of viral hepatitis.

Development of tolerance, instead of immunosuppression, has been already suggested and explored by other authors [3,4]. It is generally well established that auto-reactive T cell clones are completely deleted from the immune repertoire during fetal development [5]. Indeed rats, intra-fetally tolerated against human liver antigens, accept xeno-transplantation with hepatocytes coming from the same donor [3]. However, once again

* Corresponding author. Fax: +39 06 91093654.
E-mail address: julio_padron@merck.com (J. Padron).

0006-291X/$ - see front matter © 2004 Elsevier Inc. All rights reserved.
doi:10.1016/j.bbrc.2004.10.204

the availability of freshly isolated human hepatocytes and in this case from the same donor would be the limiting step.

Recent advances in stem cell research suggest alternative sources for many human tissues, including hepatocytes. Not only some clones of embryonic stem cells have been reported to differentiate into hepatocyte-like cells, under specific in vitro culture conditions [6,7], but also certain subpopulations of adult stem cells were shown to generate hepatocyte-like cells when transplanted into adult immunosuppressed mice [8–11]. In particular, it was shown that c-kit+ Thy-1.1 Lin-sca-1+ stem cells, isolated from healthy mouse bone marrow, were able to correct liver defects in fumarylacetoacetate hydrolase-deficient mice (Fah−/−) mice and to rescue mutant mice from death [12].

Many authors argue in favor of stem cell plasticity [13,14], but it is still controversial whether this process is due to *trans*-differentiation [15–17], presence of different adult committed stem cell subpopulations [18,19], cell fusion [20], or the coexistence of all these phenomena. In any case, it seems clear that stem cells possess enormous potential as raw material for tissue engineering.

Human cord blood $CD34^+$ cells have been extensively used to repopulate the hematopoietic system of adult sub-lethally irradiated non-obese diabetic (NOD)-severe combined immunodeficient (SCID) mice [21,22]. Moreover, hematopoietic chimerism has also been demonstrated by microinjecting $hCBCD34^+$ cells into mouse blastocysts [23] as well as into pig fetuses [24]. Finally, transplantation of human hematopoietic stem cells into adult SCID mice may result in engraftment of human hepatocyte-like cells expressing human albumin [25]. Nevertheless, the possibility of achieving non-hematopoietic chimerism and in particular liver engraftment in mice xeno-transplanted at pre-immune stages of development is much less clearly established.

In the present study, we wanted to explore whether it was possible to combine two approaches, namely the use of adult human stem cells and mouse pre-immune xeno-transplantation, to generate an immunocompetent mouse with human liver chimerism.

Materials and methods

Human cord blood cell collection and isolation. Human cord blood cells were obtained from informed mothers undergoing cesarean delivery of full-term infants. Cord blood collection and the entire study were approved by the Hospital Investigational Review Board. Only cord blood collections with a final volume higher than 70 ml and showing a $CD34^+$ cell frequency higher than 0.2%, were processed for cell isolation. Red cells were removed from cord blood by a 90-min sedimentation using hydroxyethyl starch at a ratio of 1:8 to cord blood. After washings, mononuclear cells were isolated from red cell-depleted nucleated cells by centrifugation on a Ficoll–Paque density gradient (1.077 g/ml; Pharmacia LKB, Uppsala, Sweden). Finally, isolated MCs were processed by magnetic activated cell sorting for $CD34^+$ using CD34 isolation kit (Miltenyi Biotec, Bergish Gladbach, Germany), in accordance with the manufacturer's instructions.

Intra-blastocyst microinjection. Murine blastocysts were isolated from donor C57BL/6 on day 3.5 of gestation, and 15–20 $hCBCD34^+$ cells were injected into each blastocyst. Blastocysts were then retransferred into foster mothers.

Intra-fetal transplantation. All animal housing, handling, and experimental procedures were carried out in accordance with national and company guidelines, and the criteria outlined in the "Guide for the care and use of laboratory animals." All fetuses from pregnant CD1 mice (Charles River, Italy) were individually transplanted with $hCBCD34^+$ cells, using the surgical procedure described by Rice et al. [26]. Pregnant CD1 mothers were anesthetized with Avertine. Following a single vertical abdominal incision, the uterus was exposed and *trans*-illuminated. Using a sterile Hamilton syringe, 3000–5000 $hCBCD34^+$ cells (contained in 10 μl PBS plus 1% bovine serum albumin) were intra-peritoneally inoculated into each individual fetus. At the end of the microinjection procedure, the uterus was carefully returned back into the abdominal cavity, and both peritoneal wall and skin were sutured and externally disinfected with betadine.

Immunohistochemistry. Mouse livers were collected, fixed in 10% formalin for 48 h and embedded in paraffin (Bio-plast special; melting point: 52–54 °C). Tissue sections (5 μm) were adsorbed on polylysine-coated glass slides, air-dried, deparaffinized, and then placed in a water bath. Unmasking was carried out by immersion into a pre-heated target retrieval solution (DAKO) for 15 min at 92–95 °C. Endogenous peroxidase was blocked by incubating sections with 1% H_2O_2 in methanol for 30 min. Finally, different sections were used to develop immunostaining with different human-specific antibodies: monoclonal mouse anti-HepPar1 (DAKO), rabbit anti-hAAT (DAKO), and pre-adsorbed goat polyclonal anti-HSA (Bethyl Labs). Human hepatocyte-specific antigen (HepPar1) was labeled by using DAKO ARK (Animal Research Kit). Rabbit-anti-hAAT and goat-anti-HSA, after 1 h of incubation at RT, were detected with biotinylated anti-rabbit and anti-goat IgG, respectively. Sections were then incubated with streptavidin–peroxidase, ready-to-use (Labvision) for 15 min at RT. The reaction was developed by using diaminobenzidine (DAB) (0.075 g/L) and H_2O_2 (0.003%) for 5 min at RT. Following several washes with distilled water, slides were counterstained in hematoxylin, dehydrated, and mounted in a non-aqueous permanent-mounting medium.

Reverse-transcriptase-polymerase chain reaction. Total RNA was extracted from tissue samples using Micro-to-Midy Total RNA Purification System according to manufacturer's protocols (Invitrogen life technologies, Paisley, UK) and the RNA was eluted in DEPC treated water (0.01% DEPC) and stored at −80 °C until RT-PCR analysis. Nucleic acid concentration of each sample was measured by spectrophotometry (Hewlett-Packard HP UV/VIS spectrophotometer 8450).

Reverse-transcriptase-polymerase chain reaction (RT-PCR) assay was performed using the two-step method. For the first-step RT reaction we used Superscript III Rnase H^- Reverse Transcriptase (Invitrogen life technologies) starting from 3 μg of total RNA and following manufacturer's recommendations for cDNA synthesis with oligo(dT) primers.

The second step PCR was based on three temperature cycles set-up for primers specific for human HSA. Amplification conditions were 95 °C for 9 min followed by 40 cycles of denaturation at 94 °C for 30 s, annealing at 55 °C for 30 s, and elongation at 72 °C for 1 min for HSA, followed by a final extension at 72 °C for 7 min.

The primers used were 5′-TCCTGATTACTCGTCGTG-3′ (sense) and 5′-GCAGCATTTGGTGACTCTG-3′ (antisense) for HSA [25].

PCR conditions were 10 pmol of each primer, 200 μM of each dNTP, 2.5 U *Taq* polymerase (AmpliTaq Gold DNA Polymerase, Applied Biosystems—Foster City CA), 2.5 mM $MgCl_2$ and 1× PCR buffer I (Applied Biosystems).

Identical PCR analysis was performed on RNA extracted from liver samples of control (non-transplanted) mice. The positive control

for RT-PCR was human RNA from HepG2 cells (a human hepatoma cell line).

A negative control (cDNA replaced with H_2O) was also used to rule out any contamination. The amplification product (457 bp) was examined by electrophoresis on 1.7% agarose gel stained with ethidium bromide, and the marker was pUC Mix Marker 8 (MBI, Fermentas, Vilnius, Lithuania). All samples were analyzed in triplicate.

Results

In the present study, we explored whether xeno-transplantation of hCBCD34$^+$ cells during pre-immune stages of development in immunocompetent mice might lead to human–mouse liver chimerism. We used two different approaches: in one case, female CD1 mice at 15–17th days of pregnancy were anesthetized in order to surgically expose the uterus and subsequently were intra-fetally injected with $3–5 \times 10^3$ hCBCD34$^+$ cells. In the second case, blastocysts at day 3.5, obtained from C57Bl6 pregnant mice, were microinjected with 15–20 hCBCD34$^+$ cells and re-implanted into foster mothers. One and four weeks after birth, animals were euthanized and the liver was processed for both immunohistochemistry and PCR.

Presence of human hepatocyte-like cells, in liver sections from 1-week-old mice receiving intra-fetal (IF) injections with hCBCD34$^+$ cells, was assessed by using three different specie-specific antibodies directed against human hepatocytes markers: HepPar1, HSA, and hAAT (Fig. 1). Specificity of the staining was in all cases ascertained by comparing human (Figs. 1A, E, and I) and mouse (Figs. 1B, F, and J) control liver sections,

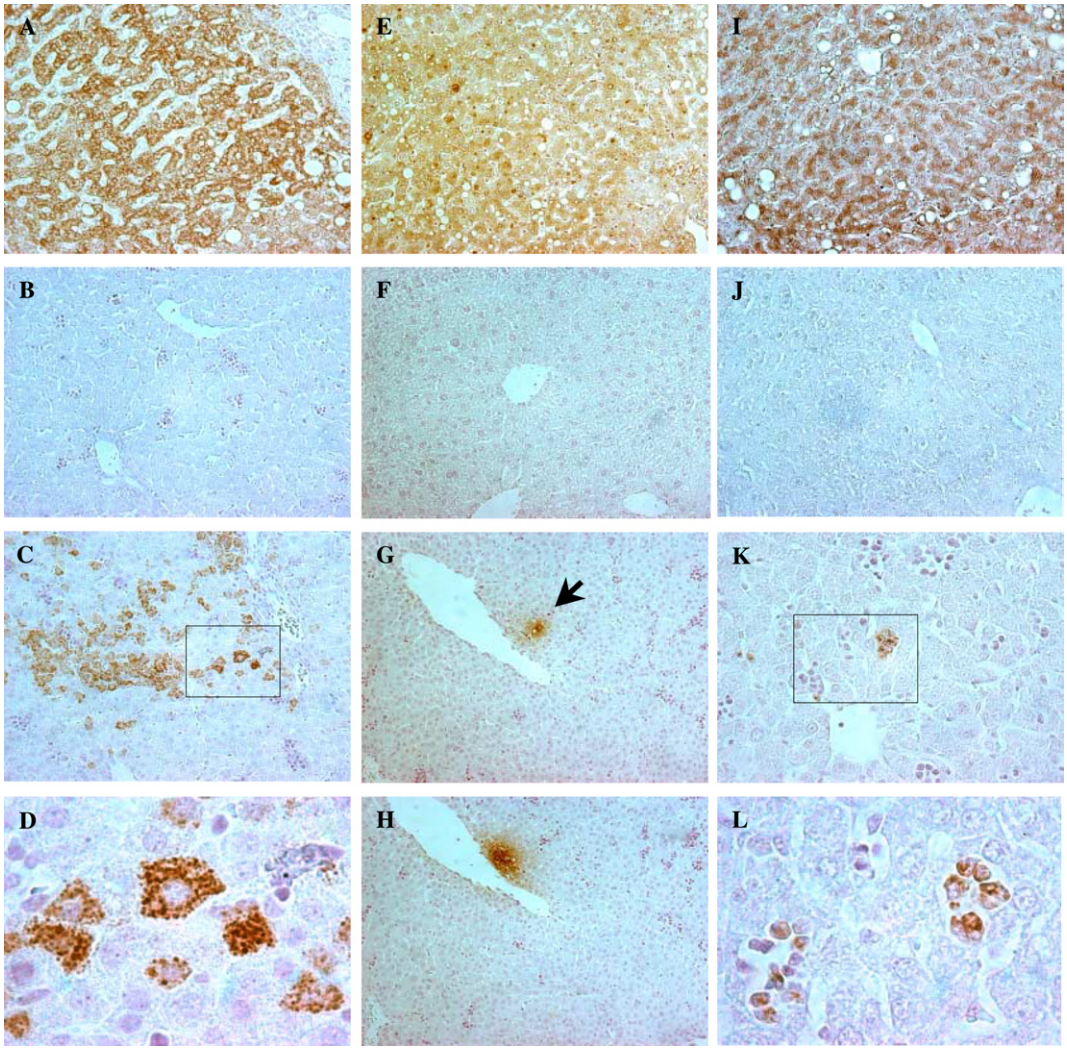

Fig. 1. Presence of human hepatocyte-like cells in the liver of 1-week-old mice receiving IF injection with hCBCD34$^+$ cells. Human hepatocyte-like cells were identified by using human-specific antibodies against HepPar1 (A–D), HSA (E–H), and hAAT (I–L). Control immunostaining using human (A, E, and I) and mouse liver sections (B, F, and J) was carried out to ascertain specificity of the antibodies. HepPar1 positive cells are dispersed all around in the liver (C). HSA positive foci were detected in serial liver sections (G,H). Clumps of small hAAT immunopositive cells were observed in the livers of chimeric mice (K). (Magnification 200×; D and L, 1000×).

and by omitting the primary antibody in a serial liver section from chimeric mice (data not shown).

HepPar1 positive cells were observed in four out of five chimeric subjects, appearing mostly dispersed all around in the liver (Fig. 1C). Greater magnification (Fig. 1D) highlighted the granular pattern of the cytoplasmatic immunostaining that typically characterizes human hepatocytes [2,27].

Next, by using a HSA polyclonal antibody pre-absorbed to mouse serum albumin, we found specific immunostaining for HSA in the livers of three out of these five mice. Although the signals appeared more sparse and diffuse as compared to HepPar1, the presence of immunostaining throughout several serial sections (Figs. 1G and H) corroborate the finding of foci of engraftment. Similarly, by using a hAAT-specific polyclonal antibody, we found four out of five mice with small clumps of immunopositive cells in the liver (Figs. 1K and L).

Immunohistochemistry was also carried out in liver sections of 1-week-old xeno-transplanted mice derived from blastocysts subjected to micro-injection with hCBCD34^{+} cells. HepPar1 immunopositive cells were found in liver sections from 7 out of 12 mice (Figs. 2A–C). Whereas, just 2 out of 12 liver samples from these mice scored immunoreactivity for HSA (Figs. 2D–F). Human AAT positive immunostaining was observed in the livers of 7 out of 12 of these mice (Figs. 2G–I).

Further to this, expression of a liver-specific human gene such as HSA was also assessed by RT-PCR in both IF and intra-blastocyst (IB) xeno-transplanted mice (Fig. 3). The specificity of the primers was ascertained by using in parallel control RNA from the human hepatoma cell line HepG2 and from non-transplanted mouse liver. Liver samples from the same mice analyzed by immunohistochemistry were used for RT-PCR analysis. In agreement with the immunostaining results, the PCR analysis confirms the presence of human hepatocyte-like cells in the liver of both IF and IB xeno-transplanted mice after 1 week from birth. Also in this case, a higher number of animals showing HSA positivity in the liver were observed in IF rather than IB xeno-transplanted groups (see Fig. 3 and Table 1).

Next, in order to explore whether engrafted cells could survive for longer periods of time, we assessed the expression of human-specific hepatic markers at four weeks after birth in both IF and IB xeno-transplanted mice with hCBCD34^{+} cells. As shown in Fig. 4 and Table 1, HepPAR1 immunostaining was still present in a relatively high proportion in liver sections from both IF- and IB-injected mice. HepPar1 positive foci were of-

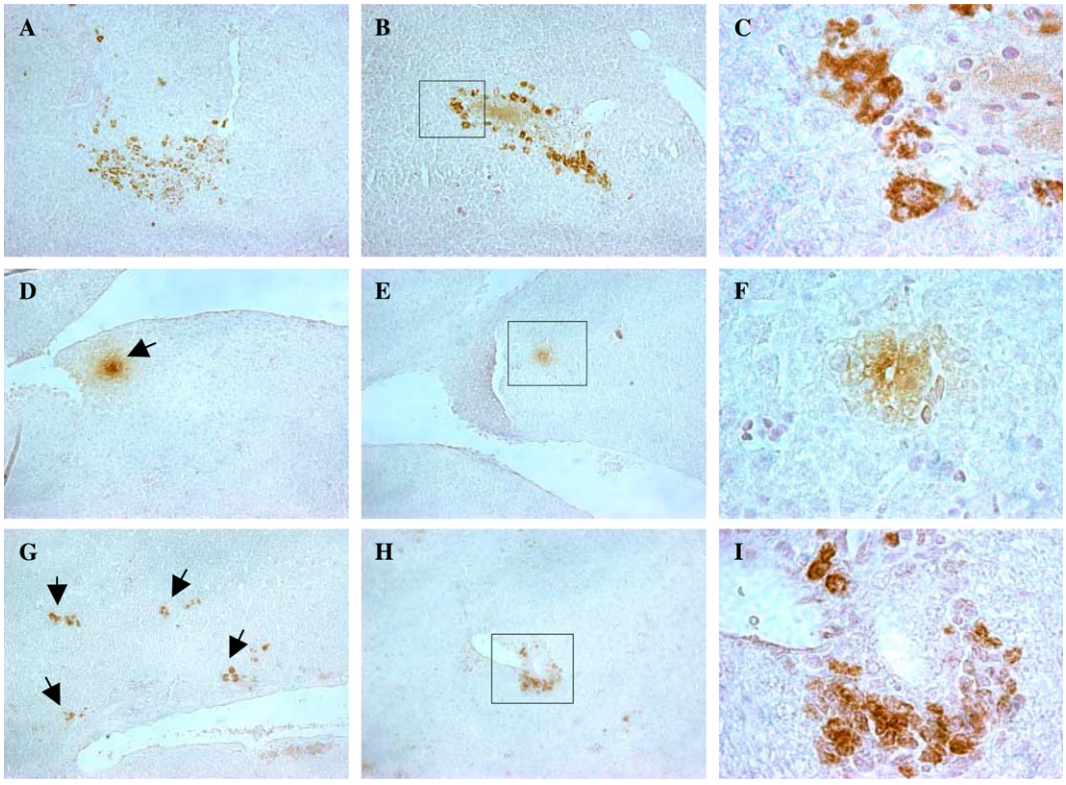

Fig. 2. Presence of human hepatocyte-like cells in the liver of 1-week-old mice receiving IB injection with hCBCD34^{+} cells. Human hepatocyte-like cells were identified by using human-specific antibodies against HepPar1 (A–C), HSA (D–F), and hAAT (G–I), (Magnification 200×; C, F, and I, 1000×).

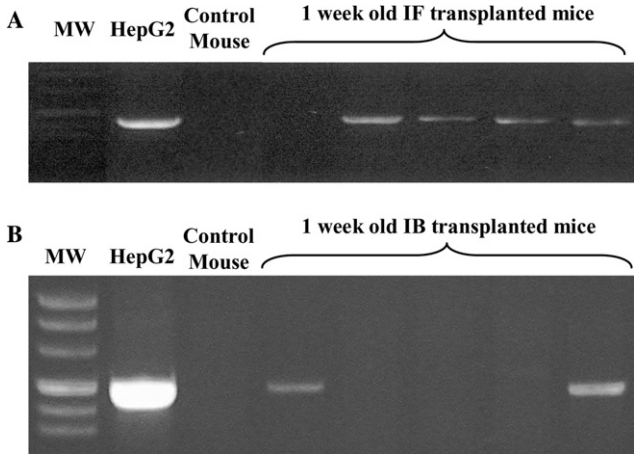

Fig. 3. RT-PCR analysis for HSA in 1-week-old mice receiving either IF or IB injection with hCBCD34$^+$ cells. Total liver mRNAs extracted 1 week after birth from mice subjected to IF (A) or IB (B) injection with hCBCD34$^+$ cells were analyzed by RT-PCR using specific probes to detect HSA. Molecular weight markers, HepG2 cells (as positive control) and non-transplanted control mouse, were assessed and depicted in both cases. HSA bands were detected in the liver extracts of four out of five IF xeno-transplanted mice (A) and in only two out of six mice derived from C57Bl6 mouse blastocyst micro-injected with hCBCD34$^+$ cells (B).

ten observed engrafted in clusters around major blood vessels (Fig. 4). Interestingly, RT-PCR demonstrated presence of HSA in the liver of three out of eight mice receiving IF injection and in just 1 out of 10 IB-injected mice with hCBCD34$^+$ cells (Fig. 5). This result is striking, considering that at four weeks after birth we never detected hAAT or HSA by either immunohistochemistry or ELISA in both IF and IB groups of animals (data not shown).

Summarizing (see Table 1), if looking at HepPar1 as an early marker of human hepatocyte differentiation, we found a quite important proportion of immunocompetent mice derived from fetuses or blastocysts injected with hCBCD34$^+$ cells that exhibited liver chimerism at both 1 and 4 weeks after birth. However, much lower figures come out when using markers of late stage of human hepatocyte lineage such as HSA. Furthermore, by observing the results in Table 1, it is also possible to deduce that the efficacy of engraftment and differentiation of hCBCD34$^+$ cells toward human hepatocytes phenotype is better in IF rather than IB-injected mice but in any case decreases with age of animals.

Discussion

Even though some reports have argued in favor of a putative immune escape mechanism for stem cells [28], other authors have already attempted additional long-term tolerance by IF allogenic [29,30] or xenogenic [26,31] stem cell transplantation. It is fairly well established that inoculating antigens during pre-immune stages of development leads to tolerance against such specific antigens [32,33].

Most studies on adult stem cell derived hepatocytes are based on the use of different subpopulations of bone marrow stem cells [34–36]. However, it has been also demonstrated that transplantation of human cord blood stem cells into adult SCID mice may result in engraftment of human hepatocyte-like cells expressing human albumin [25]. More recently, hCBCD34$^+$ cells were shown to generate human hepatocyte-like cells in pre-immune sheep [37]. In agreement with previous reports, the results of the present study add new evidence indicating that hCBCD34$^+$ cells can integrate not only into adult [11,25,28], but also into pre-immune mouse liver and give rise to liver cells expressing human hepatocyte-specific markers for several weeks after birth. The actual stage of hepatocyte maturation in these animals is less obvious, and hence difficult to judge. Indeed, whereas HepPar1 immunopositive cells were always found at any time point evaluated, HSA and hAAT liver staining was mainly observed at 1 week and much less at 4 weeks of age in both IF- and IB-injected mice. Though HepPAR1 antigen is considered an excellent marker of human hepatocyte lineage, it is known to be present not only in fully differentiated human hepatocytes, but also in very early stages of liver development such as in human hepatoblasts [38]. So even if limited, the presence of specific immunostaining for HSA and hAAT in the liver of both IF and IB xeno-transplanted mice at 1 week of age provides support in favor of a further step in hepatoblast maturation.

There are several possible explanations for the absence of a rigorous correlation between the level of

Table 1
Summary table showing total number of mice injected with hCBC34+ cells during pre-immune stages of development and being screened at 1 and 4 weeks after birth for human into mouse liver chimerism

Groups	Immunohistochemistry			RT-PCR		
	Total	HepPar1	Percentage	Total	mRNA-HSA	Percentage
Intra-fetal-1 week	5	4	80	5	4	80
Intra-fetal-4 week	20	10	50	8	3	38
Intra-blastocyst-1 week	12	7	58	6	2	33
Intra-blastocyst-4 week	10	6	60	10	1	10

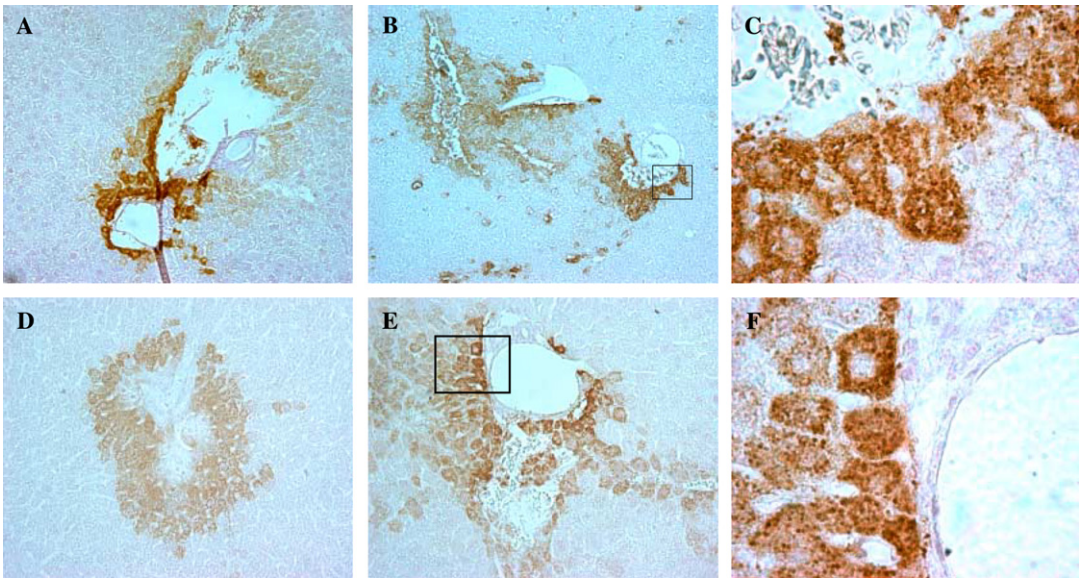

Fig. 4. HepPar1 immunohistochemistry in liver sections from four-week-old mice receiving either IF or IB injection with hCBCD34$^+$ cells. HepPar1 immunostaining was carried out in liver sections from mice that received IF (A–C) or IB (D–F) injection with hCBCD34$^+$ cells. HepPar1 positive cells engrafted always around vessels (Magnification 200×: A, B, D, and E; 1000×: C, F).

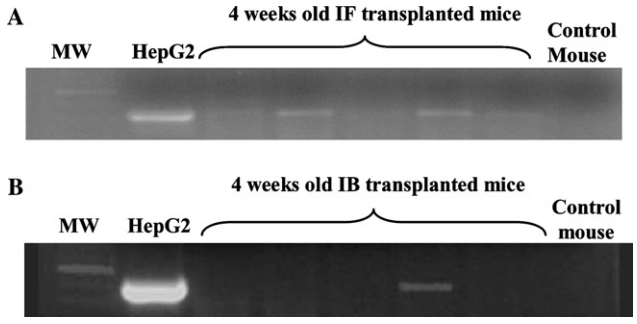

Fig. 5. RT-PCR analysis for HSA in four-week-old mice receiving either IF or IB injection with hCBCD34$^+$ cells. Total liver mRNAs extracted four weeks after birth from mice subjected to IF (A) or IB (B) injection with hCBCD34$^+$ cells were analyzed by RT-PCR using specific probes to detect HSA. Molecular weight markers, HepG2 cells (as positive control) and non-transplanted control mouse were assessed and depicted in both cases. Very weak HSA bands were detected in the liver extracts of two out of five IF xeno-transplanted mice (A) and in only one out of six mice derived from C57Bl6 mouse blastocyst micro-injected with hCBCD34$^+$ cells (B).

immunostaining for HepPAR1 and HSA or hAAT. Scarcity of HSA producing cells might indicate the existence of a rare stem cell subpopulation with a real hepatic commitment, whereas some other CD34$^+$ cell subpopulations might be able to initiate hepatocyte nuclear programming without reaching full maturation. Alternatively, the small number of HSA producing cells might be the consequence of the xeno-environment. Development of fully differentiated cells might require human specie-specific factors. This hypothesis is supported by the recent finding that HSA expression in adult SCID mice transplanted with hCBCD34$^+$ cells was significantly increased by treatment with human hepatocyte growth factor [25]. It is noteworthy that a discrepancy between the extent of liver repopulation, as assessed by HepPAR1 expression (50% of hepatocytes) and the expression of HSA (5% of normal levels in human serum), was also observed by other authors in albumin-urokinase-type plasminogen activator (Alb-uPA)-SCID mice transplanted with freshly isolated human hepatocytes [2].

Recent findings suggest that the ability of transplanted stem cells to produce differentiated cell types for other tissues is due to fusion with resident somatic cells, rather than to transdifferentiation of the original stem cell population. Indeed, extensive liver repopulation in adult Fah−/− mice transplanted with stem cells from mouse bone marrow was reported to be mostly the consequence of cell fusion with defective host hepatocytes [36]. Interestingly however, such fused cells were able to correct a metabolic defect and rescued animals from death [39]. It cannot be excluded that the results of the present study can be explained by a similar mechanism, i.e., the production of fused cells that efficiently express some human hepatocyte genes, such as HepPAR1, but express only low levels of other human genes, such as HSA. It should nonetheless be noted that cell fusion could not be detected in the livers of SCID mice transplanted at the adult stage with hCBCD34$^+$ cells [27]. In conclusion, we have demonstrated that it is possible to transplant adult human hematopoietic stem cells in early stage of mouse development. Human CBCD34$^+$ cells are, in fact, able to reach the liver environment and to partially differentiate toward human hepatocyte-like cells. While it is clear that further studies are required to elucidate the underlying mechanisms, our results show that even in absence of immunosup-

pression, chronic selective advantage, or treatment with human growth/differentiating factors, both IF- and IB-injected hCBCD34$^+$ cells are able to integrate and express some human-specific hepatocyte markers for up to 4 weeks after birth in the liver of recipient mice.

It remains to be determined whether the procedure of pre-immune xeno-transplantation described here could be applied for complete liver repopulation of genetically hepatopathic mice (Alb-uPA or Fah−/−) and whether it could be further improved by treatment of mice with human growth/differentiating factor such as HGF [25], or by using alternative stem cell subpopulations such as MAPC [40] or C1qR [9] with a more committed hepatic lineage potential or early stem/progenitor stem cells from fetal human liver [41]. It is hoped that the current findings may be exploited to develop new strategies for generating immunocompetent mice harboring a humanized liver with an enormous potential for human metabolism and HCV infection studies.

Acknowledgments

This work was supported in part by a grant from the MIUR. The authors thank Maria Luisa Orlando for her excellent secretarial assistance, Janet Clench for English revision, Manuela Emili for artwork, Dr. Giovanni Migliaccio, and Prof. Riccardo Cortese for reviewing the manuscript and providing critical input.

References

[1] M. Dandri, M.R. Burda, E. Torok, J.M. Pollok, A. Iwanska, G. Sommer, et al., Repopulation of mouse liver with human hepatocytes and in vivo infection with hepatitis B virus, Hepatology 33 (2001) 981–988.

[2] D.F. Mercer, D.E. Schiller, J.F. Elliott, D.N. Douglas, C. Hao, A. Rinfret, et al., Hepatitis C virus replication in mice with chimeric human livers, Nat. Med. 7 (2001) 927–933.

[3] E.C. Ouyang, C.H. Wu, C. Walton, K. Promrat, G.Y. Wu, Transplantation of human hepatocytes into tolerized genetically immunocompetent rats, World J. Gastroenterol. 7 (2001) 324–330.

[4] C.H. Wu, E.C. Ouyang, C.M. Walton, G.Y. Wu, Human hepatocytes transplanted into genetically immunocompetent rats are susceptible to infection by hepatitis B virus in situ, J. Viral Hepat. 8 (2001) 111–119.

[5] J.M. Cruse, R.E. Lewis Jr., The immune system victorious: selective preservation of self, Immunol. Res. 12 (1993) 101–114.

[6] T. Hamazaki, Y. Iiboshi, M. Oka, P.J. Papst, A.M. Meacham, L.I. Zon, N. Terada, Hepatic maturation in differentiating embryonic stem cells in vitro, FEBS Lett. 497 (2001) 15–19.

[7] N. Lavon, O. Yanuka, N. Benvenisty, Differentiation and isolation of hepatic-like cells from human embryonic stem cells, Differentiation 72 (2004) 230–238.

[8] B.E. Petersen, W.C. Bowen, K.D. Patrene, W.M. Mars, A.K. Sullivan, N. Murase, et al., Bone marrow as a potential source of hepatic oval cells, Science 284 (1999) 1168–1170.

[9] G.H. Danet, J.L. Luongo, G. Butler, M.M. Lu, A.J. Tenner, M.C. Simon, D.A. Bonnet, C1qRp defines a new human stem cell population with hematopoietic and hepatic potential, Proc. Natl. Acad. Sci. USA 99 (2002) 10441–10445.

[10] R.E. Schwartz, M. Reyes, L. Koodie, Y. Jiang, M. Blackstad, T. Lund, et al., Multipotent adult progenitor cells from bone marrow differentiate into functional hepatocyte-like cells, J. Clin. Invest. 109 (2002) 1291–1302.

[11] S. Kakinuma, Y. Tanaka, R. Chinzei, M. Watanabe, K. Shimizu-Saito, Y. Hara, et al., Human umbilical cord blood as a source of transplantable hepatic progenitor cells, Stem Cells 21 (2003) 217–227.

[12] E. Lagasse, H. Connors, M. Al-Dhalimy, M. Reitsma, M. Dohse, L. Osborne, et al., Purified hematopoietic stem cells can differentiate into hepatocytes in vivo, Nat. Med. 6 (2000) 1229–1234.

[13] M.A. Goodell, Stem-cell "plasticity": befuddled by the muddle, Curr. Opin. Hematol. 10 (2003) 208–213.

[14] J.E. Grove, E. Bruscia, D.S. Krause, Plasticity of bone marrow-derived stem cells, Stem Cells 22 (2004) 487–500.

[15] G. Condorelli, U. Borello, L. De Angelis, M. Latronico, D. Sirabella, M. Coletta, et al., Cardiomyocytes induce endothelial cells to *trans*-differentiate into cardiac muscle: implications for myocardium regeneration, Proc. Natl. Acad. Sci. USA 98 (2001) 10733–10738.

[16] A. Vescovi, A. Gritti, G. Cossu, R. Galli, Neural stem cells: plasticity and their transdifferentiation potential, Cells Tissues Organs 171 (2002) 64–76.

[17] J.C. Howell, W.H. Lee, P. Morrison, J. Zhong, M.C. Yoder, E.F. Srour, Pluripotent stem cells identified in multiple murine tissues, Ann. N. Y. Acad. Sci. 996 (2003) 158–173.

[18] M.D. Dabeva, P.M. Petkov, J. Sandhu, R. Oren, E. Laconi, E. Hurston, D.A. Shafritz, Proliferation and differentiation of fetal liver epithelial progenitor cells after transplantation into adult rat liver, Am. J. Pathol. 156 (2000) 2017–2031.

[19] C.J. Hogan, E.J. Shpall, G. Keller, Differential long-term and multilineage engraftment potential from subfractions of human CD34$^+$ cord blood cells transplanted into NOD/SCID mice, Proc. Natl. Acad. Sci. USA 99 (2002) 413–418.

[20] X. Wang, H. Willenbring, Y. Akkari, Y. Torimaru, M. Foster, M. Al-Dhalimy, et al., Cell fusion is the principal source of bone-marrow-derived hepatocytes, Nature 422 (2003) 897–901.

[21] J. Cashman, K. Bockhold, D.E. Hogge, A.C. Eaves, C.J. Eaves, Sustained proliferation, multi-lineage differentiation and maintenance of primitive human haemopoietic cells in NOD/SCID mice transplanted with human cord blood, Br. J. Haematol. 98 (1997) 1026–1036.

[22] C.J. Hogan, E.J. Shpall, O. McNulty, I. McNiece, J.E. Dick, L.D. Shultz, G. Keller, Engraftment and development of human CD34(+)-enriched cells from umbilical cord blood in NOD/LtSz-scid/scid mice, Blood 90 (1997) 85–96.

[23] F. Harder, R. Henschler, I. Junghahn, M.C. Lamers, A.M. Muller, Human hematopoiesis in murine embryos after injecting human cord blood-derived hematopoietic stem cells into murine blastocysts, Blood 99 (2002) 719–721.

[24] Y. Fujiki, K. Fukawa, K. Kameyama, O. Kudo, M. Onodera, Y. Nakamura, et al., Successful multilineage engraftment of human cord blood cells in pigs after in utero transplantation, Transplantation 75 (2003) 916–922.

[25] X. Wang, S. Ge, G. McNamara, Q.L. Hao, G.M. Crooks, J.A. Nolta, Albumin expressing hepatocyte-like cells develop in the livers of immune-deficient mice transmitted with highly purified human hematopoietic stem cells, Blood 101 (2003) 4201–4208.

[26] H.E. Rice, M.H. Hedrick, A.W. Flake, In utero transplantation of rat hematopoietic stem cells induces xenogeneic chimerism in mice, Transplant. Proc. 26 (1994) 126–128.

[27] P.N. Newsome, I. Johannessen, S. Boyle, E. Dalakas, K.A. McAulay, K. Samuel, et al., Human cord blood-derived cells can

differentiate into hepatocytes in the mouse liver with no evidence of cellular fusion, Gastroenterology 124 (2003) 1891–1900.
[28] W. Beerheide, M.A. von Mach, M. Ringel, C. Fleckenstein, S. Schumann, N. Renzing, et al., Downregulation of beta2-microglobulin in human cord blood somatic stem cells after transplantation into livers of SCID-mice: an escape mechanism of stem cells?, Biochem. Biophys. Res. Commun. 294 (2002) 1052–1063.
[29] D.R. Jones, In utero stem cell transplantation: two steps forward but one step back? Expert Opin. Biol. Ther. 1 (2001) 205–212.
[30] S. Hayashi, W.H. Peranteau, A.F. Shaaban, A.W. Flake, Complete allogeneic hematopoietic chimerism achieved by a combined strategy of in utero hematopoietic stem cell transplantation and postnatal donor lymphocyte infusion, Blood 100 (2002) 804–812.
[31] S.A. Tanaka, T. Hiramatsu, T. Oshitomi, Y. Imai, H. Koyanagi, Induction of donor-specific tolerance to cardiac xenografts in utero, J. Heart Lung Transplant. 17 (1998) 888–891.
[32] E.D. Zanjani, G. Almeida-Porada, J.L. Ascensao, F.R. MacKintosh, A.W. Flake, Transplantation of hematopoietic stem cells in utero, Stem Cells 15 (1) (1997) 79–92.
[33] C.T. Albanese, M.R. Harrison, Prenatal cellular transplantation, Semin. Pediatr. Surg. 8 (1999) 101–108.
[34] D.S. Krause, N.D. Theise, M.I. Collector, O. Henegariu, S. Hwang, R. Gardner, et al., Multi-organ, multi-lineage engraftment by a single bone marrow-derived stem cell, Cell 105 (2001) 369–377.
[35] H. Fujii, T. Hirose, S. Oe, K. Yasuchika, H. Azuma, T. Fujikawa, et al., Contribution of bone marrow cells to liver regeneration after partial hepatectomy in mice, J. Hepatol. 36 (2002) 653–659.
[36] G. Vassilopoulos, P.R. Wang, D.W. Russell, Transplanted bone marrow regenerates liver by cell fusion, Nature 422 (2003) 901–904.
[37] G. Almeida-Porada, C.D. Porada, J. Chamberlain, A. Torabi, E.D. Zanjani, Formation of human hepatocytes by human hematopoietic stem cells in sheep, Blood 104 (2004) 2582–2590.
[38] Y. Haruna, K. Saito, S. Spaulding, M.A. Nalesnik, M.A. Gerber, Identification of bipotential progenitor cells in human liver development, Hepatology 23 (1996) 476–481.
[39] H. Willenbring, A.S. Bailey, M. Foster, Y. Akkari, C. Dorrell, S. Olson, et al., Myelomonocytic cells are sufficient for therapeutic cell fusion in liver, Nat. Med. 10 (2004) 744–748.
[40] Y. Jiang, B. Vaessen, T. Lenvik, M. Blackstad, M. Reyes, C.M. Verfaillie, Multipotent progenitor cells can be isolated from postnatal murine bone marrow, muscle, and brain, Exp. Hematol. 30 (2002) 896–904.
[41] H. Malhi, A.N. Irani, S. Gagandeep, S. Gupta, Isolation of human progenitor liver epithelial cells with extensive replication capacity and differentiation into mature hepatocytes, J. Cell Sci. 115 (2002) 2679–2688.