Characterization of two distinct liver progenitor cell subpopulations of hematopoietic and hepatic origins

Corcelle, Véronique; Stieger, B; Gjinovci, Aslan; Wollheim, Claes; Gauthier, Benoît

EXPERIMENTAL CELL RESEARCH 312 (2006) 2826–2836

available at www.sciencedirect.com

ScienceDirect

www.elsevier.com/locate/yexcr

Research Article

Characterization of two distinct liver progenitor cell subpopulations of hematopoietic and hepatic origins

V. Corcelle[a], B. Stieger[b], A. Gjinovci[a], C.B. Wollheim[a], B.R. Gauthier[a,*]

[a]Department of Cell Physiology and Metabolism, University Medical Center, 1211 Geneva 4, Switzerland
[b]Division of Clinical Pharmacology and Toxicology, Zurich, Switzerland

ARTICLE INFORMATION

Article Chronology:
Received 10 February 2006
Revised version received
7 April 2006
Accepted 16 May 2006
Available online 19 May 2006

Keywords:
Hematopoietic stem cells
Oval cells
Liver regeneration

ABSTRACT

Despite extensive studies, the hematopoietic versus hepatic origin of liver progenitor oval cells remains controversial. The aim of this study was to determine the origin of such cells after liver injury and to establish an oval cell line. Rat liver injury was induced by subcutaneous insertion of 2-AAF pellets for 7 days with subsequent injection of CCl_4. Livers were removed 9 to 13 days post-CCl_4 treatment. Immunohistochemistry was performed using anti-c-*kit*, OV6, Thy1, CK19, AFP, vWF and Rab3b. Isolated non-parenchymal cells were grown on mouse embryonic fibroblast, and their gene expression profile was characterized by RT-PCR. We identified a subpopulation of OV6/CK19/Rab3b-expressing cells that was activated in the periportal region of traumatized livers. We also characterized a second subpopulation that expressed the HSCs marker c-*kit* but not Thy1. Although we successfully isolated both cell types, OV6/CK19/Rab3b$^+$ cells fail to propagate while c-*kit*$^+$-HSCs appeared to proliferate for up to 7 weeks. Cells formed clusters which expressed c-*kit*, Thy1 and albumin. Our results indicate that a bona fide oval progenitor cell population resides within the liver and is distinct from c-*kit*$^+$-HSCs. Oval cells require the hepatic niche to proliferate, while cells mobilized from the circulation proliferate and transdifferentiate into hepatocytes without evidence of cell fusion.

Introduction

The existence of a hepatic stem cell compartment has been a subject of interest for over a century [1]. Indeed, in rodent models in which chronic liver injury is provoked by a carcinogenic regimen such as 2-acetylaminofluorene (2-AAF) combined with CCl_4, regeneration is accomplished predominantly by a rapidly growing population of distinct epithelial cells [2,3]. These cells are small in size (approximately 10 μm), with a large nuclear-to-cytoplasm ratio and with an oval-shaped nucleus (hence their name) [2]. Although oval cells are thought to emanate from the periportal area [4–8], their lineage remains controversial. Immunofluorescence studies revealed that oval cells express cytokeratin 19 (CK19), a marker specific for the biliary tree that includes bile ducts as well as the canal of Hering [8,9]. OV6, the most widely used monoclonal antibody to identify oval cells in rat liver [10], stains similar structures [11], indicating that epithelial cells residing within the canal of Hering are the most probable candidates for resident liver stem cells [12].

Hepatic progenitor oval cells also express stem cell factor (SCF) and its receptor c-*kit* [13] as well as Thy1 and CD34 [14,15], indicating a potential hematopoietic origin. Consistent with this premise, hematopoietic stem cells (HSCs) have been shown to transdifferentiate into cells of the hepatocytic lineage in both rodents and humans [16–23]. However, these

* *Corresponding author*. Fax: +41 22 379 55 43.
E-mail address: Benoit.Gauthier@medecine.unige.ch (B.R. Gauthier)

0014-4827/$ – see front matter © 2006 Elsevier Inc. All rights reserved.
doi:10.1016/j.yexcr.2006.05.007

findings have been challenged by studies showing that cell fusion rather than transdifferentiation of HSCs is involved in regeneration [24,25] and that bone marrow (BM) does not contribute as source of expanding oval cells [26]. Thus, it remains to be determined to what degrees HSCs as opposed to endogenous-derived liver oval cells participate in regeneration. Recently, the small GTPase Rab3b and the nuclear hormone receptor Ear2 were shown to be upregulated in rat oval cells subsequent to 2-AAF treatment and partial hepatectomy (PH) providing potential novel markers for the identification of bona fide hepatic progenitor cells [27].

Independent of their origin, hepatic progenitor cells have been reported to be isolated, propagated and differentiated into hepatic- and pancreatic-islet-like cells in culture [28–31], thus providing an attractive renewable source of stem cells for cellular therapy. The goal of the current study was to further characterize the potential origin of hepatic progenitor cells in 2-AAF/CCl_4-treated rat livers and to subsequently isolate and establish a stable oval cell line. Our study reveals the potential existence of two distinct cell populations which are induced subsequent to liver injury and are likely involved in hepatic regeneration. One population is endogenous to the hepatic tissue and, in our hands, is not prone to replication in vitro, while the second population would be derived from HSCs and able to transiently proliferate in culture. These data support the concept of the contribution of both resident liver stem cells as well as HSCs to hepatocellular regeneration [32]. However, caution should be taken on conclusions regarding the potential 'oval cell' origin of hepatic stem cell lines.

Materials and methods

Animals and treatment

Male Sprague–Dawley rats (180 to 200 g) were obtained from Harlan Netherlands (Horst, Netherlands) and kept under standard conditions. All experiments were performed in accordance with the guidelines of the local regulatory authorities. Inhibition of hepatocyte proliferation was attained through subcutaneous insertion of 2-AAF (70 mg pellet, 2.5 mg/day release; Innovative Research of America, Sarasota, FL). On day 7, hepatic injury was induced by a single intraperitoneal injection of CCl_4 (1.9 ml/kg; CCl_4/corn oil 1:1) [33]. Treated and control animals were sacrificed and liver extracted at 9, 11 and 13 days post-CCl_4 treatment.

Cell isolation

Isolation of hepatic small cells was performed as described by Seglen [34] with modifications. Briefly, after a two-step collagenase IV perfusion (Roche, Rotkreuz, CH), liver tissue was mechanically dissociated in RPMI-1640 medium (Invitrogen, Basel, CH) supplemented with 10% fetal calf serum (FCS), penicillin (100 U/ml), streptomycin (100 μg/ml) and tobramycin (100 μg/ml) (Sigma, Buchs, CH). Large hepatocytes were eliminated by low-speed centrifugation and remaining non-parenchymal cells were consecutively filtered through 100 and 30-μm nylon meshes (Milian, Plan-les-Ouates, CH). Small cells were further purified on a Histopaque (Sigma) density gradient (density of 1.077 ± 0.001; 2500 rpm, 20 min). Cells located at the interphase were collected, washed and resuspended in IMDM (Sigma) supplemented with leukemia inhibitory factor (LIF, 10 ng/ml, VWR, CH), IL-3 and SCF (each 10 ng/ml, VWR). Alternatively, cells were seeded on irradiated mouse embryonic fibroblast (iMEFs) and placed in a humidified atmosphere saturated with 5% CO_2 at 37°C. Fresh media were added every other day. iMEFs were obtained from embryos of 13-day pregnant female CF-1 mice.

BM cells were isolated from control and treated rat thighbones. Cells were flushed in RPMI-1640 medium supplemented with 10% FCS and penicillin/streptomycin (100 U/ml/100 μg/ml). Cells were collected on SuperFrost Plus slides (50,000 cells/slide) by cytocentrifugation using a Shandon cytospin® 3 centrifuge (Thermo Electron Corp., USA).

Immunohistochemistry

Control and treated rat liver fragments were frozen in liquid nitrogen and embedded in OCT compound® (Digitana AG, Lausanne, CH). Semi-thin 5 μm cryostat sections or cytocentrifuged cells were fixed in either 4% paraformaldehyde/phosphate-buffered saline (PBS) or in acetone and permeabilized with 0.1% Triton/PBS. Preparations were pre-incubated for 30 min in 1% Bovine Serum Albumin (BSA)/PBS and then exposed overnight at 4°C to the following primary antibodies: CK19 (Sigma), OV6 (kindly provided by Dr. S. Sell, Albany Medical College, USA), Thy1, c-*kit* and α-fetoprotein (AFP) (Santa Cruz, USA). Primary antibodies were detected using Alexa 488- or Alexa 568-conjugated secondary antibodies. Cells were then stained with DAPI (4′,6-diamidino-2-phenylindole) to reveal the nuclei and mounted using Dako fluorescent mounting medium. Proliferation was measured by BrdU incorporation as described previously [35].

Polymerase chain reaction

Total RNA was extracted from liver samples and pooled cell clusters using the RNAqueous™-Micro kit (Ambion). cDNA was synthetized with Superscript II reverse transcriptase (Invitrogen) as described by Gauthier et al. [36] and was

Table 1 – Primers used for PCR analysis

Primer name	Sequence
Thy1	S: 5′-TGA CAG CCT GCC TGG TGA A-3′
	A: 5′-GCT GGA TGG GCA AGT TGG T-3′
c-*kit*	S: 5′-GGA TGC GGT CTG ACA AAT TCA-3′
	A: 5′-GAC TTG TTT CGG GCA CAG ACA-3′
CK19	S: 5′-AGG ACT TGC GCG ACA ACA TC-3′
	A: 5′-GGG CAT TGT CGA TCT GTA GGA-3′
CTGF	S: 5′-GGT GTG TGA TGA GCC CAA GG-3′
	A: 5′-CTC TAG GTC AGC TTC ACA GG-3′
Albumin	S: 5′-GCT GAT GAG AAT GCC GAA AAC-3′
	A: 5′-TGG AAT GGC GCA TAA CTT GTC-3′
Glucokinase (GK)	S: 5′-GCA GGC TGA CAC CCA ACT G-3′
	A: 5′-TGC CCT CCT CTG ATT CGA TG-3′
Cyclophilin	S: 5′-TCA CCA TCT CCG ACT GTG GA-3′
	A: 5′-AAA TGC CCG CAA GTC AAA GA-3′

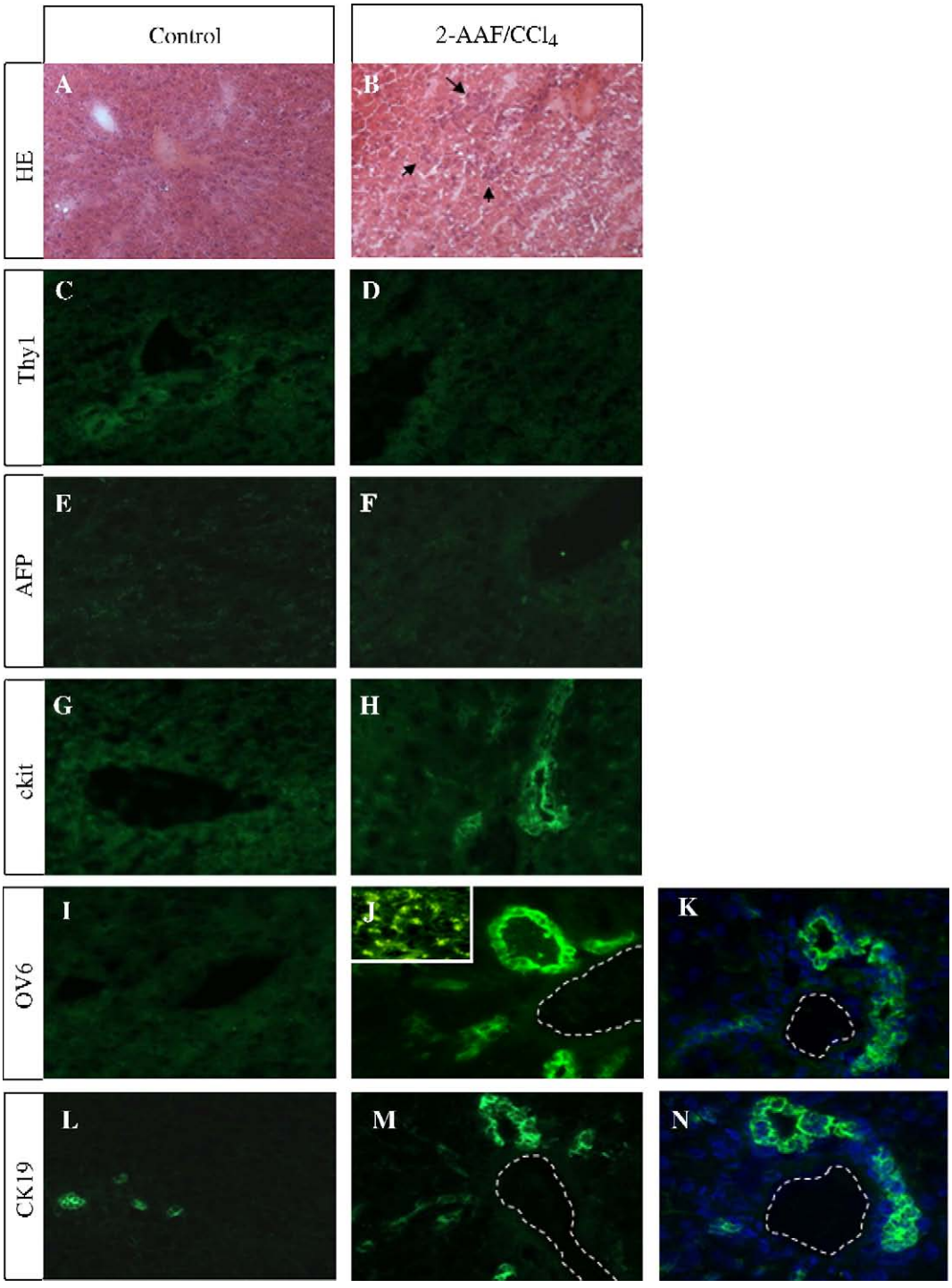

Fig. 1 – c-*kit*, OV6 and CK19 but not Thy1 nor AFP-positive cells are induced in 2-AAF/CCl_4-treated rat livers. Cryostat sections of control and treated rat livers were stained with hematoxylin–eosin (A, B). Alternatively, cryostat sections of control and treated rat livers were fixed in either 4% paraformaldehyde/phosphate-buffered saline (PBS) or in acetone and subsequently processed for immunofluorescence localization of various proteins. Absence of Thy1 and AFP staining in both normal (C, E) and treated rat livers (D, F). The HSC marker c-*kit* was not detected in control animals (G), while the protein was induced in treated liver in blood-vessel-like structures (H). No $OV6^+$ cells were observed in normal rat livers (I), whereas the periportal region of treated livers was strongly stained (J). $OV6^+$ cells were observed infiltrating the hepatic parenchyma from a biliary like-structure (J, inset). Few $CK19^+$ cells appeared in control liver sections (L). The proportion of positive cells largely increased after the treatment (M) and co-localized with OV6 staining (compare K and N). Cryostat sections represented in panels K and N were also stained with DAPI to reveal nuclei. The portal vein in panels J, K, M and N is delineated by a dashed line. Magnification [A–N] ×40; [J, inset] ×63.

subjected to PCR amplification (30 cycles) with specific primers (Table 1) using a Stratagene Robocycler Gradient 96.

Results

Immunohistochemical characteristics of activated hepatic progenitor cells

We initially evaluated the expression pattern of Thy1, c-*kit*, OV6, CK19 and AFP in 2-AAF/CCl_4-treated rat livers. This model of hepatotoxicity has revealed massive oval cell proliferation by day 9 post-CCl_4 injection with activation continuing persistently through days 11 and 13 [33]. Hematoxylin–eosin staining clearly confirmed the efficacy of the treatment in destroying the classical hepatic tissue architecture in treated animals as compared to controls (Figs. 1A and B). Astonishingly, Thy1 could not be detected in either control or treated rat liver sections (Figs. 1C and D) whereas it was expressed in rat brain (data not shown). To exclude a time-dependent activation of Thy1, we harvested the livers 13 days post-treatment. Additionally, various fixing procedures (acetone versus paraformaldehyde) as well as 3 different commercially available antibodies were evaluated. All conditions failed to reveal Thy1 expression (data not shown). Consistent with the latter, the AFP previously shown to co-localize with Thy1 in progenitor cells [14] was also absent in 2-AAF/CCl_4-treated rat livers (Figs. 1E and F). In contrast, expression of the hematopoietic marker c-*kit* was induced in 2-AFF/CCl_4-treated rat liver and localized to luminal side of blood vessels, suggesting the presence of HSCs in the injured liver (Figs. 1G and H). We next investigated the expression of the oval-cell-specific marker OV6 [10]. Whereas no staining was present in normal liver sections (Fig. 1I), periportal regions of treated livers were labeled for OV6 (Fig. 1J). Reminiscent to cholongiocytes forming the canal of Hering, $OV6^+$ cells appeared as strings of cuboidal cells organized in clusters, or extensions thereof, located in close proximity to periportal veins (Fig. 1J). Furthermore, $OV6^+$ cells could be discerned infiltrating the parenchyma (Fig. 1J, inset). Expression of the biliary/stem cell marker CK19 localized to similar structures as OV6 in treated rats, while it was barely detectable in control animals (Figs. 1L and M). Consecutive cryostat sections confirmed co-localization of OV6 and CK19 to identical cells of the canal of Hering (compare Figs. 1K and N). In contrast, OV6 labeling did not co-localize with Von Willebrand factor (endothelial cell marker) (Fig. 2A), suggesting that $OV6^+$ cells do not stem from blood vessels and are distinct from c-*kit*-expressing cells. Recently, Rab3b mRNA levels were shown to be induced in hepatic progenitor cells of 2-AAF/PH-treated rats [27]. We found that Rab3b and OV6 co-localized to identical cells in injured livers (Fig. 2B).

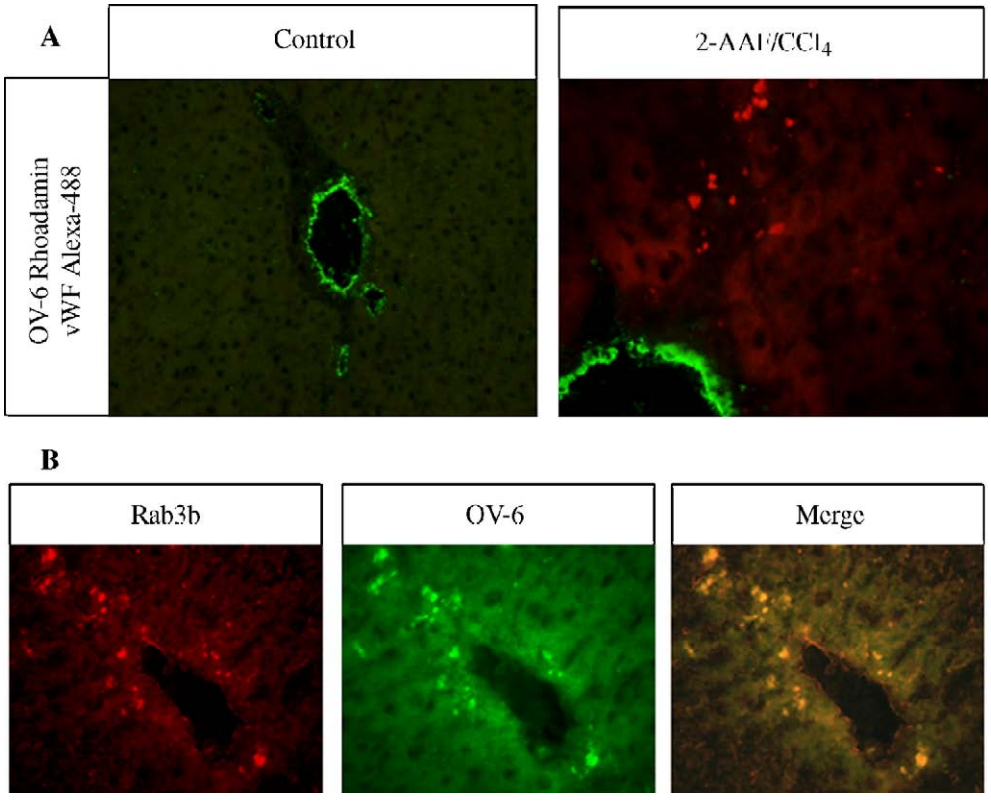

Fig. 2 – Co-immunolabeling experiments reveal distinct localization of vWF and OV6, while Rab3b is found in OV6-expressing cells. (A) Control and 2-AAF/CCl_4-treated liver sections were co-immunolabeled with anti-vWF (green) and anti-OV6 (red) or (B) with anti-Rab3b (red) as well as with anti-OV6 (green). Magnification [A, left panel] ×20; [A, right panel and B] ×63. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

Omission of primary antibodies revealed no specific staining (data not shown).

OV6/CK19/Rab3b$^+$ cells do not express Thy1 and are distinct from c-kit$^+$-HSCs

Our inability to detect Thy1 expression in cryostat sections prompted us to re-evaluate the expression pattern of this marker using an alternative method. To increase the probability of capturing all hepatic progenitor cells, control and 2-AFF/CCl_4-treated livers were dissociated by collagenase digestion and cells were concentrated by cytocentrifugation onto cover slips. Corroborating results of Fig. 1, Thy1 (Figs. 3A–D) as well as AFP (Figs. 3E–H) could not be detected whereas c-*kit* (Figs. 3I–L), CK19 (Figs. 3M–P) and OV6 (Figs. 3Q–T) were all found in treated liver as compared to control. Co-immunolabeling studies revealed that anti-c-*kit* stained a different cell population to that of either anti-OV6 or anti-CK19 (Fig. 4).

Human studies have shown increased levels of circulating HSCs in response to liver trauma [37,38]. These cells are thought to be activated in the BM and mobilized from the circulation in order to repopulate the damaged tissue [39]. We

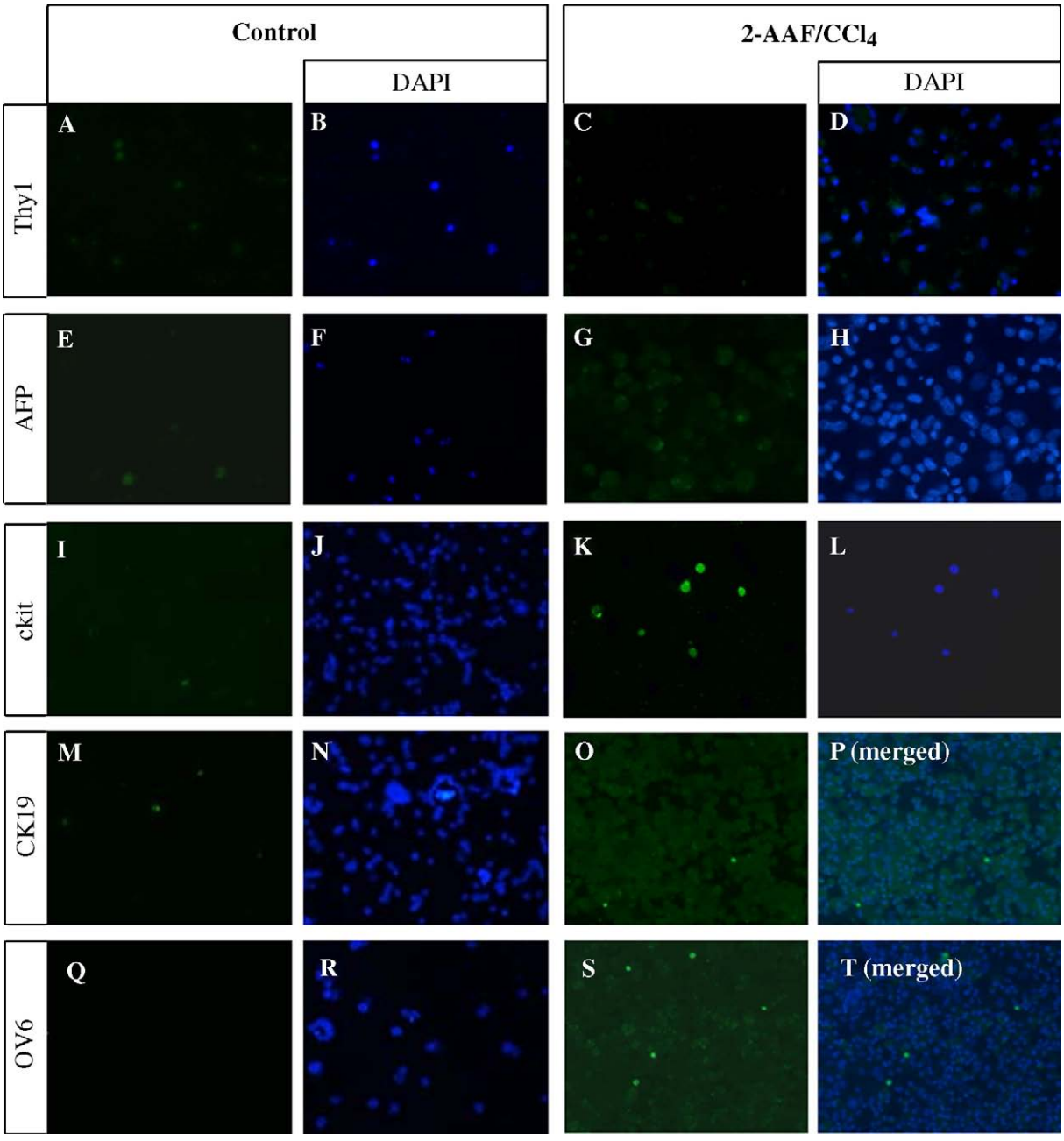

Fig. 3 – Immunohistochemical characterization of cells isolated from untreated and 2-AAF/CCl_4-treated rat livers. Isolated small hepatic cells were concentrated onto cover slips by cytocentrifugation and immunolabeled with anti-Thy1 (A, C), AFP (E, G), c-*kit* (I, K), CK19 (M, O) or OV6 (Q, S). Cells were also co-stained with DAPI to reveal cell nuclei. Panels P and T represent merged images of either CK19 or OV6 with DAPI labeling. Magnification [A–N and Q–R] ×40; [O–P and S–T] ×20.

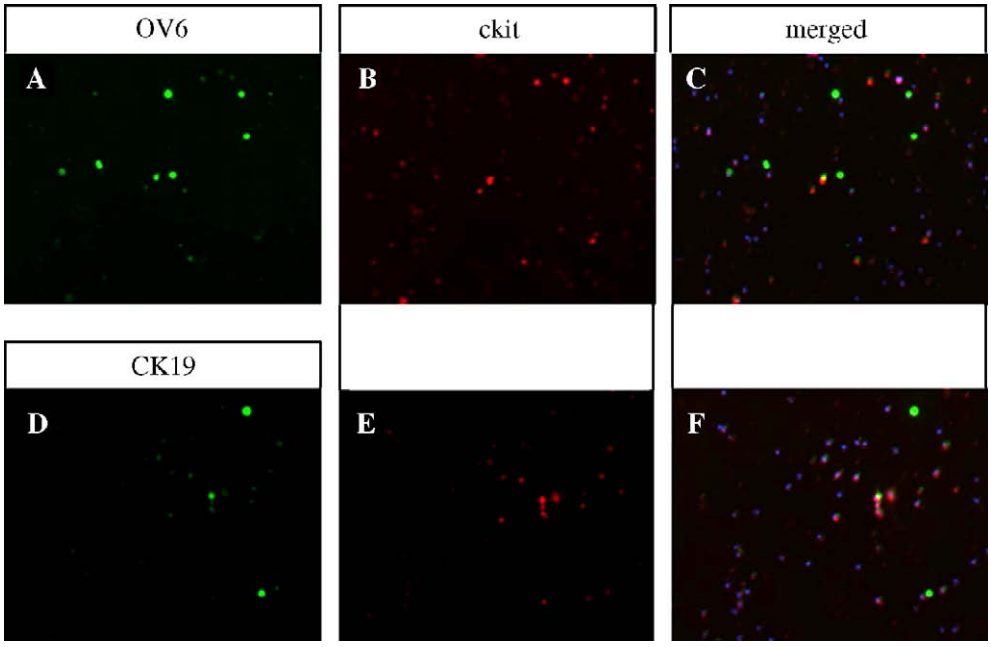

Fig. 4 – c-*kit*-expressing cells are distinct from OV6$^+$ and CK19$^+$ cells. Cytocentrifuged small hepatic cells were co-immunostained with either (A–C) anti-OV6 (green) and anti-c-*kit* (red) or (D–F) anti-CK19 (green) and anti-c-*kit* (red). Magnification ×20. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

thus investigated whether HSCs expressing c-*kit* and potentially Thy1 could be detected in the BM of 2-AAF/CCl_4-treated rats. c-*kit*-expressing cells were observed in both control and 2-AAF/CCl_4-treated rats (Figs. 5E–H). More importantly, Thy1-positive cells were also found in BM of treated animals whereas expression was undetected in cells from control thighbone (Figs. 5A–D). These cells did not co-stain for c-*kit*, indicating either distinct cell subpopulations or the same cell lineage at a different maturity stage. In contrast, neither CK19 nor OV6-expressing cells were detected in either group (Figs. 5I–P). Taken together, these results suggest that c-*kit*-positive cells, detected in injured liver, could be derived from BM while OV6-positive cells are endogenous to the hepatic tissue. Furthermore, although Thy1-expressing cells were identified in BM, these do not appear to be mobilized to the injured liver in the time frame analyzed in this study.

Isolation and growth of liver progenitor cells in culture

In order to purify and enrich OV6 and c-*kit*-expressing cells, we performed an isolation protocol to eliminate large quiescent differentiated cells from small replicating cells. Filtering through consecutively smaller size mesh successfully removed large hepatic cells (Fig. 6A). Immunofluorescent studies using hepatic and stem cell markers revealed that, prior to purification, 56% and 20% of the cells expressed glucokinase and c-*kit*, respectively, while low levels of AFP (3%) and CK19 (5%) were found. In contrast, neither Thy1 nor OV6 were detected in the cell suspension. Subsequent to the isolation protocol, a decrease in the hepatic marker glucokinase was observed, while expression of c-*kit*, CK19 and OV6 was all increased, indicating the enrichment of progenitor stem cell markers (Table 2). Small purified cells of which 30% comprised c-*kit*$^+$ cells (Fig. 6B) adhered to poly-ornithine-coated surface. Although serum-free media prevented propagation of contaminating fibroblasts (data no shown), the addition of LIF, SCF and IL-3 did not promote growth of small cells which died after 3 weeks. Exclusion of any of the factors resulted in cell death within 48 h indicating that the mixture was critical to sustain cell survival but not sufficient to expand cellular mass. In contrast, we found that iMEFs incited hepatic progenitor cell propagation, confirming the importance of feeder layers in maintaining a self-renewal phenotype [40]. Although we were able to identify small OV6$^+$ cells in culture (Fig. 6C), these did not appear to replicate. In contrast, cells morphologically larger (~25 μm) than the OV6$^+$ subpopulation (~10 μm) were replicating (Fig. 6D) and forming small clusters (Fig. 7A). These propagated for up to 6–7 weeks. Unfortunately, simultaneous staining for BrdU and OV6 was technically impossible.

Cell clusters are derived from the c-kit-expressing HSC subpopulation

To investigate the cellular origin of clusters, we performed RT-PCR on selected gene markers (Fig. 7B). Consistent with our immunohistochemistry results, c-*kit* expression was observed in 2-AAF/CCl_4-treated livers as well as in cell clusters and BM. Interestingly, although c-*kit* was not observed at the protein level, the transcript was detected in control liver. In addition, low levels of Thy1 mRNA were also found in control and injured liver, whereas cells clusters and control brain tissue exhibited higher transcript levels. In contrast, control and 2-AAF/CCl_4-treated livers but not cell clusters expressed CK19 and AFP. The connective tissue growth factor (CTGF) gene was shown to be upregulated in Thy1-expressing cells isolated from 2-AAF/PH-treated rats [41]. Corroborating these results, the CTGF transcript

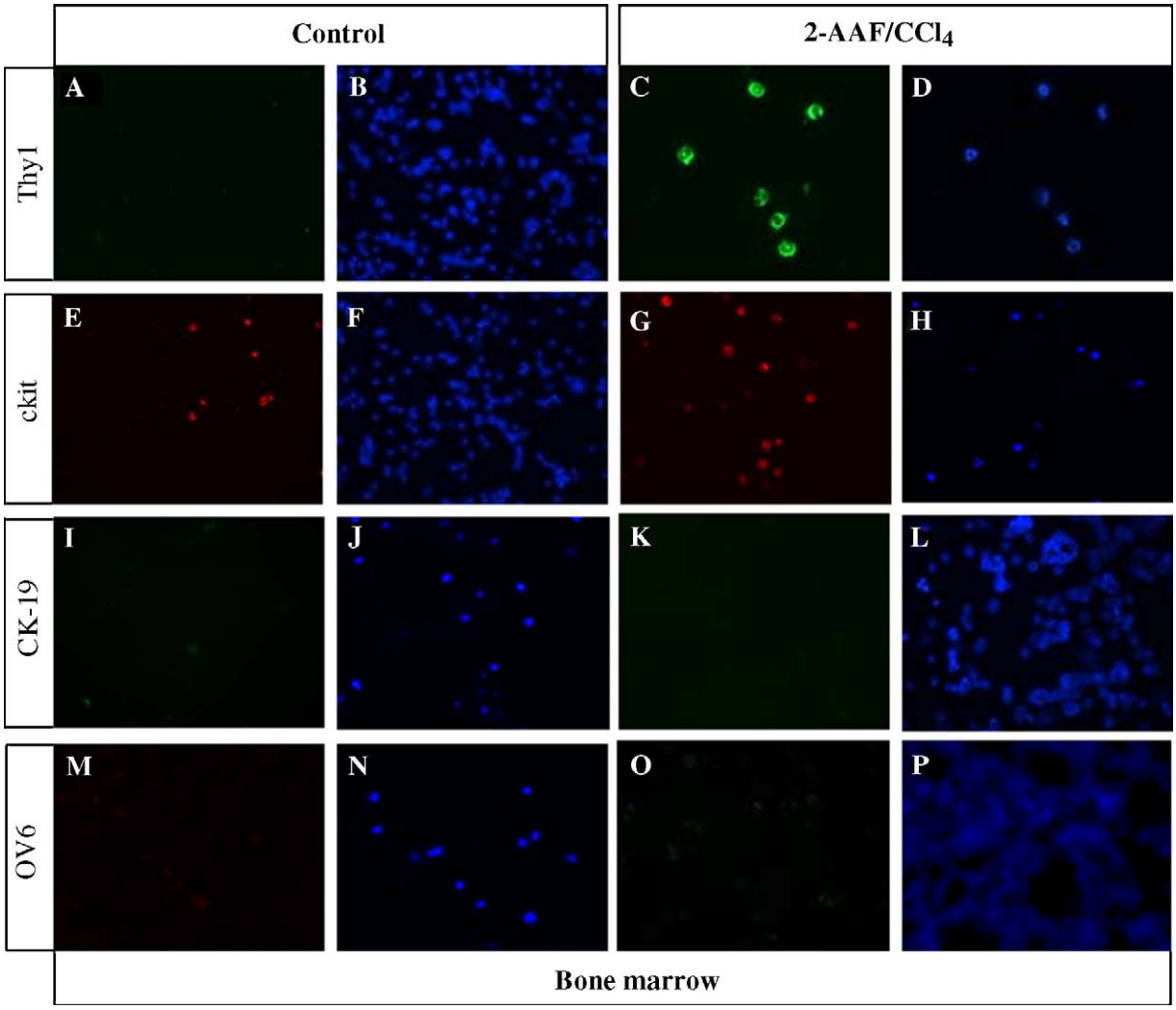

Fig. 5 – Thy1 and c-*kit* but not OV6 or CK19-expressing cells are found in the bone marrow of 2-AAF/CCl_4-treated rats. Cells isolated from the thighbone marrow were cytocentrifuged onto cover slips and processed for immunohistochemistry. (A–D) Anti-Thy1 (green), (E–H) anti-c-*kit* (red), (I–L) anti-CK19 (green) and (M–P) OV6 (green). Cells were also co-stained with DAPI to reveal cell nuclei. Magnification [A–D; I–P] ×63; [E–H] ×40. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

was detected in treated liver, while control-hepatic tissue and cell clusters did not express this gene. Finally, albumin but not glucokinase mRNA was observed in cell clusters, suggesting differentiation towards the hepatic lineage.

Discussion

Oval-cell-mediated regeneration in chronic liver injury is a well-established physiological process [42]. Yet, the origin of these cells remains obscure, a predicament most likely attributable to the diversity of experimental models to investigate liver regeneration. Herein, we induced liver damage using a combination of 2-AAF and CCl_4 which has been shown to elicit an oval cell response in rats [14]. This regimen has the advantage of being surgically non-invasive as compared to partial hepatectomy which provokes a robust mobilization of HSCs to assist in the regeneration of the damaged tissue. The latter may provide a justification for the identification of HSCs as primary hepatic progenitors while marginalizing the authenticity of resident liver oval cells. Our results confirm this premise by demonstrating that the hepatic tissue but not the BM possesses an endogenous OV6/CK19/Rab3b-expressing cell subpopulation forming ductular structures representing extensions of the canals of Hering [8,43]. Our findings substantiate the importance of Rab3b as a novel marker to distinguish endogenous liver stem cells from invading HSCs [27]. Nonetheless, a distinct subpopulation of c-*kit*-expressing cells was also detected in liver as well as in the BM of 2-AAF/CCl_4 animals, indicating that HSCs may be important in liver regeneration. The distinctive origin of c-*kit* and OV6-expressing cells is substantiated by a study demonstrating that Ws/Ws rats, whose c-*kit* kinase activity is severely impaired, still exhibited oval cell proliferation in response to 2-AAF/PH [44].

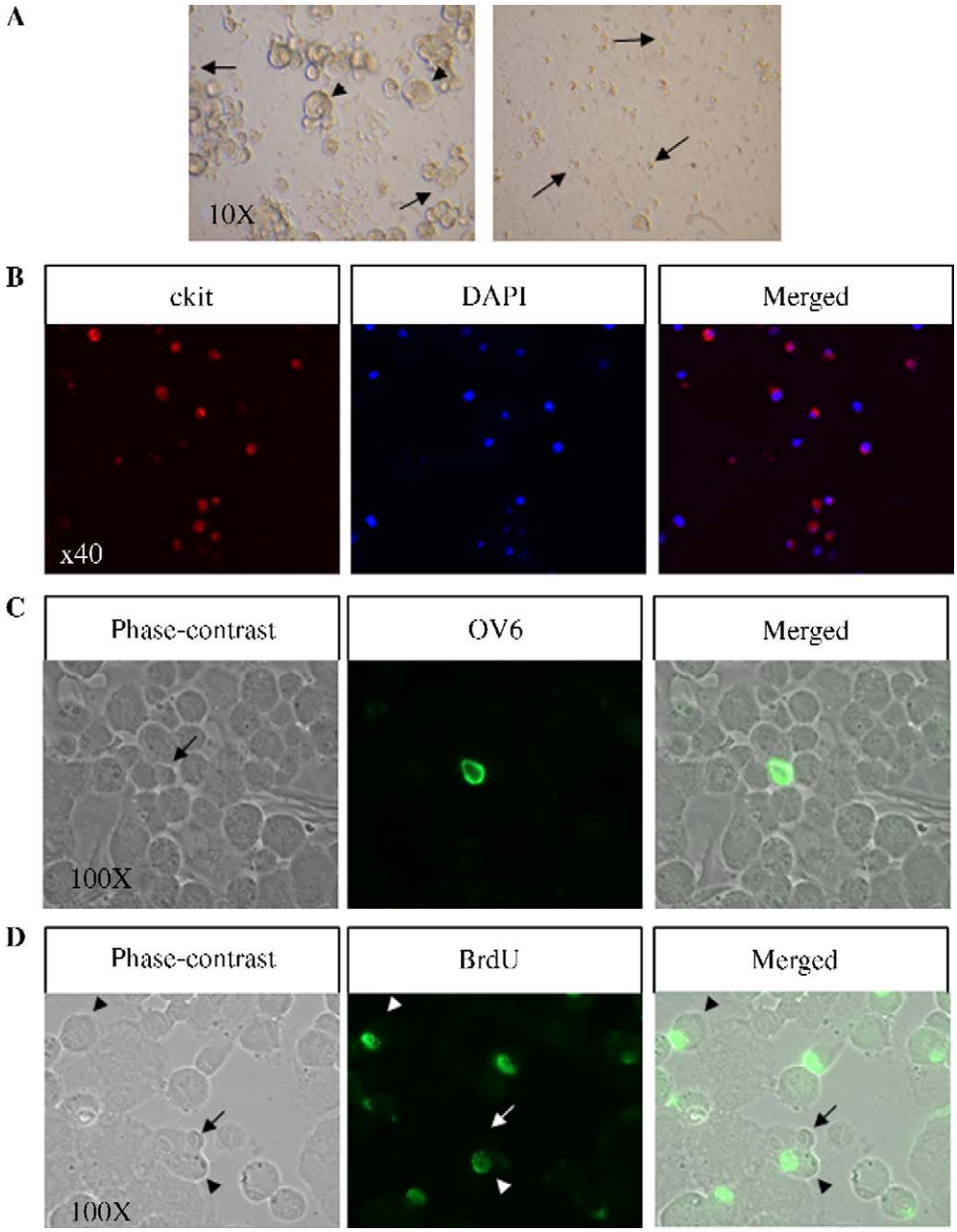

Fig. 6 – Isolation and proliferation of small hepatic progenitor cells in culture. (A) Phase-contrast photomicrograph showing hepatic cells subsequent to collagenase digestion (left panel). Small non-parenchymal cells were then purified (see Materials and methods) and maintained in culture (right panel). Arrow heads depict large hepatocytes, while arrows identify small non-parenchymal cells. Purified cells were cytocentrifuged and immunolabeled with anti-c-*kit* (B) or anti-OV6 (C). (D) Immunocytochemical detection of BrdU incorporation (green) was performed on isolated cells. Arrow heads depict large BrdU-positive cells, while arrows mark small non-replicating OV6 cells. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

In contrast to c-*kit*, we were unable to detect Thy1$^+$ cells in 9- and 13-day CCl_4-treated rat livers. Expression of this HSC marker has been reported as a hallmark of the hepatic progenitor cell phenotype and has been used to isolate 'oval cells' [14,31]. A systematic analysis revealed induction of the Thy1 protein in BM of treated rats, while RT-PCR detected low levels of the transcript in both control and damaged liver. CTGF, a growth factor induced in Thy1$^+$ cells subsequent to PH, was also detected by RT-PCR in our experimental model [41]. Thus, a restricted number of Thy1-expressing cells are present in the traumatized liver at day 9 and 13 post-treatment but do not constitute the predominant type of hepatic progenitors. However, pending the severity of the injury, Thy1$^+$ cells may be recruited from the BM. Consistent with this hypothesis, initial experiments performed by Petersen et al. demonstrated that Thy1 was not detected prior to day 21 post-2-AFF/CCl_4 treatment [14]. Subsequent experiments were performed using PH resulting in 70% removal of hepatic tissue. This

Table 2 – Percentage of cells in 2-AAF/CCl_4-treated livers expressing hepatic and/or progenitor markers before and after enrichment

Markers	Before (%)	After (%)
Glucokinase	56	18
AFP	3	5
c-*kit*	20	30
CK19	3	9
Thy1	ND[a]	ND[a]
OV6	ND[a]	9

[a] Not detected.

model results in a massive invasion of blood cells including Thy1-expressing cells within 7 days [41]. Interestingly, subsequent to isolation, Thy1 mRNA levels appeared to increase in c-*kit*-positive cell clusters in culture, indicating that Thy1 may be induced in these cells. In agreement with the latter, all functional repopulating HSCs were shown to be contained within a c-*kit*$^+$/lin$^-$/Sca-1$^+$ BM population [45] and liver progenitor cells cloned from adult rat livers following allyl alcohol injury co-expressed both Thy1 and c-*kit* [46]. Thus, expression of Thy1 may be induced in c-*kit*$^+$ cells subsequent to injury to promote transdifferentiation of HSCs towards a hepatic progenitor phenotype. Consistent with the absence of AFP from our endogenous hepatic progenitor cells, human studies have shown that the intermediate hepatobiliary cells corresponding to the rodent hepatic oval cells expressed CK19 and OV6 whereas AFP is not detected [47]. Furthermore, the latter cells were also characterized in hamster liver during cholangiocarcinogenesis following *C. sinensis* infection and dimethylnitroasime treatment [11]. Thus, discrepancies in detecting both Thy1 and AFP in hepatic progenitors may arise from differences in the type of treatment as well as time of phenotypic analysis. Corroborating previous studies, we propose that, during liver injury, two different cell types may be involved in repopulating the regenerating organ: (1) c-*kit*$^+$ (and likely Thy1$^+$) HSCs which are mobilized from the BM and invade the liver through the circulation [39] and (2) endogenous OV6/CK19/Rab3b$^+$ oval cells which emanate from biliary ducts [12,32].

We found that following purification OV6-expressing cells were unable to proliferate in culture and eventually died. However, a distinct cell subpopulation retained the ability to propagate in the presence of iMEFs, a prerequisite shared by several hepatic progenitor as well as hematopoietic stem cell lines expressing c-*kit* and/or Thy1 [46,48,49]. Consistent with the latter, c-*kit* and Thy1 mRNA were detected in cell clusters, suggesting that these were derived from c-*kit*-positive HSCs. Of note, c-*kit* transcript was also found in untreated liver, indicating that circulating or resident c-*kit*-expressing cells are present in the organ but that resulting protein levels are too low to be detected. However, long-term culture of these clusters resulted in the loss of self-renewal with the concomitant appearance of the mature liver marker albumin. These data indicate that c-*kit*$^+$-HSCs were acquiring a hepatic phenotype substantiating previous findings that these cells can transdifferentiate into hepatocytes without evidence of cell fusion [50]. Notwithstanding, glucokinase, a marker of fully differentiated hepatocytes, is absent. However, we were unable to sustain the undifferentiated phenotype of HSCs in vitro, indicating that novel culture conditions and–perhaps yet unidentified–cytokines or other microenvironment-related factors may be required to allow these cells to exhibit their expected self-renewal potential. Consistent with this premise, tissue progenitor cells such as HSCs are localized within stem cell niches which favor interactions with other cell types and extracellular substrates [51]. These microenvironments promote self-renewal while inhibiting differentiation. Removal of HSCs from this niche may induce a genetic program promoting cell lineage commitment to the detriment of replication. Interestingly, low levels of c-*myc* were recently shown to be indispensable for sustaining undifferentiated HSCs within the bone marrow niche while high levels induced a transient-amplifying cell population which proliferated while differentiating [52]. Likewise, we found that c-*myc* mRNA levels were higher in cell clusters as compared to control or treated rat samples (data not show), thus providing a potential explanation for the loss of self-renewal competence of the cells. OV6/CK19/Rab3b$^+$ hepatic progenitors may be more susceptible to the microenvironment, rendering growth of

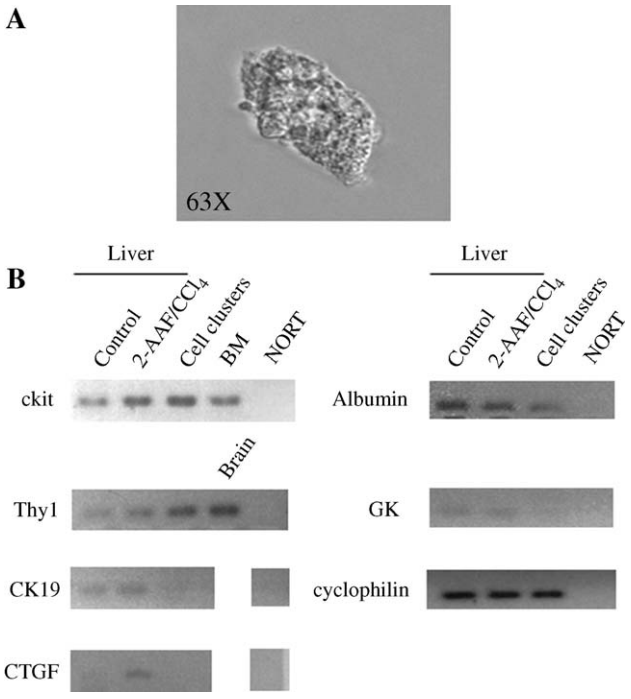

Fig. 7 – Replicating hepatic progenitors form small cells clusters and co-express the HSC markers Thy1 and c-*kit* as well as the mature liver marker albumin. (A) Phase-contrast photomicrograph showing a cell cluster 2 weeks after plating. (B) Expression pattern of genes encoding c-*kit*, Thy1, CK19, CTGF, albumin, glucokinase (GK) and cyclophilin in control and 2-AAF/CCl_4-treated rat liver as well as in cell clusters was assessed by RT-PCR (30 cycles). As controls, cDNAs derived from bone marrow (BM) and brain RNAs were used to amplify c-*kit* and Thy1, respectively. NORT, no reverse transcriptase reaction.

these cells in culture more fastidious. A recent study has demonstrated that a TNF family member called TWEAK (TNF-like weak inducer of apoptosis) specifically stimulated endogenous hepatic oval cell proliferation both in vivo and in vitro [53]. This selective mitogen may provide a valuable tool to discriminate between endogenous and HSCs-derived hepatic progenitor cells as well as potentially permitting expansion of the former in culture.

In conclusion, we have identified a bona fide oval progenitor cell population within the liver which appears to be distinct from c-*kit*$^+$-HSCs. These cells require the hepatic niche in order to proliferate in vitro while the identified c-*kit*$^+$-HSCs may have a limited capacity to replicate and transdifferentiate towards the hepatic lineage.

Acknowledgments

We would like to acknowledge Dr. S. Sell for kindly providing the OV6 antibody. We would also like to thank Drs. P. Halban, B. Thorens and M. Donath for helpful advice. The technical assistance of Stéphanie Hausler is gratefully acknowledged. This study was supported by grants from the Roche Research Foundation (C.B.W.), the JDRF (#4-2002-461 to C.B.W.) and from the Swiss National Science Foundation (#32-66907.01 to C.B.W./B.R.G and #4046-58638 to B.S.). This work was performed within the framework of the Geneva program in metabolic disorders (GeMet).

REFERENCES

[1] W.G. MacCallum, Regenerative changes in the liver after acute yellow atrophy, Johns Hopkins Hosp., Rep. 10 (1902) 375–379.
[2] E. Farber, Similarities in the sequence of early histological changes induced in the liver of rat by ethionine, 2-acetylaminofluorene, and 3′-methyl-4-dimethylaminoazobenzene, Cancer Res. 16 (1956) 142–148.
[3] S. Sell, Is there a liver stem cell? Cancer Res. 50 (1990) 3811.
[4] R. Evarts, P. Nagy, E. Marsden, S. Thorgeirsson, A precursor–product relationship exists between oval cells and hepatocytes in rat liver, Carcinogenesis 8 (1987) 1737–1740.
[5] R. Evarts, P. Nagy, H. Nakatsukasa, E. Marsden, S. Thorgeirsson, In vivo differentiation of rat liver oval cells into hepatocytes, Cancer Res. (1989) 1541–1547.
[6] M. Golding, C. Sarraf, E. Lalani, T. Anilkumar, R. Edwards, P. Nagy, S. Thorgeirsson, M. Alison, Oval cell differentiation into hepatocytes in the acetylaminofluorene-treated regenerating rat liver, Hepatology 22 (1995) 1243–1253.
[7] M.R. Alison, M.H.C. Golding, C.E. Sarraf, Pluripotential liver stem cells: facultative stem cells located in the biliary tree, Cell Proliferation 29 (1996) 373–402.
[8] S. Paku, J. Schnur, P. Nagy, S. Thorgeirsson, Origin and structural evolution of the early proliferating oval cells in rat liver, Am. J. Pathol. (2001) 1313–1323.
[9] P. van Eyken, R. Sciot, B. van Damme, C. de Wolf-Peeters, V.J. Desmet, Keratin immunohistochemistry in normal human liver. Cytokeratin pattern of hepatocytes, bile ducts and acinar gradient, Virchows Arch. A. Pathol. Anat. Histopathol. 412 (1987) 63–72.
[10] H.A. Dunsford, S. Sell, Production of monoclonal antibodies to preneoplastic liver cell populations induced by chemical carcinogens in rats and to transplantable Morris hepatomas, Cancer Res. 49 (1989) 4887–4893.
[11] J.H. Lee, H.J. Rim, S. Sell, Heterogeneity of the “oval-cell” response in the hamster liver during cholangiocarcinogenesis following *Clonorchis sinensis* infection and dimethylnitrosamine treatment, J. Hepatol. 26 (1997) 1313–1323.
[12] S. Sell, The hepatocyte: heterogeneity and plasticity of liver cells, Int. J. Biochem. Cell Biol. 35 (2003) 267–271.
[13] K. Fujio, R. Evarts, Z. Hu, E. Marsden, S. Thorgeirsson, Expression of stem cell factor and its receptor, c-*kit*, during liver regeneration from putative stem cells in adult rat, Lab. Invest. 70 (1994) 511–516.
[14] B. Petersen, J. Goff, J. Greenberger, G. Michalopoulos, Hepatic oval cells express the hematopoietic stem cell marker Thy-1 in the rat, Hepatology 27 (1998) 433–445.
[15] N. Omori, M. Omori, R.P. Evarts, T. Teramoto, M.J. Miller, T.N. Hoang, S.S. Thorgeirsson, Partial cloning of rat CD34 cDNA and expression during stem cell-dependent liver regeneration in the adult rat, Hepatology 26 (1997) 720–727.
[16] B.E. Petersen, W.C. Bowen, K.D. Patrene, W.M. Mars, A.K. Sullivan, N. Murase, S.S. Boggs, J.S. Greenberger, J.P. Goff, Bone marrow as a potential source of hepatic oval cells, Science 284 (1999) 1168–1170.
[17] D. Krause, N. Theise, M. Collector, R. Gardner, S. Neutzel, S. Sharkis, Multi-organ, multi-lineage engraftment by a single bone marrow-derived stem cell, Cell 105 (2001) 369–377.
[18] S.H. Oh, M. Miyazaki, H. Kouchi, Y. Inoue, M. Sakaguchi, T. Tsuji, N. Shima, K. Higashio, M. Namba, Hepatocyte growth factor induces differentiation of adult rat bone marrow cells into a hepatocyte lineage in vitro, Biochem. Biophys. Res. Commun. 279 (2000) 500–504.
[19] E. Lagasse, H. Connors, M. Al-Dhalimy, M. Reitsma, M. Dohse, L. Osborne, X. Wang, M. Finegold, I.L. Weissman, M. Grompe, Purified hematopoietic stem cells can differentiate into hepatocytes in vivo, Nat. Med. 6 (2000) 1229–1234.
[20] N.D. Theise, M. Nimmakayalu, R. Gardner, P.B. Illei, G. Morgan, L. Teperman, O. Henegariu, D.S. Krause, Liver from bone marrow in humans, Hepatology 32 (2000) 11–16.
[21] N.D. Theise, S. Badve, R. Saxena, O. Henegariu, S. Sell, J.M. Crawford, D.S. Krause, Derivation of hepatocytes from bone marrow cells in mice after radiation-induced myeloablation, Hepatology 31 (2000) 235–240.
[22] G.G. Wulf, K.L. Luo, K.A. Jackson, M.K. Brenner, M.A. Goodell, Cells of the hepatic side population contribute to liver regeneration and can be replenished with bone marrow stem cells, Haematologica 88 (2003) 368–378.
[23] Y.Y. Jang, M.I. Collector, S.B. Baylin, A.M. Diehl, S.J. Sharkis, Hematopoietic stem cells convert into liver cells within days without fusion, Nat. Cell Biol. 6 (2004) 532–539.
[24] G. Vassilopoulos, D.W. Russell, Cell fusion: an alternative to stem cell plasticity and its therapeutic implications, Curr. Opin. Genet. Dev. 13 (2003) 480–485.
[25] X. Wang, H. Willenbring, Y. Akkari, Y. Torimaru, M. Foster, M. Al-Dhalimy, E. Lagasse, M. Finegold, S. Olson, M. Grompe, Cell fusion is the principal source of bone-marrow-derived hepatocytes, Nature 422 (2003) 897–901.
[26] A. Menthena, N. Deb, M. Oertel, P.N. Grozdanov, J. Sandhu, S. Shah, C. Guha, D.A. Shafritz, M.D. Dabeva, Bone marrow progenitors are not the source of expanding oval cells in injured liver, Stem Cells 22 (2004) 1049–1061.
[27] D.S. Batusic, V. Cimica, Y. Chen, K. Tron, T. Hollemann, T. Pieler, G. Ramadori, Identification of genes specific to “oval cells” in the rat 2-acetylaminofluorene/partial hepatectomy model, Histochem. Cell Biol. 124 (2005) 245–260.

[28] R. Pack, R. Heck, H.P. Dienes, F. Oesch, P. Steinberg, Isolation, biochemical characterization, long-term culture, and phenotype modulation of oval cells from carcinogen-fed rats, Exp.Cell Res. 204 (1993) 198–209.
[29] H.A. Crosby, D.A. Kelly, A.J. Strain, Human hepatic stem-like cells isolated using c-*kit* or CD34 can differentiate into biliary epithelium, Gastroenterology 120 (2001) 534–544.
[30] Z.P. He, W.Q. Tan, Y.F. Tang, H.J. Zhang, M.F. Feng, Activation, isolation, identification and in vitro proliferation of oval cells from adult rat livers, Cell Proliferation 37 (2004) 177–187.
[31] L. Yang, S. Li, H. Hatch, K. Ahrens, J.G. Cornelius, B.E. Petersen, A.B. Peck, In vitro trans-differentiation of adult hepatic stem cells into pancreatic endocrine hormone-producing cells, Proc. Natl. Acad. Sci U. S. A. 99 (2002) 8078–8083.
[32] S. Sell, Heterogeneity and plasticity of hepatocyte lineage cells, Hepatology 33 (2001) 738–750.
[33] B.E. Petersen, V.F. Zajac, G.K. Michalopoulos, Hepatic oval cell activation in response to injury following chemically induced periportal or pericentral damage in rats, Hepatology 27 (1998) 1030–1038.
[34] P.O. Seglen, Preparation of isolated rat liver cells, Methods Cell Biol. 13 (1976) 29–83.
[35] T. Brun, I. Franklin, L. St-Onge, A. Biason-Lauber, E. Schoenle, C.B. Wollheim, B.R. Gauthier, The diabetes-linked transcription factor Pax4 promotes beta-cell proliferation and survival in rat and human islets, J. Cell Biol. 167 (2004) 1123–1135.
[36] B. Gauthier, M. Robb, R. McPherson, Cholesteryl ester transfer protein gene expression during differentiation of human preadipocytes to adipocytes in primary culture, Atherosclerosis 142 (1999) 301–307.
[37] G. De Silvestro, M. Vicarioto, C. Donadel, M. Menegazzo, P. Marson, A. Corsini, Mobilization of peripheral blood hematopoietic stem cells following liver resection surgery, Hepatogastroenterology 51 (2004) 805–810.
[38] C.E. Lamming, L. Augustin, M. Blackstad, T.C. Lund, R.P. Hebbel, C.M. Verfaillie, Spontaneous circulation of myeloid-lymphoid-initiating cells and SCID-repopulating cells in sickle cell crisis, J. Clin. Invest. 111 (2003) 811–819.
[39] E. Dalakas, P.N. Newsome, D.J. Harrison, J.N. Plevris, Hematopoietic stem cell trafficking in liver injury, FASEB J. 19 (2005) 1225–1231.
[40] M.J. Evans, M.H. Kaufman, Establishment in culture of pluripotential cells from mouse embryos, Nature 292 (1981) 154–156.
[41] L. Pi, S.H. Oh, T. Shupe, B.E. Petersen, Role of connective tissue growth factor in oval cell response during liver regeneration after 2-AAF/PHx in rats, Gastroenterology 128 (2005) 2077–2088.
[42] S.H. Oh, H.M. Hatch, B.E. Petersen, Hepatic oval 'stem' cell in liver regeneration, Semin. Cell Dev. Biol. 13 (2002) 405–409.
[43] S. Paku, P. Nagy, L. Kopper, S.S. Thorgeirsson, 2-acetylaminofluorene dose-dependent differentiation of rat oval cells into hepatocytes: confocal and electron microscopic studies, Hepatology 39 (2004) 1353–1361.
[44] S. Matsusaka, T. Tsujimura, A. Toyosaka, K. Nakasho, A. Sugihara, E. Okamoto, K. Uematsu, N. Terada, Role of c-*kit* receptor tyrosine kinase in development of oval cells in the rat 2-acetylaminofluorene/partial hepatectomy model, Hepatology 29 (1999) 670–676.
[45] I.L. Weissman, D.J. Anderson, F. Gage, Stem and progenitor cells: origins, phenotypes, lineage commitments, and transdifferentiations, Annu. Rev. Cell Dev. Biol. 17 (2001) 387–403.
[46] L. Yin, M. Sun, Z. Ilic, H.L. Leffert, S. Sell, Derivation, characterization, and phenotypic variation of hepatic progenitor cell lines isolated from adult rats, Hepatology 35 (2002) 315–324.
[47] T.A. Roskams, N.D. Theise, C. Balabaud, G. Bhagat, P.S. Bhathal, P. Bioulac-Sage, E.M. Brunt, J.M. Crawford, H.A. Crosby, V. Desmet, M.J. Finegold, S.A. Geller, A.S. Gouw, P. Hytiroglou, A.S. Knisely, M. Kojiro, J.H. Lefkowitch, Y. Nakanuma, J.K. Olynyk, Y.N. Park, B. Portmann, R. Saxena, P.J. Scheuer, A.J. Strain, S.N. Thung, I.R. Wanless, A.B. West, Nomenclature of the finer branches of the biliary tree: canals, ductules, and ductular reactions in human livers, Hepatology 39 (2004) 1739–1745.
[48] K. Klarmann, M. Ortiz, M. Davies, J.R. Keller, Identification of in vitro growth conditions for c-Kit-negative hematopoietic stem cells, Blood 102 (2003) 3120–3128.
[49] N.M. Bilko, I.A. Votyakova, S.V. Vasylovska, D.I. Bilko, Characterization of the interactions between stromal and haematopoietic progenitor cells in expansion cell culture models, Cell Biol. Int. 29 (2005) 83–86.
[50] M. Grompe, Bone marrow-derived hepatocytes, Novartis Found Symp. 265 (2005) 20–27.
[51] E. Fuchs, T. Tumbar, G. Guasch, Socializing with the neighbors: stem cells and their niche, Cell 116 (2004) 769–778.
[52] A. Wilson, M.J. Murphy, T. Oskarsson, K. Kaloulis, M.D. Bettess, G.M. Oser, A.C. Pasche, C. Knabenhans, H.R. Macdonald, A. Trumpp, c-Myc controls the balance between hematopoietic stem cell self-renewal and differentiation, Genes Dev. 18 (2004) 2747–2763.
[53] A. Jakubowski, C. Ambrose, M. Parr, J.M. Lincecum, M.Z. Wang, T.S. Zheng, B. Browning, J.S. Michaelson, M. Baestcher, B. Wang, D.M. Bissell, L.C. Burkly, TWEAK induces liver progenitor cell proliferation, J. Clin. Invest. 115 (2005) 2330–2340.