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Activation, isolation, identification and *in vitro* proliferation of oval cells from adult rat livers

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Abstract. Oval cells, putative hepatic stem cells, could potentially provide a novel solution to the severe shortage of donor livers, because of their ability to proliferate and differentiate into functional hepatocytes. We have previously demonstrated that oval cells can be induced to differentiate into cells with morphologic, phenotypic, and functional characteristics of mature hepatocytes. In this study, we have established a new model combining ethionine treatment with partial hepatectomy to activate oval cells, then developed a procedure utilizing selective enzymatic digestion and density gradient centrifugation to isolate and purify such cells from heterogeneous liver cell population. We identified oval cells by their morphological characteristics and phenotypic properties, thereby providing definitive evidence of the presence of hepatic stem-like cells in adult rat livers. Viewed by transmission electron microscopy, they were small cells with ovoid nuclei, a high nucleus/cytoplasm ratio and few organelles, including mitochondria and endoplasmic reticulum. Flow cytometric assay showed that these cells highly expressed OV-6, cytokeratin-19 (CK-19) and albumin. Reverse transcriptase-polymerase chain reaction (RT-PCR) analysis displayed that the freshly isolated cells co-expressed albumin, cytokeratin-7 (CK-7) and CK-19 mRNA, indicating that they were essentially bipotential hepatic stem-like cells. Furthermore, we set up a culture system containing growth factors and a fibroblast feeder layer, to provide nourishment to these cells. Thus, we were able to culture them *in vitro* for more than 3 months, with the number of cells doubling 100 times. Gene expressions of albumin, CK-7 and CK-19 in the cells derived from the expanding colonies at day 95 were confirmed by RT-PCR analysis. These data suggested that the hepatic oval cells derived from adult rat livers possess a high potential to proliferate *in vitro* with a large increase in number, while maintaining the bipotential nature of hepatic stem cells.

INTRODUCTION

Hepatic stem cells have aroused considerable interest because of their developmental importance and therapeutic potential, including cell transplantation, tissue engineering and gene therapy for

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liver-related diseases (He *et al*. 2003). The existence of hepatic stem cells was first postulated in 1958 (Wilson & Leduc 1958). Although there was some evidence for the presence of hepatic stem cells in the fetal mouse livers (Suzuki *et al*. 2000a; Taniguchi *et al*. 2000), the existence of such cells in adult livers remained equivocal. The identification of hepatic stem cells has also been hampered by the lack of specific markers to isolate them. Liver explant culture, an attractive method to expand haematopoietic stem cells, has also been used to isolate hepatic stem cells (Monga *et al*. 2001). However, no pure population of hepatic stem cells had yet been obtained from adult livers. In this paper, we describe a new isolation and purification procedure involving perfusion of the liver with collagenase, treatment of the cell suspension with ethylene diamine tetraacetic acid (EDTA), selective removal of mature hepatocytes by the digestion of Pronase E, and cell separation through centrifugation in a Percoll density gradient to eliminate mesenchymal cells.

Hepatic stem cell transplantation is not widespread due to the lack of sufficient cells. A feeder layer composed of mouse embryonic fibroblasts has been shown to play an important role in the establishment of human embryonic stem cell line (D'Amour & Gage 2000). We have attempted to ascertain whether a feeder layer of fibroblasts derived from adult liver tissues could help expand oval cells isolated from adult rat livers. Therefore, a specific culture system consisting of certain growth factors and a fibroblast feeder layer was established to support the growth and proliferation of our hepatic oval cells.

MATERIALS AND METHODS

Animals

Male Wistar rats (120 g body wt) were obtained from the specific pathogen-free (SPF) laboratory animal breeding centre, Institute of Zoology, Chinese Academy of Sciences. The rats were fed standard laboratory chow *ad libitum*. All animal care and procedures were approved by the institutional animal care committee.

Establishing a model of oval cell activation

0.07% wt/wt ethionine (Sigma, St Louis, MO, USA) was administered to the rats for 1 week, after which a two-thirds partial hepatectomy (PH) was performed. After a 5-day recovery period, liver tissues from treated rats were used to isolate hepatic oval cells.

Histological slice and haematoxylin staining

The livers of treated rats were fixed in formaldehyde on day 6 after partial hepatectomy. Liver tissue was dehydrated in ethanol, rendered transparent with chloroform and embedded in paraffin. Sections were cut and examined by light microscopy after staining with haematoxylin.

Isolation and purification of oval cells

Liver tissues were minced in cold Hank's balanced salt solution (HBSS) (Gibco BRL, Grand Island, NY, USA) and digested in Dulbecco's minimum Eagle's medium (DMEM) (Gibco BRL) containing 0.10% wt/v collagenase IV (Gibco Invitrogen Corporation Products, NY, USA) and 0.025% wt/v EDTA for 15 min at 37 °C in a shaking bath when a two-step collagenase perfusion was accomplished. After centrifugation at 50 g for 5 min, the supernatant was re-centrifuged at 350 g for 10 min. Pellets were suspended in 50 ml of DMEM including 0.10% wt/v Pronase E (Sigma) and 0.005% wt/v Dnase I (Sigma), and the cell suspension was incubated for 30 min at 37 °C. Thereafter, the suspension was centrifuged at 350 g for 5 min at 4 °C, and

pellets were re-suspended in 2 ml HBSS. The density gradient was as follows: 1.20 g/ml Percoll (Sigma), 1.12 g/ml Percoll, cell suspension, and HBSS. After centrifugation at 350 g for 20 min at 4 °C, the interface between 1.12 and 1.20 g/ml Percoll was decanted and re-centrifuged at 350 g for 5 min. Viability of the cells was estimated by the staining of 0.25% wt/v trypan blue.

Transmission electron microscopy (TEM)

The freshly isolated cells were fixed in 2.5% wt/v glutaraldehyde in 0.1 M cacodylate buffer (PH 7.4). After extensive washings in phosphate-buffered saline (PBS) (Sigma), the cells were post fixed in 1% wt/v osmium tetroxide (OsO_4) for 30 min, dehydrated in graded solutions of ethanol and embedded in Epon. Ultrathin sections were cut and examined under a Philips EM301 electron microscope after staining with uranyl acetate and lead citrate.

Scanning electron microscopy (SEM)

The freshly isolated oval cells were rinsed with PBS without Ca^{2+} or Mg^{2+}, and fixed in 3.5% v/v glutaraldehyde (0.1 M phosphate buffer, 2% v/v sucrose, PH 7.3) for 2 h. After several washes in distilled water, the cells were dehydrated in alcohol and dimethoxypropane, and sputter-coated with 10 nm gold. SEM examination was carried out with a JSM-5600LV electron microscope.

Flow cytometric analysis of freshly isolated cells

A total of 1×10^5 oval cells were fixed in acetone for 10 min at 4 °C. After centrifugation at 350 g for 5 min at 4 °C, the pellets were suspended in 0.1 ml PBS and incubated with anti-OV-6 antibody (a kind gift from Prof. Stewart Sell, Albany Medical College) for 60 min at 37 °C. Centrifugation was performed at 350 g for 15 min and followed by extensive washes with PBS. Pellets were re-suspended in 0.1 ml PBS in preparation for cell suspension. Binding of primary antibody was detected by a phycoerythrin (PE)-labelled IgG (Dako, Kyoto, Japan). Cells were assessed by flow cytometry (Becton-Dickinson, San Jose, CA, USA), and the data were analysed with Cell-Quest software (Becton-Dickinson). Replacement of the primary antibody with PE-labelled IgG served as a negative control.

Double staining by flow cytometry was performed using primary antibody combinations containing albumin with CK-19 to verify hepatobiliary phenotype of oval cells. Antibodies to albumin and CK-19 were purchased from Dako. Goat anti-mouse fluorescein isothiocyanate (FITC)-labelled IgG (Dako) to recognize CK-19 and goat ant-rabbit PE-labelled IgG (Dako) for albumin served as the secondary antibodies. The protocol for double labelling was similar to the procedure for single staining (except, of course, for the differences in primary and second antibodies).

Oval cell proliferation *in vitro*

Freshly isolated cells were plated at a density of 1×10^4 cells per 35 mm per Petri dish (Becton Dickinson, NJ, USA) with or without a feeder layer composed of fibroblasts which had been treated with mitomycin C (MMC) overnight at 37 °C. DMEM medium was supplemented with 100 μg/ml penicillin G and 100 μg/ml streptomycin. Growth factors used in this culture system were 20 ng/ml stem cell factor (SCF), 10 ng/ml hepatocyte growth factor (HGF), 20 ng/ml hepatic stimulator substance (HSS), 10 ng/ml epidermal growth factor (EGF), and 10 ng/ml leukaemia inhibitory factor (LIF). All these growth factors except for HSS were purchased from Sigma. Cultures were maintained in a humidified incubator in a mixture of 95% air and 5% CO_2 at 37 °C. Fresh medium was provided every 3 days and cell growth was carefully examined under a phase-contrast

microscope. The purification and identification of HSS was performed according to previously published methods (Fleig & Hoss 1989; Liu *et al*. 1998).

RNA extraction and reverse transcriptase-polymerase chain reaction (RT-PCR) analysis
Total RNA was prepared from the freshly isolated cells, those derived from colonies at day 95, mature hepatocytes and bile epithelial cells by using TRIzol (Life Technologies, Rockville, MD, USA). To eliminate genomic DNA contamination, mRNA was purified by using oligo(dT) cellulose (MicroFastTrack 2.0 kit, Invitrogen) according to the manufacturer's protocol. One microgram of total RNA was used for the first-strand cDNA, and the cDNA samples were subjected to PCR amplification with specific primers under linear conditions. The cycling parameters were as follows: denaturation at 94 °C for 1 min, annealing at 56 °C for 1 min, and elongation at 72 °C for 1 min (35 cycles). The PCR primers used for amplification were as follows: albumin (forward 5′-CTGGGAGTGTGCAGATATCAGAGT-3′, and reverse 5′-GAGAAGGTCAC-CAAGTGCTGTAGT-3′, 618 bp); CK-19 (forward 5′-ACCATGCAGAACCTGAACGAT-3′, and reverse 5′-CACCTCCAGCTCGCCATTAG-3′, 425 bp); CK-7 (forward 5′-GCTCATTCTTCT-TCAGGTAG-3′, and reverse 5′-CGAGCTGATGACTTCAGAACC-3′, 398 bp). The PCR products were separated by electrophoresis in 1.0% agarose gel.

RESULTS

Activation of the oval cells
In the ethionine/PH regime, massive proliferation of small cells with oval nuclei and a large nucleus/cytoplasm ratio was seen on day 6 after the partial hepatectomy (Fig. 1). Hepatic stem cells, including oval cells, remain dormant (G_0 phase) in the normal adult livers. The activation of oval cells in this model might therefore be due to the mito-inhibitory effect of ethionine on the mature hepatocytes the regenerative stimulus of partial hepatectomy on potential stem cells.

Morphological features of freshly isolated cells
We obtained about 2×10^7 cells/rat with a purity of 95%, as evaluated by transmission electron microscopy. The freshly isolated cells were oval in shape and assumed a cobblestone-like

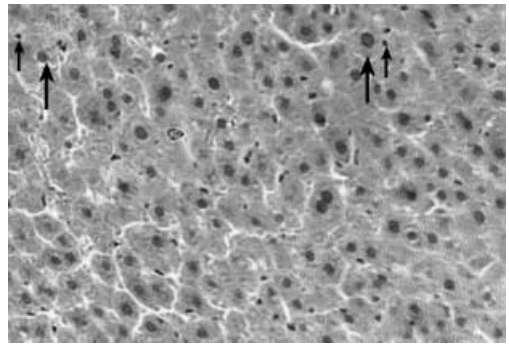


Figure 1. Histological changes in livers from adult rats fed with ethionine and followed by partial hepatectomy. A rapid and extensive proliferation of oval cells (denoted by short arrows) with ovoid nuclei and a relatively large nucleus:cytoplasm ratio was observed around mature hepatocytes (denoted by long arrows). No apparent lesions were seen in these livers. Original magnification: ×_200.

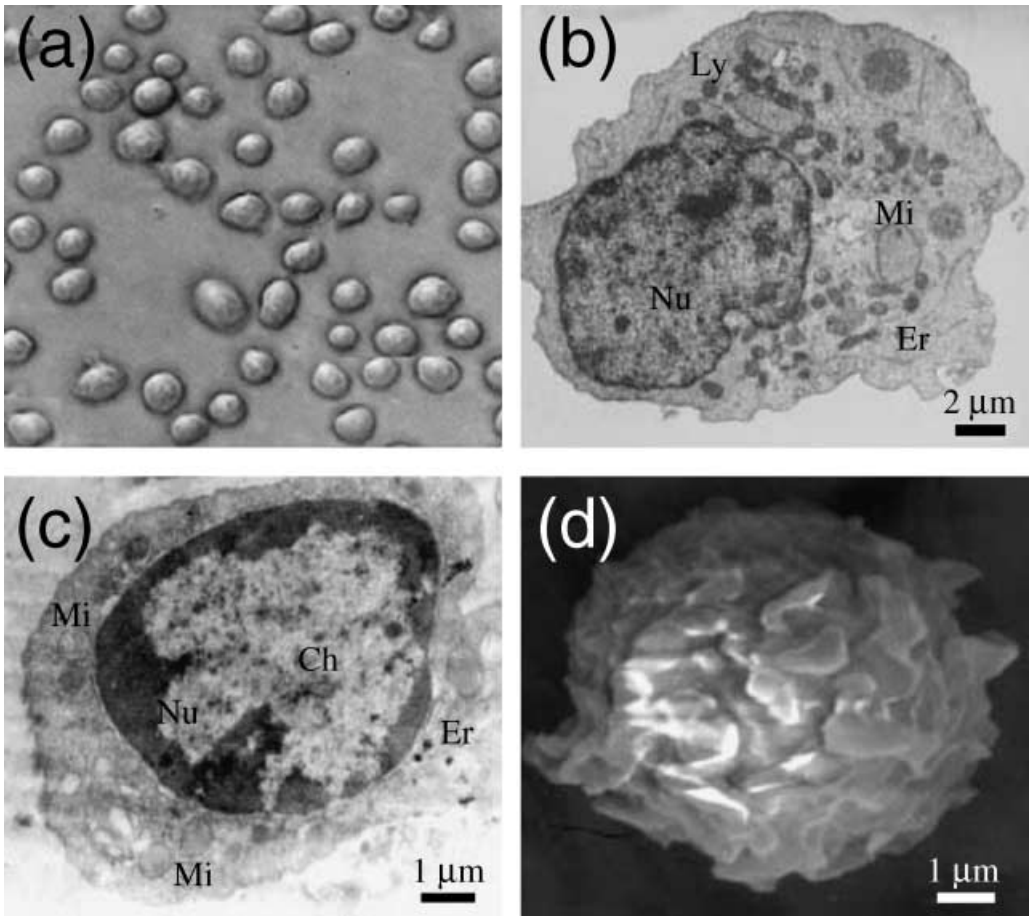


Figure 2. The morphological characteristics of the isolated oval cells. Freshly isolated cells showed a cobblestone appearance (a). Note that there were oval nuclei (Nu), a small proportion of cytoplasm, condensed chromatin (Ch), immature organelles such as mitochondria (Mi) and endoplasmic reticulum (Er) in these cells (c). A further morphological characteristic was that frequent protuberances were present on their surfaces (d). In contrast, mature hepatocytes showed dense spacious cytoplasm with well-developed organelles, including mitochondria (Mi), smooth and rough endoplasmic reticulum (Er) and lysosomes (Ly) (b). Original magnifications: (a) ×_400; (b) ×_4000; (c) ×_10 000; (d) ×_9000.

appearance under phase-contrast microscopy (Fig. 2a). Viability of freshly isolated cells was 99%, as estimated by their ability to exclude trypan blue.

Transmission electron microscope examination showed that the freshly isolated cells had ovoid nuclei, a high nucleus/cytoplasm ratio, immature mitochondria, endoplasmic reticulum and condensed chromatin (Fig. 2c). Oval cells were relatively small, their median diameter ranged between 6 and 8 μm, less than half that of mature hepatocytes (Fig. 2b). Scanning electron microscopy confirmed that these cells were ovoid in shape. In addition, this revealed that many protuberances existed on the surface of these oval cells (Fig. 2d).

Phenotypic properties of freshly isolated cells

We analysed the phenotypic characteristics of the isolated cells in order to confirm their identity. Flow cytometric assay revealed that 90.06% of the isolated cells were positive for OV-6, an

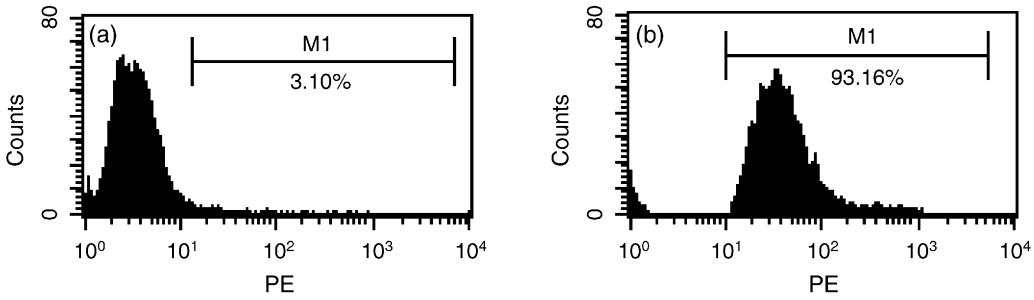


Figure 3. Phenotypic characteristics of freshly isolated cells by flow cytometric analysis. The isolated oval cells had been incubated with anti-OV-6. The majority of cells were positively stained (b). Replacement of primary antibody with PE-labelled IgG served as a negative control (a).

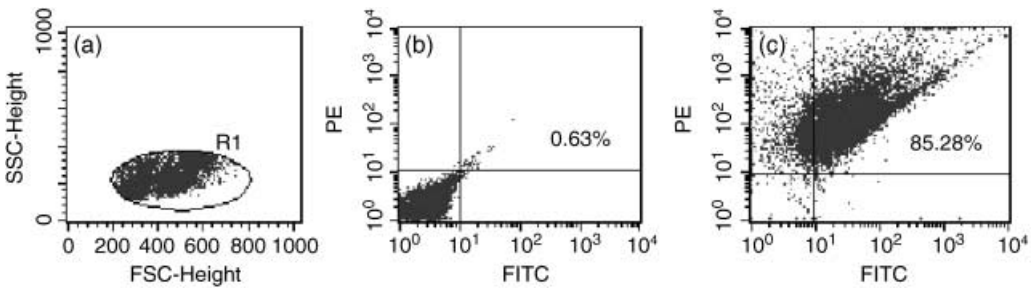


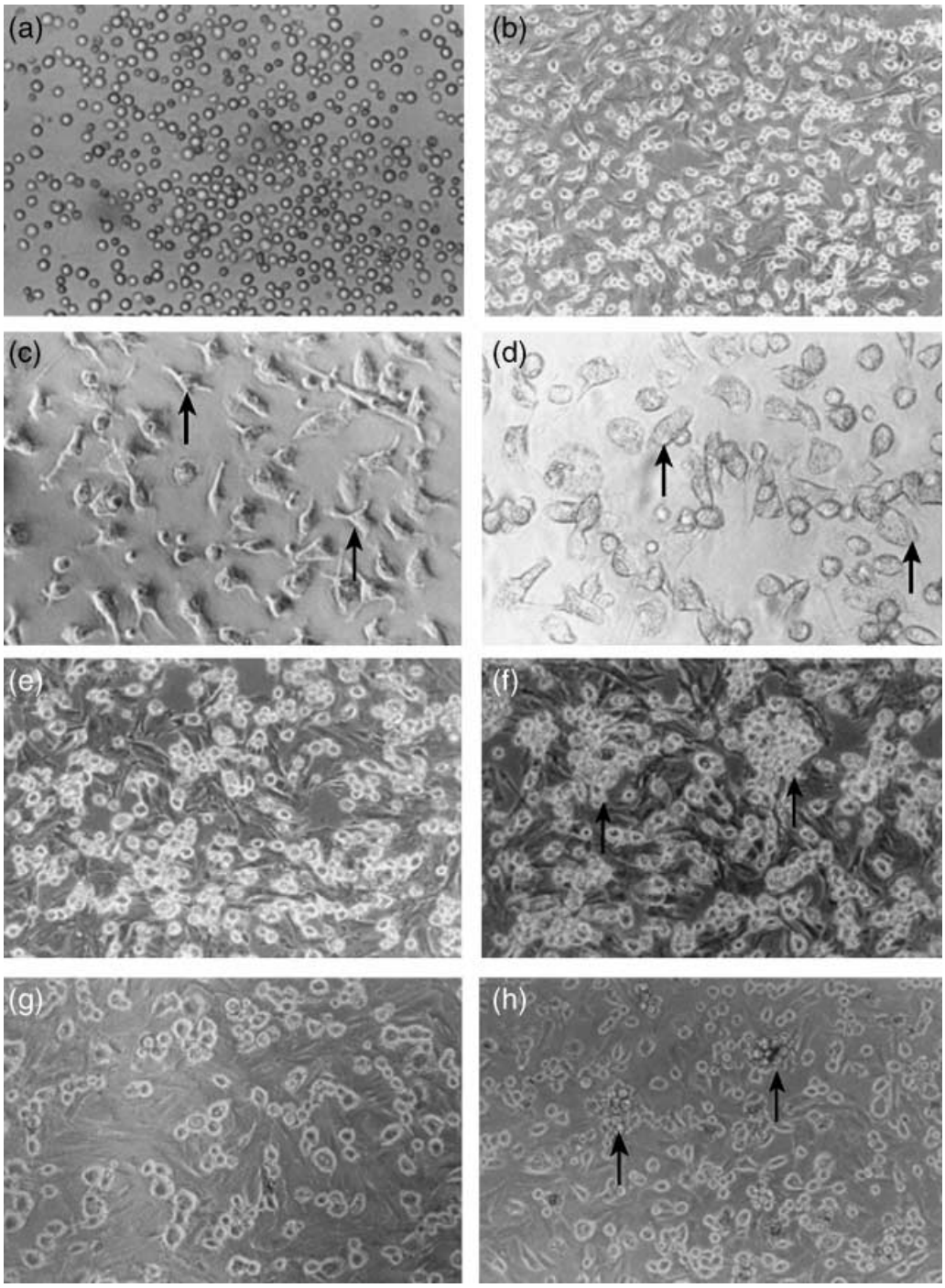
Figure 4. Double labelling with hepatocyte and cholangiocyte specific markers and flow cytometric analysis to characterize hepatic oval cells/mixed hepatobiliary phenotype. Oval cells were incubated with the primary antibodies against (i) albumin and (ii) CK-19. Replacement of the primary antibodies with PE-labelled and FITC-labelled IgG served as a negative controls (a, b). The majority of freshly isolated oval cells co-expressed albumin and CK-19 (c).

antigen specific to rodent oval cells (Fig. 3a and b). This provided conclusive evidence that most of these cells were indeed oval cells. Double staining displayed that 84.65% of the cells were co-expressing CK-19 and albumin (Fig. 4). To further clarify the characteristic phenotypes of the freshly isolated cells, we also analysed the gene expressions of hepatocytic and cholangiocytic specific markers by RT-PCR analysis. The isolated oval cells co-expressed albumin, CK-7 and CK-19 mRNA at high levels (Fig. 6a). Taken together, these tests showed that the freshly isolated cells expressed both hepatocytic and cholangiocytic markers, indicating that they were essentially bipotential hepatic stem-like cells.

Proliferative potential of hepatic oval cells

Freshly isolated cells showed an ovoid appearance when seeded on the dish (Fig. 5a). In the presence of the fibroblast feeder layer, oval cells attached to the dishes within 24 h after plating.

Figure 5. The proliferation potential of oval cells *in vitro*. Freshly isolated cells assumed a cobblestone-like appearance under the phase-contrast microscope (a). In the presence of a fibroblast feeder layer and the addition of growth factors in the medium, oval cells multiplied rapidly with the number of cells doubling twice in 48 h (b). The cells spontaneously differentiated into a variety of cell types, including bile epithelial cells (c) and hepatocytes (d) by the removal of the fibroblast feeder layer. Under the culture system mentioned in MATERIALS AND METHODS, oval cells were subcultured for a second passage at day 6 (e). Relatively larger colonies (indicated by arrows) were seen following a period of oval cell proliferation (f), and the cells were able to be subcultured for the third passage by day 14 (g). It should be noted that these cells cultured *in vitro* for more than 3 months were still capable of expanding and aggregating to form colonies (h). Original magnifications: (a, b) ×_100; (c, d) ×_400; (e, f, g, h) ×_100.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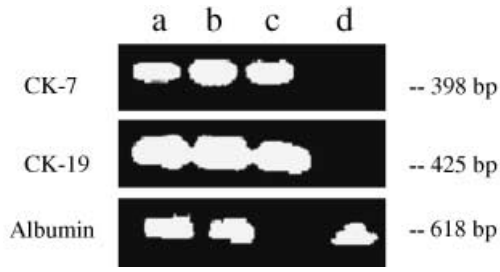

Figure 6. RT-PCR analysis of albumin, CK-7 and CK-19 mRNA expression in the freshly isolated oval cells (a), cells from expanding colonies (b), bile epithelial cells (c) and hepatocytes (d). *Albumin* gene was highly expressed in both freshly isolated cells and the cells from expanding colonies at 95 days after initiation of the culture, whereas it was not detected in bile epithelial cells. Furthermore, the freshly isolated oval cells and the cells derived from expanding colonies had high levels of CK-7 and CK-19 mRNA expression. In contrast, hepatocytes expressed albumin mRNA only but not CK-7 or CK-19 mRNAs.

These cells began to proliferate and scatter, while maintaining their oval shape, and the number of cells doubled by day 2 (Fig. 5b). In contrast, cells differentiated into a variety of cell lineages including bile epithelial cells (Fig. 5c) or hepatocytes (Fig. 5d) in the absence of the fibroblast feeder layer. On day 6 post isolation, oval cells were subcultured for the second passage (Fig. 5e) after which they multiplied more rapidly than those in primary culture. It was worth noting that oval cells aggregated to form relatively larger colonies by day 9 (Fig. 5f). When cultured for a 2-week period, the cells could be subcultured for a third passage (Fig. 5g). After three passages, these oval cells still had the ability to clonally expand and congregate to form discrete colonies (Fig. 5h). Under this culture system, as described in the MATERIALS AND METHODS section, the oval cells were maintained in culture for more than 3 months, with the number of cell population doublings reaching a hundred times.

Expression of mRNA for albumin, CK-7 and CK-19 in cells from the expanding colonies

To estimate the differentiation potential of the cells constituting the expanding colonies, we also examined the mRNA expression of differentiation markers including albumin, CK-7 and CK-19. RT-PCR analysis showed that mature hepatocytes expressed only the albumin gene (Fig. 6d), whereas bile epithelial cells expressed CK-7 and CK-19 mRNA but not albumin mRNA (Fig. 6c). In contrast, the cells derived from the expanding colonies had high levels of albumin, CK-7 and CK-19 mRNA expression (Fig. 6b), suggesting that these cells retained the bipotential nature of hepatic stem cells.

DISCUSSION

Oval cell transplantation could potentially offer an alternative to liver transplantation in the treatment of acute liver failure. A major obstacle in the study of oval cells is the lack of specific surface markers to obtain pure cell populations. In addition, shortage of sufficient cells remains a major limiting factor for their medical application. One attractive solution to this problem would be to be able to expand certain numbers of oval cells *in vitro*. The development of effective methods for the activation, isolation and proliferation of oval cells is thus essential for the use of such cells in both basic research and clinical therapy.

It has been reported that mature hepatocytes can restore the liver after partial hepatectomy (Michalopoulos & DeFrances 1997). Proliferation of hepatic oval cells occurs only when the ability of mature hepatocytes to replicate is compromised (Fujio *et al*. 1994). To date, several models using toxins or carcinogens have been utilized to activate hepatic oval cells, but these often cause liver damage. Galactosamine, for example, causes liver injury in the pericentral region, and allyl alcohol engenders periportal hepatocyte death (Dabeva *et al*. 1993). In the present study, a low dose of ethionine was used to inhibit hepatocyte replication, and partial hepatectomy resulted in liver regeneration with extensive proliferation of hepatic oval cells. It is noteworthy that there were no obvious lesions in the liver following these procedures. The ethionine/PH model therefore appears to be an excellent approach of activating oval cells.

It seems it is difficult to isolate and purify oval cells using a fluorescence-activated cell sorter due to paucity of specific surface antibodies. Density gradient centrifugation has been shown to be an effective method of separating oval cells from liver cell fractions (Sirica *et al*. 1990). However, centrifugation in different media and various density gradients results affects cell purity. For example, isopyknic centrifugation in metrizamide density gradient could obtain oval cells with a final cell purity of less than 80% (Sells *et al*. 1981; Ledda *et al*. 1983), whereas the cell separation technique using velocity sedimentation in isokinetic Ficoll gradient has led to a final oval cell purity of 32.3% (Miller *et al*. 1983). In this study, we isolated and purified oval cells from adult rat livers using specific enzymatic digestion and density gradient centrifugation in Percoll. The cell suspension was first treated with EDTA to remove contaminating fibroblasts. Non-parechymal cell suspension was then obtained by incubation with Pronase E that selectively digested mature hepatocytes into undetectable debris. and finally, oval cells were further purified from the non-parechymal cell suspension by density gradient centrifugation in Percoll. Using this isolation procedure, we have obtained essentially pure oval cells with a high viability. The morphological characteristics of the oval cells we obtained were consistent with findings reported by Pack *et al*. 1993 and Yasui *et al*. 1997, however, our cells were smaller than those isolated by Yasui *et al*. This may have occurred because Yasui *et al*. used different densities of Percoll (1.35 and 1.70 g/ml, cf. 1.12 and 1.20 g/ml in this experiment) to purify their cells. This difference in oval cell size, to some extent might illustrate the heterogeneity of the hepatic oval cell population.

The existence of hepatic stem cells in adult liver tissue was first postulated by Arber & Zajicekg Ariel 1988, however, convincing evidence has been required to demonstrate this supposition. The accurate identification of oval cells by flow cytometric assay, transmission electron microscopy, scanning electron microscopy, and RT-PCR analysis provides strong support for the presence of these hepatic stem-like cells in adult rat livers. Albumin is specifically located in mature hepatocytes, and CK-19 and CK-7 are typical markers of bile epithelial cells. Our freshly isolated cells were co-expressing these phenotypic markers, thereby demonstrating their bipotency. These cells also expressed OV-6, a specific antigen of rodent oval cells, which provides further direct evidence for the existence of hepatic stem-like cells in the adult rat livers.

Previously, oval cells seemed to lose their ability to multiply and differentiate *in vitro*. In order to expand the isolated oval cells and culture them for lengthy periods, a suitable culture system consisting of growth factors and a fibroblast feeder layer was established. Mouse embryonic fibroblast feeder layers have been shown to support the growth of haematopoietic stem cells and liver progenitor cells from mice (Sakamoto *et al*. 2000). We found that a feeder layer of fibroblasts derived from rat livers was essential for the self-renewal of oval cells; they differentiated into various phenotypes (e.g. hepatocytes and bile epithelial cells) by the removal of the fibroblast feeder layer. Certain important growth factors were employed in this culture system because of their special function. Stem cell factor (SCF), for instance, has been believed to play a fundamental role in the survival and proliferation of stem/progenitor cells (Fujio *et al*. 1994).

EGF can significantly enhance DNA synthesis of bile epithelial cells and induced the colony-formation of oval cells (Braun *et al*. 1987; Sirica *et al*. 1990), and HGF has been shown to be up-regulated during oval cell proliferation (Thorgeirsson *et al*. 1993). The combination HGF and SCF has been demonstrated to expand hepatic stem-like cells obtained from foetal livers (Monga *et al*. 2001). LIF in our culture system was used to ensure that oval cells were maintained in the undifferentiated state. Our preliminary experiments revealed that oval cells differentiated into the cultures containing hepatocytes and bile epithelial cells in the absence of LIF (data not shown). In addition, it was confirmed that hepatic stimulator substance promoted hepatoma cell and normal hepatocyte proliferation due to its modulation of EGF receptor expression and its signalling pathway (Fleig & Hoss 1989; Liu *et al*. 1998).

In contrast, the intimate contact of fibroblasts with oval cells indicated a fundamental role for hepatic oval cell expansion, probably through production of essential growth factors and extracellular matrix components including laminin, fibronectin, collagen IV and collagen I. Embryonic fibroblast conditioned medium has been shown to promote proliferation and colony formation of mouse hepatic stem cells more effectively than HGF (Suzuki *et al*. 2000b). However, the specific growth factors that account for the expansion of hepatic stem cells are still unknown. We speculate that a fibroblast feeder layer might produce soluble growth factors, including SCF, ciliary neurotrophic factor (CNTF), acidic fibroblast growth factor (aFGF), basic fibroblast growth factor, HGF, transforming growth factor-β (TGF-β) and transforming growth factor-α (TGF-α). In an earlier study, it was noted that SCF and CNTF produced by mouse fibroblast feeder layers could stimulate the proliferation and development of chicken primordial germ cells (PGC) (Karagenc & Petitte. 2000). Basic fibroblast growth factor has been shown to support the continuous growth of human embryonic stem cells while maintaining them in an undifferentiated stage (Amit *et al*. 2000). Levels of growth factors including SCF, HGF, aFGF, TGF-β and TGF-α have been shown to be up-regulated during oval cell proliferation *in vivo* (Thorgeirsson *et al*. 1993; Fujio *et al*. 1994), and hepatic oval cells expressed the receptors of these growth factors (Alison *et al*. 1996). The close association of oval cells and fibroblast feed layers places these cells in a situation that mimicks the hepatic microenvironment or niche, making it possible for the cells to maintain their undifferentiated state *in vitro*.

The generation of the cell colonies appeared to consist of three phases: hepatic oval cell proliferation with the generation of more oval cells, migration and interaction of the cells to form discrete clusters, and finally the fusion and aggregation of oval cell clusters to develop large colonies. It should be noted that the cells from expanding colonies still possessed dual differentiation potential. The establishment of an *in vitro* colony-forming assay as described here will allow us to develop techniques for the culture of liver tissue. These advances in the activation, isolation, and proliferation of oval cells from adult livers provide an abundant source of cells for further investigation into hepatic stem-like cells.

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