

Differential hepatic stem cell proliferation and differentiation after partial hepatectomy in rats

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Received February 2, 2013; Accepted June 24, 2013

DOI: 10.3892/mmr.2013.1606

Abstract. Stem cell-derived hepatocyte precursor cells represent a promising model for clinical transplantation to diseased livers, as well as for establishment of *in vitro* systems for drug metabolism and toxicology studies. The present study aimed to establish a new method of induction of hepatocyte differentiation using various factors and evaluate the effect of different partial hepatectomies and the duration of collagenase perfusion on hepatic stem cell proliferation and differentiation. A rat model of hepatic oval cell proliferation was established by partial hepatectomy (PH). Following 73.1 and 83.4% PH, rats underwent perfusion with IV collagenase for 10, 20 and 30 min. Density gradient centrifugation was performed and cells in the supernatant were cultured in various combinations of factors to induce oval cells to differentiate into mature hepatocytes. Cells were characterized for hepatocyte marker expression by morphology, flow cytometry, immunofluorescence and western blot analysis. Hepatic oval cells isolated from rats at 7 and 14 days post-PH exhibited properties of hepatic stem/progenitor cells. Following culturing in RPMI-1640 medium with hepatocyte growth factor and fibroblast growth factor-4, the cells resembled primary human hepatocytes with regard to morphology and expression of the hepatocyte markers, cytokeratin 18 (CK-18) and α -1-fetoprotein (AFP). Optimal differentiation of hepatic stem cells to CK-18- and AFP-positive cells was observed when stem cells isolated from 83.4% PH rats (7 days following surgery) were perfused with IV collagenase for 20 min. The results of this study provide novel insights into characteristics of rat hepatic stem cells.

Introduction

Hepatitis, cirrhosis, hepatoma and end-stage liver disease caused by multiple factors still lack effective treatment proto-

cols. Treatment of chronic liver disease had become a major problem for clinicians due to the poor efficacy of clinical treatments and high mortality. It has been reported that ~10% of patients with liver disease succumb to their condition while waiting for liver sources each year (1). Liver transplantation is currently the only therapeutic option for patients with end-stage chronic liver disease and for severe acute liver failure. Due to limited donor availability, surgical injury, a higher incidence of surgical complications and the expensive cost of treatment, the development of liver transplantation has been restricted (2).

Recent studies have focused on methods to restore liver mass and function through cell transplantation. Stem cells are a promising source for liver repopulation following cell transplantation (3). Several studies have reported that the administration of *in vitro* expanded stem cells may promote liver regeneration (4,5). In addition, hepatic stem cells have been identified in adult liver tissues (6,7) and oval cells derived from rodent and human livers have been demonstrated to be hepatic stem cells (8-10). Hepatic stem cells may participate in liver regeneration and restoration during serious liver injury (11-14). Thus, studies on the proliferation and differentiation of these cells are extremely useful for elucidating the molecular mechanisms of liver development.

In the present study, partial hepatectomy (PH) was performed to activate hepatic oval cells in rat livers, and oval cells undergoing proliferation and differentiation were identified by analysis of specific morphological and phenotypical characteristics. We sought to optimize hepatic stem cell proliferation and differentiation in *in vitro* culture by comparing various conditions, including different percentage PH and duration of collagenase perfusion.

Materials and methods

Animals. Male Wistar rats (age, 8-10 weeks old; weight, 150-180 g) were purchased from the Laboratory Animal Center of Dalian Medical University and maintained on standard laboratory chow and daily cycles of alternating 12 h of light and dark.

PH model. All experiments were performed in accordance with the Principles of Laboratory Animal Care and approved by the Local Committee for Experimental Animal Research.

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Key words: stem cells, differentiation, proliferation, hepatocytes

PH (73.1 and 83.4%) of rat livers was performed according to the procedure described previously (15,16). Sham-operated control animals were treated in an identical manner with the omission of hepatectomy.

Hepatic stem cell isolation. At 0, 7 and 14 days following PH, rat liver cells were isolated by a two-step collagenase IV digestion method, as described previously (17). The liver was perfused via a cannula in the inferior vena cava with 250 ml buffer [142 mM NaCl, 6.7 mM KCl and 10 mM HEPES (pH 7.4)] followed by 250 ml buffer containing additional 5.7 mM CaCl₂ and 0.5 mg/ml IV collagenase (Sigma-Aldrich, St. Louis, MO, USA) for 10, 20 or 30 min.

Hepatic stem cell culture and differentiation. Isolation and purification of oval cells were performed according to the protocol of Pack *et al.* (18) with specific modifications. Briefly, the hepatocytes were dispersed and washed twice with cold Ca²⁺-free perfusion buffer and resuspended in RPMI-1640 medium (Gibco-BRL, Carlsbad, CA, USA) supplemented with 10% FBS (Hyclone Laboratories, Inc., Logan, UT, USA), 5 ng/ml M-CSF, 0.4 ng/ml IL-3 and 140 μ M β -mercaptoethanol (all PeptoTech Inc., Rocky Hill, NJ, USA) to stimulate stem cell proliferation. Following a 6-day culture period, culture medium was replaced with RPMI-1640 medium containing 10% FBS, 20 μ g/l hepatocyte growth factor (HGF) and 10 μ g/l fibroblast growth factor-4 (FGF-4; both PeptoTech, Inc.) to stimulate stem cell differentiation. Cell growth was observed under a contrast phase microscope and recorded at days 0, 7 and 14. Viability was determined by trypan blue exclusion and only preparations with >90% viability were used. Cell number was determined with a hemocytometer (Bio-Rad, Hercules, CA, USA).

Flow cytometric analysis. The presence of CD34⁺ Thy-1⁺ cells was determined at 0, 7 and 14 days after PH. Freshly isolated cells (1x10⁵) were fixed in cold acetone for 8 min at 4°C. Following centrifugation at 141 x g for 5 min at 4°C, pellets were suspended in 0.1 ml PBS and incubated with anti-CD34 and Thy-1 (Santa Cruz Biotechnology, Inc., Santa Cruz, CA, USA) antibodies for 60 min at 37°C. Centrifugation was performed at 141 x g for 15 min and followed by extensive washes in PBS. Pellets were suspended in 0.1 ml PBS in preparation for cell suspension. Binding of primary antibody was detected by phycoerythrin (PE)-labeled IgG (Dako, Carpinteria, CA, USA). Cells were assayed by flow cytometry (Becton Dickinson, San Jose, CA, USA) and data were analyzed using Cell-Quest software (Becton Dickinson). Replacement of primary antibody with PE-labeled IgG served as a negative control.

Immunofluorescence assay of oval cells. Immunofluorescence analysis of oval cells in culture was performed on days 0, 7 and 14. Cells on glass slides were fixed in cold acetone for 5 min, blocked with normal goat serum following extensive washes in PBS (pH 7.4) and incubated with primary antibodies including anti-cytokeratin-18 (CK-18) and α -1-fetoprotein (AFP) at 4°C overnight. Following washing three times in PBS, cells were cultured with PE-labeled IgG for 30 min at 37°C, washed three times again in PBS and observed microscopically for

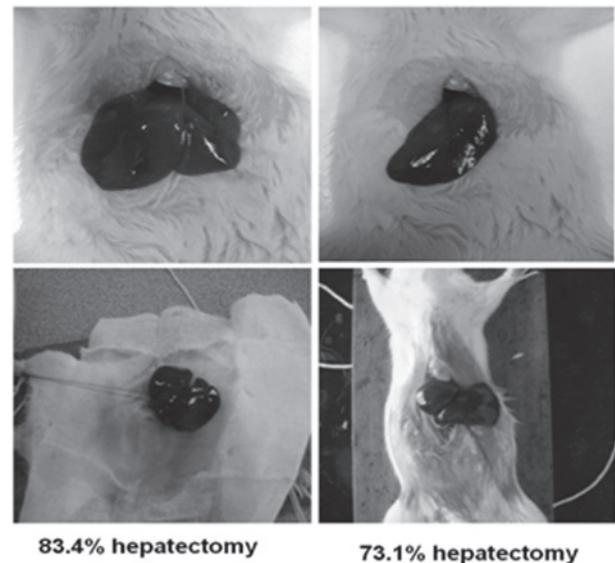


Figure 1. Images of PH rats. PH, partial hepatectomy.

fluorescence. Replacement of the primary antibody with PE-labeled IgG served as a negative control.

Western blot analysis. Rat liver tissue was homogenized (TissueRuptor; Qiagen, Hilden, Germany). Proteins were lysed with lysis buffer containing 25 mM Tris-HCl (pH 7.6), 150 mM NaCl, 1% NP-40, 1% sodium deoxycholate, 0.1% SDS, 1 mM PMSF and protease and phosphatase inhibitor cocktails (all from Sigma-Aldrich), as described previously (19). Proteins were separated by SDS-PAGE and transferred to a PVDF membrane (Bio-Rad, Hercules, CA, USA) using standard techniques. Antibodies used for immunoblotting were as follows: CK-18, AFP and β -catenin, and horseradish peroxidase-conjugated IgG secondary antibodies (all Santa Cruz Biotechnology, Inc.). An ECL plus western blotting detection kit (Amersham Biosciences, Piscataway, NJ, USA) was used for development of the membrane.

Statistical analysis. All results are expressed as the mean \pm SD. Statistical differences were determined by Student's t-test. P<0.05 was considered to indicate a statistically significant difference.

Results

Morphological features of freshly isolated cells from hepatectomized rats. Hepatectomy in rat livers was performed at 83.4 and 73.1% (Fig. 1). Collagenase perfusion was used to extract and isolate hepatic cells. In the control group, the newly separated hepatic cells were observed to exhibit a round shape, homogeneous size and strong diffraction. The viability of freshly isolated cells was >95%, as estimated by their ability to exclude trypan blue (data not shown). Following culture for 4-6 h, phase-contrast microscopy revealed that cells attaching to the culture dishes were round in shape (Fig. 2). After culture for 7 days, cells were observed to grow individually and no colonies were noted. Parts of the cells gradually stretched, causing fusiform or spindle-shaped growth. At day 14,

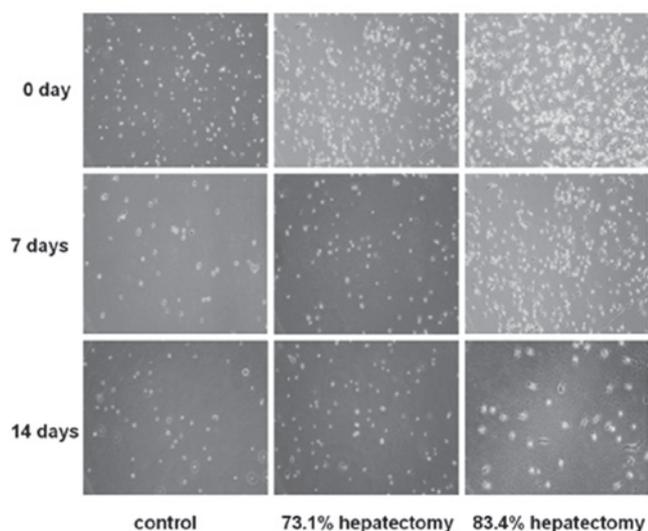


Figure 2. Histological features of rat livers following partial hepatectomy. Phase-contrast microscopy demonstrated the morphology of hepatic cells.

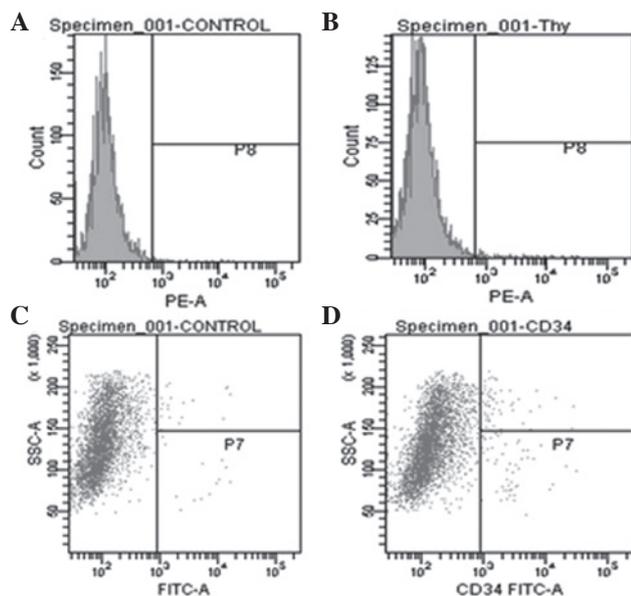


Figure 3. Phenotypic characteristics of oval cells indicated by flow cytometric analysis. (A) PE-labeled IgG secondary antibody, (B) Thy-1 primary antibody, (C) FITC-labeled IgG and (D) CD34. PE, phycoerythrin.

the majority of the cells were fibroblast-like (Fig. 2). In the 73.1% hepatectomy group, colony-like growth was observed following culture for 6 days and a number of cells were fused, indicative of hepatic stem cell proliferation (Fig. 2). In the 83.4% hepatectomy group, cells did not exhibit colony-like growth (Fig. 2).

Phenotypic characteristics of freshly isolated cells from hepatectomized rats. Next, the phenotypic characteristics of differentiated cells derived from isolated cells from PH rats were analyzed to confirm biochemical identity. Flow cytometry indicated that all the cells isolated from PH rats were positive for hepatic progenitor markers, CD-34 and Thy-1 (Fig.3). In 83.4% PH rats, a significant increase in

CD-34 and Thy-1 levels were observed compared with 73.1% PH rats.

In addition, when phenotypic characteristics were compared at 0, 7 and 14 days following 83.4% PH surgery, cells isolated 7 days following PH were observed to express CD-34 and Thy-1 at higher levels than cells at 0 and 14 days. In addition, the effect of various durations of IV collagenase perfusion on CD-34 and Thy-1 levels (Fig. 3) was analyzed. Perfusion of IV collagenase for 20 min resulted in a significant increase in CD-34 and Thy-1 levels, higher than that of 10 and 30 min collagenase perfusion.

Immunofluorescence analysis of proliferative oval cells. Hepatocyte differentiation was evaluated further by immunofluorescence analysis for AFP and CK-18 (markers of hepatocytes). Cells in the control group were negative for AFP and CK-18 (Fig. 4). By contrast, cells isolated from hepatectomized rats expressed AFP and CK-18, indicating that the cells had differentiated into hepatocyte-like cells. In the control group, expression of AFP and CK-18 was negative.

The effect of different PH procedures and duration of collagenase perfusion on differentiation was analyzed *in vitro*. As demonstrated in Fig. 4, immunofluorescence staining revealed that isolated cells on day 7 following 73.1 and 83.4% PH surgery expressed AFP and CK-18 at high levels. Expression was highest in cells isolated from 83.4% PH rats. Levels of AFP and CK-18 increased in cells isolated from PH rats perfused with collagenase for 20 min compared with 10 and 30 min perfusion. In addition, cells isolated 7 days following PH expressed AFP and CK-18 at higher levels than cells at 0 and 14 days (data not shown).

Analysis of AFP protein expression by western blot analysis. To further evaluate the phenotypic properties of differentiated cells, the expression of differentiation marker, AFP, was analyzed by western blot analysis in 83.4% PH rats. Differentiated cells were observed to express AFP protein at high levels (Fig. 5). These results further indicated that oval cells were specified towards a hepatocyte lineage and that fully differentiated cells possess the genetic characteristics of mature hepatocytes following the isolation of cells from PH rats. In addition, consistent with results of immunofluorescence, cells isolated from rats 7 days following PH with collagenase perfusion for 20 min revealed stronger ATP expression.

Discussion

In recent years, there have been a number of advances in liver stem cell biology and studies have hypothesized that fetal liver stem cells (hepatoblasts) and adult liver stem cells (oval cells) may be useful for the generation of primary hepatocytes. These hepatic progenitors have the potential to give rise to hepatocytes or cholangiocytes (20-22). However, it has also been demonstrated that the number of hepatic progenitor cells in tissues are low, leading to difficulties in isolation, purification and expansion for large-scale expansion (23).

A large number of growth factors regulate cell proliferation and differentiation. Oh *et al* (24) previously reported that HGF promotes hepatic stem cell differentiation into hepatic cells *in vitro*. Fiegel *et al* (25) reported that CD34⁺ bone

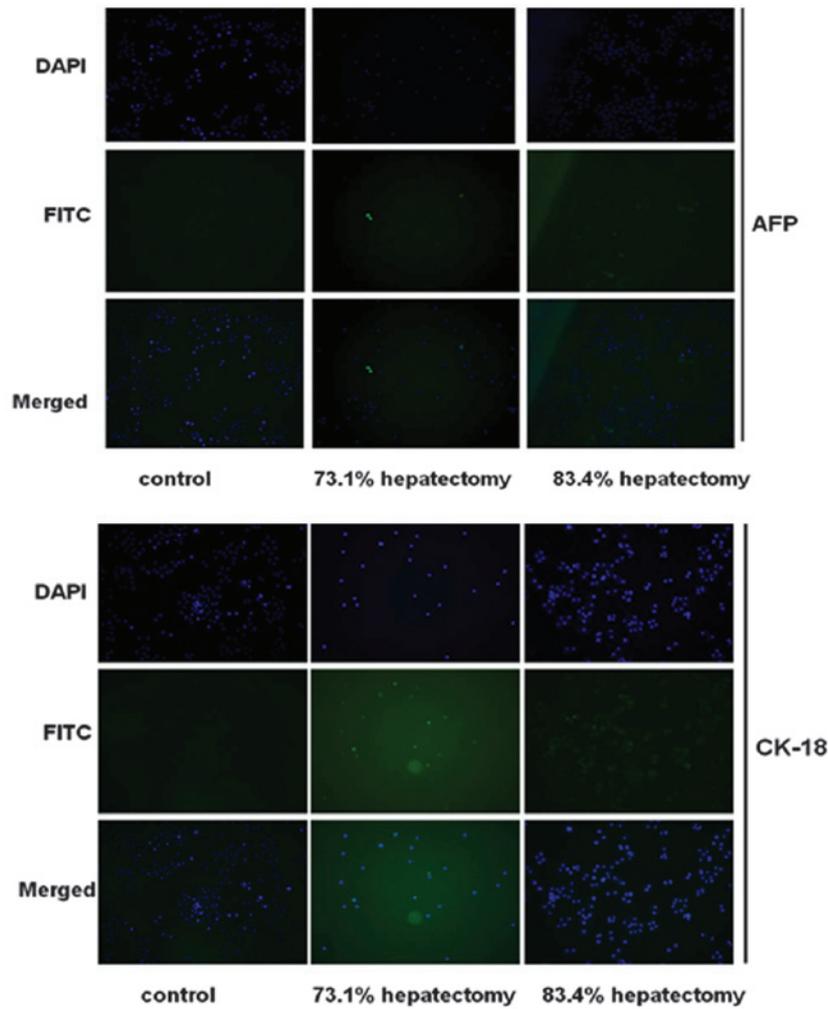


Figure 4. Characterization of the phenotypes of rat oval cells stained by immunofluorescence analysis. Immunofluorescence revealed the expression of AFP and CK-18 in oval cells following culture for 7 days. AFP, α -1-fetoprotein; CK-18, cytokeratin 18.

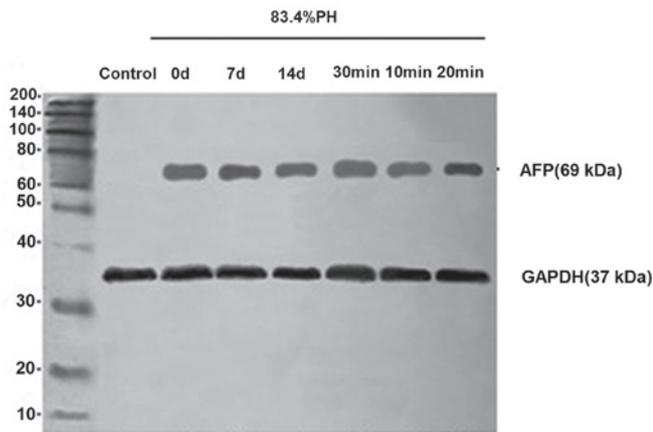


Figure 5. Western blot analysis of AFP protein expression. Blots presented are representative of typical results. GAPDH was applied to determine equal loading. AFP, α -1-fetoprotein.

marrow hematopoietic stem cells express specific hepatic cell markers, including AFP and CK-18, when HGF is added to the culture medium. Additional studies demonstrated that stem cells originating from the liver are able to be differentiated

into hepatocyte-like cells under HGF and epidermal growth factor (EGF) induction (25,26). To date, HGF, FGF, EGF and transforming growth factor have been extensively applied in stem cell studies worldwide (28,29).

Jang *et al* (30) found that damaged hepatic cells excrete specific factors when co-cultured with purified hematopoietic stem cells, inducing stem cells to differentiate into hepatocyte-like cells expressing hepatoid cells markers, including CK-18 and hepatocyte nuclear factor-3 β (27). The ability of a number of factors to induce multipotent adult progenitor cell (MAPC) differentiation into functional hepatocyte-like cells was analyzed and optimal differentiation was observed when cells were cultured with FGF-4 and HGF. Human, mouse and rat MAPCs cultured with FGF-4 and HGF differentiated into epithelioid cells expressing hepatic cell markers, acquired functional characteristics of hepatocytes and maintained the accordant metabolic activity of humans (27). Ruhnke *et al* (31) reported that peripheral blood monocytes cultured with M-CSF and IL-3 expressed the hematopoietic stem cell marker, CD90, and monocyte marker, CD14, and then differentiated into hepatocyte-like (NeoHap) cells following induction by FGF-4 and HGF. The NeoHep cells resembled primary human hepatocytes with regard to morphology, expression of hepatocyte

markers, various secretory and metabolic functions and drug detoxification activities. Following transplantation of NeoHep cells into the liver of severe combined immunodeficiency disease/non-obese diabetic mice, neohepatocytes integrated well into the liver tissue and revealed a morphology and albumin expression level similar to that of primary human hepatocytes transplanted under identical conditions (32).

In this context, to optimize hepatocyte formation, we first induced hepatic stem cell proliferation by PH, and the liver was removed 0, 7 and 14 days following PH. To obtain viable cells from the liver, two-step collagenase perfusion was performed. The first step consists of an isotonic buffer to flush blood cells and platelets from the vasculature. The second perfusate contained collagenase, which digests the extracellular matrix, allowing for the collection of a single cell suspension suitable for the isolation of discrete cell populations. IV collagenase was perfused for 10, 20 or 30 min.

Next, 5 $\mu\text{g/l}$ M-CSF, 0.4 $\mu\text{g/l}$ IL-3 and 140 $\mu\text{mol/l}$ β -mercaptoethanol was added to RPMI-1640 medium for 6 days, followed by the addition of 20 $\mu\text{g/l}$ HGF and 10 $\mu\text{g/l}$ FGF-4. Cell morphology was analyzed and proteins levels were determined by immunohistochemistry and western blot analysis. The results revealed that putative hepatic stem cells (oval cells) differentiate *in vitro* into cells that are morphologically, phenotypically and functionally representative of hepatocytes via a 2-step induction protocol. HGF and FGF-4 are likely to initiate a stable hepatic phenotype and are key to oval cell specification toward hepatocytes, as indicated by the observation that these substances induced oval cells to change into small hepatocytes with hepatic characteristics. These results further confirmed that HGF and FGF-4 are important for hepatocyte differentiation.

Hematopoietic markers, including CD34 and Thy-1, although restricted to hematopoietic stem cells, have been used in a number of previous studies to identify and isolate hepatic progenitors (33,34). The results of our study demonstrated that rat-derived stem cells differentiate *in vitro* into an endodermal cell type with hepatocyte phenotype. Hepatectomy is necessary to trigger CD34⁺ stem cells. The CD34 and Thy-1 contents of 83.4% PH cells were higher than that of 73.1% PH cells. However, in the control group, cells were negative for CD34 and Thy-1, indicating that the difference of percentage in PH had an effect on the stem cell quantity. By contrast, differences in the isolation times following surgery were found to be associated with stem cell generation. At day 0, no expression of CD34 and Thy-1 was observed, as the sorted cells were immature. Over time, the expression of CD34 and Thy-1 increased as the cells underwent maturation, with higher expression of CD34 and Thy-1 observed at day 7. However, on day 14, CD34 and Thy-1 expression began to decrease.

The expression of CD34 and Thy-1 was also analyzed under various durations of collagenase perfusion. Following 10 min perfusion, cells exhibited a polygonal or circle appearance and were negative for the hepatic stem cell markers, CD34 and Thy-1. By contrast, after 20 min perfusion, cells exhibited a stretched fibroblast-like appearance and expressed hepatic markers. However, the expression of CD34 and Thy-1 decreased following perfusion for 30 min. These results indicated that the optimal conditions for generation of hepatic cells following PH include 83.4% PH in rats followed by cell

isolation at 7 days using perfusion for 20 min. Long-term collagenase perfusion may inhibit stem cell formation.

Following cell culture in medium containing HGF and FGF-4, cells were characterized by the presence of hepatic markers, including AFP and CK-18. The expression of AFP and CK-18 was highest in cells isolated under perfusion for 20 min at day 7 following PH, indicating that the optimal differentiation of hepatic stem cells to CK-18- and AFP-positive cells is observed in stem cells isolated from 83.4% PH rats (7 days following surgery) perfused with IV collagenase for 20 min.

In conclusion, results of the current study indicate that cells from PH were able to proliferate and differentiate into cells of the hepatic lineage. Differences in the isolation times following surgery and duration of perfusion were observed to affect stem cell generation and differentiation. The establishment of an *in vitro* protocol for the optimization of hepatic stem cell culture as described in this study is likely to be useful for the development of *in vitro* techniques for liver tissue or organ culture.

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