J. Biochem. 2009;145(1)51–58
doi:10.1093/jb/mvn146

Establishment and Characterization of Hepatic Stem-like Cell lines from Normal Adult Rat Liver

Mitsuhi Hirata, Kumiko Amano, Ayumi Miyashita, Mayu Yasunaga, Tomoko Nakanishi and Kenzo Sato*

Division of Molecular Biology, School of Life Science, Tottori University Faculty of Medicine, 86 Nishicho, Yonago 683-8503, Japan

Received September 4, 2008; accepted October 16, 2008; published online October 30, 2008

The liver is a unique organ with the potential to regenerate from injury. Hepatic stem cells contribute to liver regeneration when surviving hepatocytes in injured liver are unable to proliferate. To investigate the mechanism of liver regeneration *in vitro*, we established hepatic stem cell lines named HY1, HY2 and HY3, derived from a healthy liver of adult rat. HY cells showed an expression pattern similar to oval cells, and efficiently induced hepatic differentiation following sequential treatment with type I collagen, transforming growth factor-β1 (TGF-β1), and hepatocyte growth factor (HGF) or oncostatin M (OSM). These results suggested that HY cells are liver stem cells representing an excellent tool for *in vitro* studies on liver regeneration.

Key words: hepatic stem cell, hepatic differentiation, type I collagen, TGF-β1, HGF.

Abbreviations: TGF-β1, transforming growth factor-β1; HGF, hepatocyte growth factor; OSM, oncostatin M; ECM, extracellular matrix.

The liver has an extremely strong ability to regenerate from injury; even if two-thirds of the liver is surgically lost, the survived cells rapidly proliferate to restore the liver mass. In addition, some kinds of stem cells are also known to contribute to liver regeneration when the proliferation of mature hepatocytes is inhibited in injured liver (*1–3*). Among these stem cells, oval cells are shown to participate in the regeneration (*2*, *3*), and to differentiate into hepatocytic or biliary lineages both *in vivo* and *in vitro* (*3–9*). It has been suggested that oval cells are derived from the canals of Hering (*1–3*). However, a recent report has indicated that bone marrow is a potential source of oval cells (*10*, *11*) that can differentiate into hepatocytes (*12–15*). Other several candidate hepatic stem cells have been reported (*16–26*). Small hepatocytes (SHs) derived from the healthy livers of adult and foetal rats can differentiate into hepatocytes and ductal cells, and are thought to be progenitor cells with hepatic characteristics (*22*). However, it remains unclear what cells are involved in such regeneration in injured liver. The potential of these stem cells to differentiate into hepatocytes has been shown by *in vitro* and *in vivo* studies; however, their induction into hepatocytes has been performed by various growth factors and an extracellular matrix (ECM) proteins cocktail (*22–26*). As it is not known which factors are involved in hepatic differentiation, it is essential for an understanding of liver regeneration to examine the roles of these differentiation factors.

To activate the oval cells in adult rat liver, all experimental models to date have used such as toxins or carcinogens. Isolation and culture of hepatic stem cells, including oval cells, were performed by use of the Percoll density gradient technique, several growth factors and a feeder layer. To investigate the mechanism of liver regeneration *in vitro*, we attempted to develop a novel method for the isolation of liver stem cells that would meet the following requirements: a simple isolation method, maintenance of proliferative activity without any growth factors and maintenance of differentiated traits over the long-term. We established oval-like stem cell lines derived from the healthy livers of adult rats, named HY1, HY2 and HY3. These cells retain their morphology, gene expression patterns and proliferative capacity over a long period of time. In this study, we analysed the differentiation of HY cells into hepatocytes following treatment with various growth factors, demonstrating the importance of hepatic regeneration.

MATERIALS AND METHODS

Establishment of Stem-like Cell Lines: HY1, HY2 and HY3—Liver cells were purified from an 8-week-old male Wistar rat (Crea Japan, Tokyo, Japan) weighing 160 g by the two-step liver perfusion method of Seglen with slight modification (*27*). Briefly, a collagenase-digested liver cell suspension was centrifuged at 50*g* for 1 min. The pellet was resuspended in minimum essential medium and centrifuged at 50*g* for 1 min. After this procedure was repeated four times, the supernatant was centrifuged at 150*g* for 5 min. The pellet was resuspended in MEM and centrifuged at 150*g* for 5 min. After this procedure was repeated, the pellet was resuspended in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% foetal calf serum (FCS), 150 U/ml penicillin, and 100 mg/ml streptomycin. Then, 2×10^5 cells/ml were incubated on culture dishes (10-cm diameter) and cultured at 37°C in a 5% CO_2/95% air incubator. Three days after seeding, three propagating colonies were isolated and named HY1, HY2

*To whom correspondence should be addressed. Tel: 81-859-38-6201, Fax: +81-859-38-6200, E-mail: kensato@med.tottori-u.ac.jp

and HY3 cells. These cells were constantly grown and subcultured at 80% confluence. Cells were passaged every 3–4 days at a splitting ratio of 1:4 in DMEM with 10% FCS.

Cell Lines—The rat myofibroblast cell line MFBY2 was isolated in our previous study (*28*). The rat hepatoma cell line Reuber cells were given by Dr M. Nanba, Okayama University. The rat hepatoma cell line C2 was obtained from Dr Mary C. Weiss, Institute Pasteur, France (*29–31*). These cell lines were maintained in DMEM with 10% FCS at 37°C in a 5% CO_2/95% air incubator.

RNA Extraction and Reverse Transcription–polymerase Chain Reaction (RT–PCR)—Total RNA was extracted from cell lines and foetal rat liver as described previously (*32*). One microgram of total RNA was converted to complementary DNA and amplified using a GeneAmp RNA PCR kit (Applied Biosystems Inc. Foster City, CA). The number of cycles and annealing temperature were optimized for each primer pair. The following forward and reverse primers were used for specific amplification; α-fetoprotein (AFP): GATGGAGTGCCTACAGGATG and TTAAACCCCCAAAGCCTCAC, albumin (ALB): GACCC CAGTGAGCGAGAAGG and CAGTGGCGAAGCAGTT ATCC, Tyrosine amino transferase (TAT): ATGATGCTG GAGGCTTGTAG and AGGGGTGGGGAGTGGTGACG, Sca-1: TCTGTGCACCCCTTCTCTGA, and GGTCTCC AGGAGGACTGAGC, c-kit: CATCATGGAAGATGACG AGC and CAAATGTGTACACGCAGCTG, Cytokeratin19 (CK19): GTCCTACAGATCGACAATGC and CACACCCT GGATGTGTGACAG, α1-antitrypsin (α1-AT): CTGGGGA GCAAGGGTGACAC and GGGCTCTGAGTTTCTGATTC, neural cell adhesion molecule (NCAM): AGAACATCCCT CCCAGCC and GCGTTGTAGATGGTCAGGGT, proliferating cell nuclear antigen (PCNA): GTTGATAAAGA AGAGGAAGC and CAGTATTTGTAAGGCAGAGA, Glyceraldehydes-3-phosphate dehydrogenase (GAPDH) an internal control: AGGCTGTGGGCAAGGTCAT and CACCACCCTGTTGCTGTAGC. PCR products were separated by electrophoresis through 5% polyacrylamide gels and stained with ethidium bromide. Reuber and C2 cells were used as controls for differentiated and undifferentiated hepatocytes, respectively. Foetal liver was used as immature liver.

Immunofluorescence Analysis—Cell cultures were fixed with methanol at −20°C for 15 min, and washed in 0.05% Tween 20-PBS (0.05% T-PBS) three times, followed by blocking with normal goat serum for 1 h at room temperature (RT). The cells were incubated with mouse anti-rat CK19 monoclonal antibody (Chemicon International, Temecula, CA, USA) for 1 h at RT, washed in 0.05% T-PBS at three times, incubated with Alexa Fluor-488-conjugated goat anti-mouse for 30 min at RT, washed in 0.05% T-PBS three times, and mounted. All antibodies were diluted in 0.05% T-PBS. Labelled cells were observed with a fluorescence microscope.

Treatment of HY1 Cells with Various Growth Factor—HY1 cells (1×10^5) were cultured in a 60-mm dishes coated with type I collagen (IWAKI Glass Co, Tokyo, Japan) or noncoated dishes for 24 h; subsequently these cells were treated with various growth factors, including human hepatocyte growth factor (HGF; R&D Systems Inc, Minneapolis, MN, USA), human basic fibroblast growth factor (b-FGF; PeproTech Inc. Rocky Hill, NJ, USA), mouse epidermal growth factor (EGF; Takara, Kyoto, Japan), heparin-binding EGF-like growth factor (HB-EGF; R&D Systems Inc), human transforming growth factor β1 (TGF-β1; R&D Systems Inc), mouse oncostatin M (OSM; R&D Systems Inc).

RESULTS

Establishment of the Stem-likes Cell Lines: HY1, HY2 and HY3—To investigate the mechanism of liver regeneration *in vitro*, we attempted to isolate and establish stem cells derived from the healthy liver of an adult rat by collagenase perfusion and differential centrifugation. Three days after seeding of isolated cells, several colonies composed of small-sized cells appeared. Ten days later, these colonies consisted of small and uniformly shaped cells composed of more than 1,000 cells. Most of the cells in the colony showed a small cobblestone appearance and had ovoid nuclei and a high nucleus/cytoplasm ratio following long-term culture (Fig. 1A). Isolated clones of HY1, HY2 and HY3 cells have a high capacity to proliferate for a long time. The population doubling times of these cells in culture medium containing 10% FCS were about 48 h (Fig. 1B). This doubling time was similar to those of WB-344 cells and RLE cells, well-documented liver epithelial cell lines (*16–21*). Proliferation was halted by confluence of cultures, and confluent cultures were maintained for 1 week without piling up of cells, indicating contact inhibition.

To identify the characteristics of these cells, we next examined the expression of various differentiation markers for liver cell by RT–PCR analysis (Fig. 1C). HY1, HY2 and HY3 cells expressed the hematopoietic stem cell marker c-kit, but not CD34. Furthermore, these cells expressed the cholangiocytic marker CK19, but not the hepatocyte makers ALB, TAT, α1-AT and AFP. The liver epithelial cell lines, WB-344 cells and RLE cells, showed a cobblestone appearance and had the potential to proliferate and differentiate into hepatocytes. However, these cell lines were positive for AFP and ALB and negative for CK19, while HY1, HY2 and HY3 cells were negative for AFP and ALB and positive for CK19, implying that HY1, HY2 and HY3 cells differ from liver epithelial cell lines. Moreover, the small hepatocytes isolated from nonparenchymal cells of normal adult liver had potential to differentiate into hepatocytes (*22*); however, the proliferation of small hepatocytes is low (*22*). The small hepatocytes expressed ALB but not CK19, indicating that the small hepatocytes are precursors of mature hepatocytes rather than hepatic stem cells; therefore, HY1, HY2 and HY3 cells also differ from small hepatocytes.

Oval cells are known to participate in the regeneration process after the administration of several chemical agents. The cells were characterized by ovoid nuclei and a high/cytoplasm ratio, and had the potential to differentiate into hepatocytic or biliary lineage cells (*3–9*). Oval cells characteristically co-expressed AFP and CK19, as well as c-kit. NCAM (*33–35*) and Sca-1 (*36*) have been reported as a specific maker for ductular cells and oval cells, although stellate cells also express NCAM. Interestingly, HY1, HY2 and HY3 cells expressed c-kit and

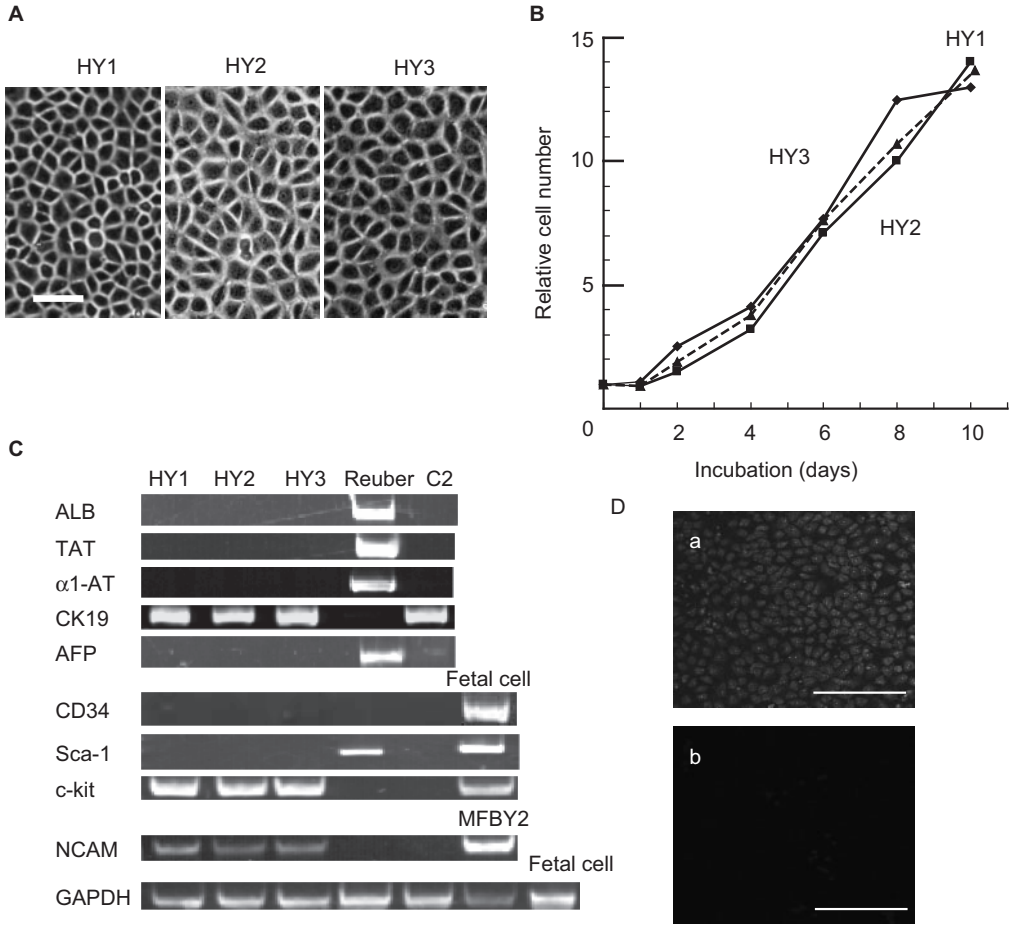

Fig. 1. **Isolation and characterization of hepatic stem cell lines.** (A) Phase-contrast microphotographs of HY1 cell colony cultured for 10 days. Scale bar shows 0.1 mm. (B) Growth curve of HY cells. (C) Expression of hepatic and stem cell markers in HY cells were determined by RT–PCR. Reuber, C2, MFBY2 and fetal cells were used as controls. (D) Immunofluorescence staining of CK19 of HY1 (a) and Reuber cells (b). Scale bar shows 0.5 mm.

NCAM, but not Sca-1. To further evaluate the phenotypic properties of these cells, we also carried out immunofluorescent staining for CK19, demonstrated to be an oval cell marker. HY cells were positive for CK19 (Fig. 1D). These results indicated that HY1, HY2 and HY3 cells possess similar phenotypic properties to oval cells, although the expression profiles of HY cells were not completely consistent with those of oval cells (Table 1). Since the expression of various markers for hepatic stem cells was identical in three lines of HY cells, we used HY1 as a representative of HY cell lines hereafter.

Differentiation of HY Cells into Hepatocytes—To examine the ability of HY cells to differentiate into hepatocytes *in vitro*, representative HY1 cells were treated with HGF, EGF, HB-EGF, b-FGF and TGF-β1, which have been demonstrated to be important growth factors for hepatic regeneration (*37–39*). As a result, HY1 cells treated with these growth factors on normal dishes did not express differentiation markers, such as ALB, TAT and AFP, but maintained the expression of CK19 (premature hepatocyte marker) and c-kit (stem cell marker) (Fig. 2). Interestingly, HY1 cells cultured on type I collagen-coated dishes showed AFP, CK19 and c-kit expression, but not ALB and TAT expression. Moreover, HY1 cells treated with TGF-β1 on type I collagen-coated dishes showed expression of ALB, and showed decreased CK19 expression, whereas HY1 cells treated with other growth factors could not induce ALB and TAT expression (Fig. 2).

We next examined the time course of the effects of TGF-β1 treatment of HY1 cells (Fig. 3). Induction of the differentiation markers AFP, ALB and TAT was not observed in TGF-β1-treated HY1 cells cultured on normal dishes. However, when HY1 cells were treated with TGF-β1 on type I collagen-coated dishes, the expression of ALB was increased by 48 h, and TAT was induced at low levels at 72 h. On the other hand, CK19 levels were decreased within 24 h, and disappeared at 48 h. These results indicated that HY1 cells have the potential to differentiate into a hepatic lineage and that the cooperation of TGF-β1 and type I collagen is important to induce the differentiation of HY1 cells *in vitro*.

To examine the growth activity of HY1 cells treated with these factors, expression of proliferating cell nuclear antigen (PCNA), related to the cell cycle, and DNA replication, were examined. PCNA was strongly expressed in all cells treated with or without these factors and type I collagen. These results suggest that these growth

Table 1. **Phenotypic characteristics of liver cell, liver epithelial cell lines and oval cell lines.**

Marker	HY1, 2, 3	Hepatocyte (*37*)	Cholangiocyte (*37*)	Oval cell (*37*)	SH (*22*)	WB-F344 (*19*,*21*)	RLE (*43*)	MFBY2 (*28*)
ALB	−	+	−	+	+	±	±	−
TAT	−	+	−	−	+	±		−
CK19	+	−	+	+	−	−	−	−
AFP	−	−	−	+	±	±	±	+
c-Kit	+	−	−	+	−		+	−
CD34	−	−	−	±				−
NCAM	+	−	−	±				+

SH, small hepatocyte, WB-F344; RLE, Normal rat liver stem cell line; MFBY2, MFB-like cells; −, not detectable; +, expression; ±, not detectable or expression.

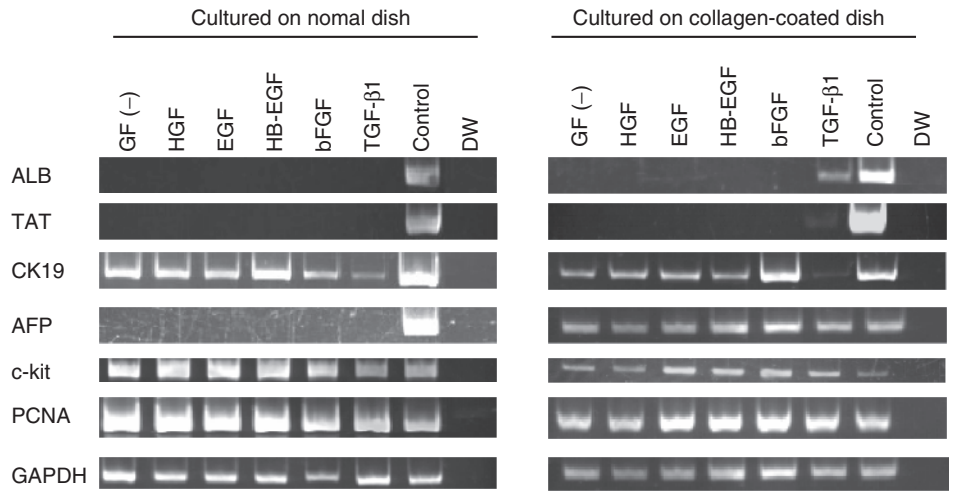

Fig. 2. **Effect of growth factors on expression of hepatic differentiation markers.** HY1 cells were grown on normal and type I collagen-coated dishes, and treated with various growth factors for 3 days. Expressions of hepatic differentiation markers were determined by RT-PCR. Controls: Reuber cells were used as positive controls for ALB, TAT, PCNA, GAPDH expression and C2 cells were used for CK19, and fetal liver was used for AFP and c-kit. DW (distilled water) was used as negative control of PCR.

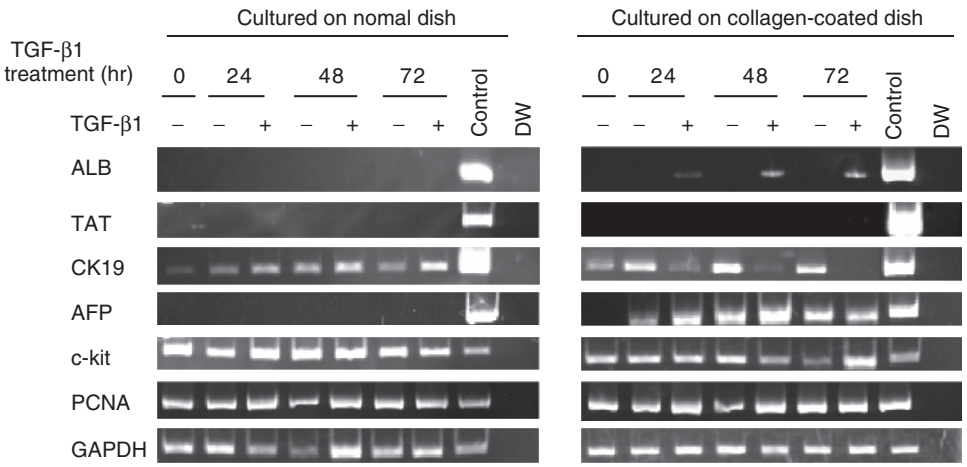

Fig. 3. **Time-course of expression of hepatic differentiation maker in HY1 cells treated with TGF-β1 on normal or type I collagen-coated dishes.** HY1 cells were grown on normal and type I collagen-coated dishes, and treated with TGF-β1 for indicating time. Expressions of hepatic differentiation markers were determined by RT–PCR. Controls were used same as Fig. 2.

factors and type I collagen did not affect the cell cycle progression of HY1 cells.

Effects of HGF or OSM on the Hepatic Differentiation of HY1 Cells Pretreated with TGF-β1—Hepatic differentiation is known to proceed stepwise during hepatogenesis in embryos responded to programmed gene expression and various cytokines from environmental tissues. To confirm this stepwise differentiation of hepatoblast cells *in vitro*, we examined the effects of HGF or OSM to further promote the maturation of HY1 cells pretreated with TGF-β1 (Fig. 4). HGF and OSM have been well documented to be essential for

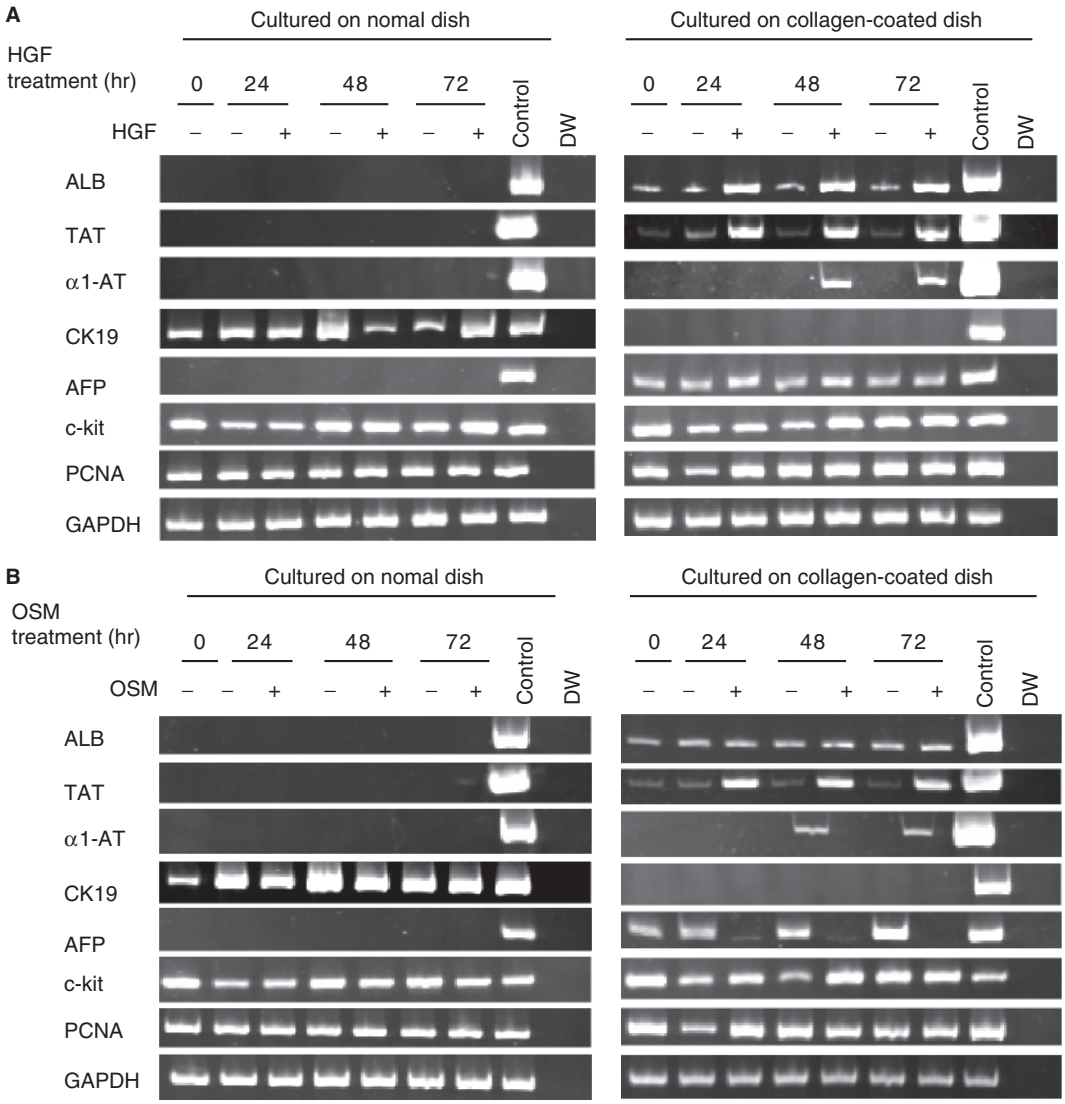

Fig. 4. **Effects of HGF or OSM on the expression of hepatic differentiation makers in HY1 cells pretreated with TGF-β1 on normal and collagen-coated dishes.** HY1 cells were grown on normal and type I collagen-coated dishes, and treated with TGF-β1 for 72 h, followed by treatment with HGF (A) or OSM (B). Expressions of hepatic differentiation markers were determined by RT–PCR. Controls were used same as Fig. 2, and Reuber cell was used as positive control for α1-AT expression.

development and regeneration (*8*, *37–39*). HY1 cells treated with TGF-β1 for 72 h were subjected to HGF and OSM for various periods. As a result, HGF strongly induced expression of ALB 24 h after treatment. By contrast OSM showed a small effect on the expression of ALB. Both HGF and OSM induced TAT- and α1-AT expression at high levels. Interestingly, OSM strongly suppressed expression of AFP by treatment for 72 h. These results indicated that HGF and OSM are involved in the hepatic maturation of HY1 cells pretreated with TGF-β1 on collagen-coated dishes, while HGF or OSM did not induce these hepatic differentiation markers without pretreatment with TGF-β1.

Further, to elucidate the importance of type I collagen on the hepatic differentiation of HY1 cells by sequential treatment with TGF-β1 and HGF or OSM, HY1 cells were sequentially treated with these cytokines on normal dishes (Fig. 4). Expression of hepatocyte markers including ALB, TAT and α1-AT was not observed to be induced by stepwise treatment with these cytokines. These results indicated that type I collagen is essential for differentiation of HY1 cells into hepatocytes.

DISCUSSION

In this study, to establish a liver regeneration model *in vitro*, we isolated hepatic stem cell lines designated HY1, HY2 and HY3 cells derived from the healthy liver of an adult rat. Consequently, the gene expression profiles of HY cells are highly similar to that of oval cells with minute differences. However, oval cells were activated by treatment with toxins and carcinogens, or in fetuses, whereas HY cells were resident in the healthy liver of an adult rat. Thus, HY cells are presumed to be

precursor cells or undifferentiated oval cells that differentiate into oval cells following stimulation induced during regeneration.

Several reports have demonstrated that bone marrow cells have trans-differentiating potential into hepatocytes in *in vitro* and *in vivo* studies (*10–15*). These results have suggested that bone marrow cells are candidate hepatic stem cells, including oval cells and small hepatocytes. In terms of the origin of HY cells, circulating cells, including blood cells, were almost excluded by liver perfusion and several centrifugation steps during cell isolation; therefore, HY cells originally inhabit the healthy livers of adult rats, and express the ductular marker CK19, suggesting that HY cells are not derivatives from bone marrow. Based on the observations that oval cells are derived from the canals of Hering (*1–3*), the HY cells were assumed to originate from the Hering canal.

To examine the possibility of HY cells differentiating into hepatocytes, HY1 cells were exposed to several growth factors, including HGF, EGF, HB-EGF, b-FGF and TGF-β1, all of which could be involved in liver hepatic regeneration. In this culture system, type I collagen was able to induce AFP expression in HY1 cells. In addition to collagen, TGF-β1 effectively induced ALB, a marker of hepatocytes, at an earlier differentiation stage, and decreased levels of the ductular marker CK19, suggesting progression of differentiation into hepatocytes. Moreover, administration of HGF or OSM to TGF-β1-treated HY1 cells on collagen effectively induced TAT and α1-AT, markers of hepatocytes at a terminal differentiation stage.

Regarding the factor promoting the differentiation of liver cells, type I collagen has been reported to be constitutively expressed during the course of oval cell-aided liver regeneration (*40*). TGF-β1 has been documented to be involved in termination of liver regeneration, acting as a growth inhibitor and pro-apoptotic factor. Also, it has been shown that TGF-β1 produced by smooth muscle actin (SMA)-positive hepatic stellate cells (HSCs) increased the proliferation of oval cells (*40*, *41*). Nagy *et al*. (*42*) showed that treatment of RLE cells (oval cell line) in monolayer cultures with TGF-β1 induced the expression of ALB mRNA. Together with the increase in oval cell number observed after *in vivo* HGF infusion, this suggests that HGF is involved in the expansion of oval cells following liver injury (*43–45*). It has been reported that HSC proliferation is closely associated with oval cell proliferation and observed around the oval cells (*44*). HSC is well documented to produce various extracellular matrix proteins and growth factors including type I collagen, TGF-β1 and HGF during liver injury and regeneration; while Kupffer cells has been reported to secrete OSM that involved in hepatocyte maturation for liver regeneration (*46–49*). Thus, these results suggested that HSCs and Kupffer cells may assist the differentiation of HY1 cells into hepatocytes and mimic the hepatic regeneration environment *in vitro*. However, the molecular mechanism by which type I collagen, TGF-β1 and HGF or OSM cooperate sequentially, or by which the differentiation of HY1 cells into hepatocytes is induced, remains unclear and needs to be further investigated. Moreover, although the experimental data are not shown, HY cells that expressed CK19 and NCAM seem to have a potential to differentiate into biliary cell (*50*). Therefore, HY cells are likely to be bipotent stem cell with differentiation into hepatocyte and bile ductular cell.

In this study, a new stem cell line, HY cells, was established and induced to differentiate into hepatocytes by the sequential cooperation of type I collagen, TGF-β1 and HGF or OSM. Therefore, if the HY cells are pre-oval cell lines, HY1, HY2 and HY3 cells may be useful as a model system with which to explore the cellular and molecular events in liver regeneration; furthermore, these cells may be useful in cell therapies for liver diseases.

ACKNOWLEDGEMENTS

We are grateful to Dr Naohiro Hori for valuable discussion.

FUNDING

KAKENHI (20590282) from JSPS.

CONFLICT OF INTEREST

None declared.

REFERENCES

1. Fausto N. (1994) Liver stem cells. (Arias, I.M., Boyer, J.L., Fausto, N., Jakoby, W.B., Schachter, D.A., and Shafritz, D.A., eds.) *The Liver: Biology and Pathobiology*, 3rd edn, pp. 1501–1515, Raven press, New York.
2. Thorgeirsson, S.S. (1996) Hepatic stem cells in liver regeneration. *FASEB J.* **10**, 1249–1256
3. Grisham, J.W. and Thorgeirsson, S.S. (1997) *Liver Stem Cells* (Potten, C.S., ed.) pp. 233–238, Academic Press Ltd, New York
4. Lazaro, C.A., Rhim, J.A., Yamada, Y., and Fausto, N. (1998) Generation of hepatocytes from oval cell precursors in culture. *Cancer Res.* **58**, 5514–5522
5. Pack, R., Heck, R., Dienes, H.P., Oesch, F., and Steinberg, P. (1993) Isolation, biochemical characterization, long-term culture, and phenotype modulation of oval cells from carcinogen-fed rats. *Exp. Cell Res.* **204**, 198–209
6. Steinberg, P., Steinbrecher, R., Radaeva, S., Schirmacher, P., Dienes, H.P., Oesch, F., and Bannasch, P. (1994) Oval cell lines OC/CDE 6 and OC/CDE 22 give rise to cholangiocarcinomas and undifferentiated carcinomas after transformation. *Lab. Invest.* **71**, 700–709
7. Radeva, V. and Steinberg, P. (1995) Phenotype and differentiation patterns of the oval cell lines OC/CDE 6 and OC/CDE 22 derived from the livers of carcinogen-treated rats. *Cancer Res.* **55**, 1028–1038
8. He, Z.P., Tan, W.Q., Tang, Y.F., and Feng, M.F. (2003) Differentiation of putative hepatic stem cells derived from adult rats into mature hepatocytes in the presence of epidermal growth factor and hepatocyte growth factor. *Differentiation* **71**, 281–290
9. He, Z.P., Tan, W.Q., Tang, Y.F., Zhang, H.J., and Feng, M.F. (2004) Activation, isolation, identification and in vitro proliferation of oval cells from adult rat livers. *Cell Prolif.* **37**, 177–187
10. Petersen, B.E., Bowen, W.C., Patrene, K.D., Mars, W.M., Sullivan, A.K., Murase, N., Boggs, S.S., Greenberger, J.S., and Go, J.P. (1999) Bone marrow as a potential source of hepatic oval cells. *Science* **284**, 1168–1170
11. Lagasse, V., Connors, H., Al-Dhalimy, M., Reitsma, M., Dohse, M., Osborne, L., Wang, X., Finegold, M.,

Weissman, I.L., and Grompe, M. (2000) Purified hematopoietic stem cells can differentiate into hepatocytes in vivo. *Nat. Med.* **6**, 1229–1234
12. Miyazaki, M., Akiyama, I., Sakaguchi, M., Nakashima, E., Okada, M., Kataoka, K., and Huh, N. (2002) Improved conditions to induce hepatocytes from rat bone marrow cells in culture. *Biochem. Biophys. Res. Commun.* **298**, 24–30
13. Okumoto, K., Saito, T., Hattori, E., Ito, J., Adachi, T., Takeda, T., Sugahara, K., Watanabe, H., Saito, K., Togashi, H., and Kawata, S. (2003) Differentiation of bone morrow cells into cells that express liver-specific genes in vitro; implication of Notch signals in differentiation. *Biochem. Biophys. Res. Commun.* **304**, 691–695
14. Saji, Y., Tamura, S., Yoshida, Y., Kiso, S., Iizuka, A. S., Matsumoto, H., Kawasaki, T., Kamada, Y., Matsuzawa, Y., and Shinomura, Y. (2004) Basic fibroblast growth factor promotes the trans-differentiation of mouse bone morrow cells into hepatic lineage cells via multiple liver-enriched transcription factors. *J. Hepatol.* **41**, 545–550
15. Thorgeirsson, S.S. and Grisham, J.W. (2006) Hematopoietic cells as hepatocyte stem cells: critical review of the evidence. *Hepatology* **43**, 2–8
16. Tsao, M.S., Smith, J.D., Nelson, K.G., and Grisham, J.W. (1984) A diploid epithelial cell line from normal adult rat liver with phenotypic properties of 'oval' cells. *Exp. Cell Res.* **154**, 38–52
17. McMahon, J.B., Richards, W.L., del Campo, A.A., Song, M.K., and Thorgeirsson, S.S. (1986) Differential effects of transforming growth factor-beta on proliferation of normal and malignant rat liver epithelial cells in culture. *Cancer Res.* **46**, 4665–4671
18. Tsao, M.S. and Liu, C. (1988) Inhibition of growth of early passage normal rat liver epithelial cell lines by epidermal growth factor. *Lab. Invest.* **58**, 636–642
19. Coleman, W.B., Smith, G.J., and Grisham, J.W. (1994) Development of dexamethasone-inducible tyrosine aminotransferase activity in WB-F344 rat liver epithelial stem-like cells cultured in the presence of sodium butyrate. *J. Cell Physiol.* **161**, 463–469
20. Coleman, W.B., McCullough, K.D., Esch, G.L., Faris, R.A., Hixson, D.C., Smith, G.J., and Grisham, J.W. (1997) Evaluation of the differentiation potential of WB-F344 rat liver epithelial stem-like cells in vivo. Differentiation to hepatocytes after transplantation into dipeptidylpeptidase IV-defcient rat liver. *Am. J. Pathol.* **151**, 353–359
21. Couchie, D., Holic, N., Chobert, M.-N., Corlu, A., and Laperche, Y. (2002) In vitro differentiation of WB-F344 rat liver epithelial cells into the biliary lineage. *Differentiation* **69**, 209–215
22. Mitaka, T., Sato, F., Mizuguchi, T., Yokono, T., and Mochizuki, Y. (1999) Reconstruction of hepatic organoid by rat small hepatocytes and hepatic nonparenchymal cells. *Hepatology* **29**, 111–125
23. Kano, J., Noguchi, M., Kodama, M., and Tokiwa, T. (2000) The in vitro differentiating capacity of nonparenchymal epithelial cells derived from adult porcine livers. *Am. J. Pathol.* **156**, 2033–2043
24. Nagai, H., Terada, K., Watanabe, G., Ueno, Y., Aiba, N., Shibuya, T., Kawagoe, M., Kameda, T., Sato, T., Senoo, H., and Sugiyama, T. (2002) Differentiation of liver epithelial (stem-like) cells into induced by coculture with hepatic stellate cells. *Biochem. Biophys. Res. Commun.* **293**, 1420–1425
25. Okumura, K., Nakamura, K., Hisatomi, Y., Nagano, Y., Tanaka, Y., Terada, K., Sugiyama, T., Umeyama, K., Matsumoto, K., Yamamoto, T., and Endo, F. (2003) Salivary gland progenitor cells induced by duct ligation differentiate into hepatic and pancreatic lineages. *Hepatology* **38**, 104–113
26. Li, W.L., Su, J., Yao, Y.C., Tao, X.R., Yan, Y.B., Yu, H.Y., Wang, X.M., Li, J.X., Yang, Y.J., Lau, J.T., and Hu, Y.P. (2006) Isolation and characterization of bipotent liver progenitor cells from adult mouse. *Stem Cells* **24**, 322–332
27. Selgen, P.O. (1973) Preparation of rat liver cells. 3 Enzymatic requirements for tissue dispersion. *Exp. Cell Res.* **82**, 391–398
28. Isono, M., Soda, M., Inoue, A., Akiyoshi, H., and Sato, K. (2003) Reverse transformation of hepatic myofibroblast-like cells by TGF-beta1/LAP. *Biochem. Biophys. Res. Commun.* **311**, 959–965
29. Deschatrette, J. and Weiss, M.C. (1974) Characterization of differentiated and dedifferentiated clones from a rat hepatoma. *Biochemie.* **56**, 1603–1611
30. Deschatrette, J., Moore, E.E., Dubois, M., and Weiss, M.C. (1980) Dedifferentiated variants of a rat hepatoma: reversion analysis. *Cell* **19**, 1043–1051
31. Clayton, D.F., Weiss, M., and Darnell, J.E. Jr. (1985) Liver-specific RNA metabolism in hepatoma cells: variations in transcription rates and mRNA levels. *Mol. Cell Biol.* **5**, 2633–2641
32. Chomzynski, P. and Sacchi, N. (1987) Single-step method of RNA isolation by acid guanidinium thiocynate-phenolchloroform extraction. *Anal. Biochem.* **162**, 156–159
33. Roskams, T., De, Vos R., Van Eyken, P., Myazaki, H., Van Damme, B., and Desmet, V. (1998) Hepatic OV-6 expression in human liver disease and rat experiments: evidence for hepatic progenitor cells in man. *J. Hepatol.* **29**, 455–463
34. Roskams, T., van den Oord, J.J., De Vos, R., and Desmet, V.J. (1990) Neuroendocrine features of reactive bile ductules in cholestatic liver disease. *Am. J. Pathol.* **137**, 1019–1025
35. Van Den Heuvel, M.C., Slooff, MJ., Visser, L., Muller, M., De Jong, K.P., Poppema, S., and Gouw, A.S. (2001) Expression of anti-OV6 antibody and anti-NCAM antibody along the biliary line of normal and diseased human livers. *Hepatology* **33**, 1387–1393
36. Wright, N., Samuelson, L., Walkup, M.H., Chandrasekaran, P., and Gerber, D.A. (2008) Enrichment of a bipotent hepatic progenitor cell from naive adult liver tissue. *Biochem. Biophys. Res. Commun.* **366**, 367–372
37. Lowes, K.N., Croager, E.J., Olynyk, J.K., Abraham, L.J., and Yeoh, G.C. (2003) Oval cell-mediated liver regeneration: Role of cytokines and growth factors. *J. Gastroenterol. Hepatol.* **18**, 4–12
38. Suzuki, A., Iwama, A., Miyashita, H., Nakauchi, H., and Taniguchi, H. (2003) Role for growth factors and extracellular matrix in controlling differentiation of prospectively isolated hepatic stem cells. *Development* **130**, 2513–2524
39. Kawasaki, T., Tamura, S., Kiso, S., Doi, Y., Yoshida, Y., Kamada, Y., Saeki, A., Saji, Y., and Matsuzawa, Y. (2005) Effects of growth factors on the growth and differentiation of mouse fetal liver epithelial cells in primary cultures. *J. Gastroenterol. Hepatol.* **20**, 857–864
40. Pi, L., Oh, S.H., Shupe, T., and Petersen, B.E. (2005) Role of connective tissue growth factor in oval cell response during liver regeneration after 2-AAF/PHx in rats. *Gastroenterology* **128**, 2077–2088
41. Park, D.Y. and Suh, K.S. (1999) Transforming growth factor-β1 protein, proliferation and apoptosis of oval cells in acetylaminofluorene-induced rat liver regeneration. *J. Korean Med. Sci.* **14**, 531–538
42. Nagy, P., Evarts, R.P., McMahon, J.B., and Thorgeirsson, S.S. (1989) Role of TGF-beta in normal differentiation and oncogenesis in rat liver. *Mol. Carcinog.* **2**, 345–354
43. Nagy, P., Bisgaard, H.C., Santoni-Rugiu, E., and Thorgeirsson, S.S. (1996) In vivo infusion of growth factors enhances the mitogenic response of rat hepatic ductal (oval) cells after administration of 2-acetylaminofluorene. *Hepatology* **23**, 71–79

44. Imai, T., Masui, T., Nakanishi, H., Inada, K., Kobayashi, K., Nakamura, T., and Tatematsu, M. (1996) Expression of hepatocyte growth factor and c-met mRNAs during rat chemically induced hepatocarcinogenesis. *Carcinogenesis* **17**, 19–24
45. Okano, J., Shiota, G., Matsumoto, K., Yasui, S., Kurimasa, A., Hisatome, I., Steinberg, P., and Murawaki, Y. (2003) Hepatocyte growth factor exerts a proliferative effect on oval cells through the PI3K/AKT signaling pathway. *Biochem. Biophys. Res. Commun.* **309**, 298–304
46. Kinoshita, T., Sekiguchi, T., Xu, M., Ito, Y., Kamiya, A., Tsuji, K., Nakahata, T., and Miyajima, A. (1999) Hepatic differentiation induced by oncostatin M attenuates fetal liver hematopoiesis. *Proc. Natl Acad. Sci. USA* **96**, 7265–7270
47. Kamiya, A. and Gonzalez, F.J. (2004) TNF-alpha regulates mouse fetal hepatic maturation induced by oncostatin M and extracellular matrices. *Hepatology* **40**, 527–536
48. Okaya, A., Kitanaka, J., Kitanaka, N., Satake, M., Kim, Y., Terada, K., Sugiyama, T., Takemura, M., Fujimoto, J., Terada, N., Miyajima, A., and Tsujimura, T. (2005) Oncostatin M inhibits proliferation of rat oval cells, OC15-5, inducing differentiation into hepatocytes. *Am. J. Pathol.* **166**, 709–719
49. Nakamura, K., Nonaka, H., Saito, H., Tanaka, M., and Miyajima, A. (2004) Hepatocyte proliferation and tissue remodeling is impaired after liver injury in oncostatin M receptor knockout mice. *Hepatology* **39**, 635–644
50. Zhou, H., Rogler, L.E., Teperman, L., Morgan, G., and Rogler, C.E. (2008) Identification of hepatocytic and bile ductular cell lineages and candidate stem cells in bipolar ductular reactions in cirrhotic human liver. *Hepatology* **45**, 716–724