Multiplicative mononuclear small hepatocytes in adult rat liver: their isolation as a homogenous population and localization to periportal zone

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Abstract

Adult rat liver contains a minor population of hepatocytes called small hepatocytes (SHs) that are smaller in size and show a higher replicative potential than conventional parenchymal hepatocytes (PHs). However, SHs have been hitherto characterized using a “SH-fraction” that was contaminated with PHs. In the present study, we isolated a PH-free SH-fraction from the adult rat liver using fluorescence-activated cell sorter combined with centrifugal elutriation and characterized the hepatocytes in the fraction. These hepatocytes were designated R3Hs in this study. R3Hs were mononuclear and of lower ploidy. They expressed at high levels genes of Cdc2, connexin 26, hydroxysteroid sulfotransferase, pancreatic secretory trypsin inhibitor, and prostaglandin E2 receptor EP3 subtype. We conclude that SHs dominate the periportal zone in the adult liver, because mRNA or proteins of these genes were exclusively expressed by periportal hepatocytes.

Key words: cyp2c22; cyp7a1; ploidy; liver progenitor cells; cDNA microarray; FACS sorting; hepatic zonation

Introduction

When adult rat hepatocytes are cultured, colonies consisting of replicating small hepatocytes (SHs) are frequently observed [1-3]. After collagenase perfusion, SHs are enriched in the nonparenchymal cell fraction by their size using a repeated centrifugation technique [2]. Cultured SHs show a high proliferative ability, and express hepatocyte markers such as albumin and transferrin as parenchymal hepatocytes (PHs). Since several SHs start to express biliary epithelial cell markers in long-term culture, the SH population might contain a bi-potential progenitor-like cell in normal adult rat liver [2]. SHs were also observed in cultured human hepatocytes [4].

SHs were isolated from adult rat liver using a repeated centrifugation technique as above. However, these SHs were still heterogeneous in both size and cell species, because we showed that the isolated SHs contained not only "true" SHs, but also PHs and other nonparenchymal cells [5]. We previously analyzed the heterogeneous SHs with a fluorescence-activated cell sorter (FACS), which showed that such SHs consist of the two major populations designated SH-R3 and SH-R2 [5]. In the present study, we rename the previous SH-R3 and SH-R2 as R3 hepatocytes (R3Hs) and R2 hepatocytes (R2Hs), respectively. Size and the in vitro proliferative potential of R3Hs were smaller and higher than those of R2Hs, respectively [5]. The size of R3Hs was homogenous as compared to the original SHs and did not contain large hepatocytes. R2Hs showed size and gene/protein expression profiles similar to PHs. Transplantation study revealed that the growth potential of R3Hs was 3 times higher than that of R2Hs [6]. Thus, R3Hs separated

from the heterogeneous SH population are regarded as “true SHs” which have a high proliferative ability both in vitro and in vivo.

FACS sorting allowed us to isolate a small amount of the true SHs (R3Hs) from the heterogeneous SH fraction [5, 6]. However, the lack of an appropriate method to prepare enough R3Hs for analytical purposes has hampered quantitative characterization of R3Hs at the gene/protein level [2]. In the present study, we succeeded in the isolation of a large amount of R3Hs from adult rat liver. We were able to characterize R3Hs using fresh preparations in terms of the expression of specific genes and the intra-lobular distributions. R3Hs expressed many hepatocyte differentiation- and proliferation-related genes, but not oval cell-related genes. From the gene expression profile, we concluded that SHs dominate the periportal zone in the adult liver.

Materials and methods

Preparation of Hepatocytes. Liver cells were isolated from 10-week-old Fischer male rats (SLC Inc., Japan) with the two-step collagenase perfusion method [7]. PHs and SHs were separated by low-speed centrifugation as previously described [2]. In the present study, we purified a large amount of R3Hs as illustrated in Fig. 1. Liver cells collected from liver by the collagenase perfusion were 3 times pelleted by centrifugation at 50 g for 2 min, placed on 45% Percoll, and centrifuged at 50 g for 24 min [6]. The pellets containing both SHs and PHs were suspended in DMEM containing 1% FBS and was fractionated by centrifugal elutriation using a CR21 high-speed refrigerated centrifuge (Hitachi, Tokyo,

Japan) with a R5E rotor and a chamber (4.7 ml). Cells (1-4.5 x 10^7) were suspended in 10-20 ml DMEM containing 1% FBS, poured into the chamber spinning at 1,000 rpm at 4 ˚C at a flow rate of 14 ml/min, and fractions at flow rates from 16 to 44 ml/min in 4-ml/min gradients were collected. The fractions were analyzed with FACS VantageTM (Becton Dickinson, Mountain View, CA) with a 100-μm nozzle following the previously reported criteria [5]. Fluorescence excited at 488 nm was measured through a 530-nm filter (FL1) with a 4-decade logarithmic amplification. To measure physical characteristics of cells, a 4-decade logarithmic amplification was used for the side scatter (SSC), which is a measure of cytoplasmic complexity. Viable cells were distinguished by a propidium iodide exclusion test. We sorted R3Hs and R2Hs from the fractions of “16 to 28 ml/min” and “40 to 44 ml/min” by FACS, respectively (Fig. 1), and obtained R3Hs and R2Hs. The mean diameter of the fractionated cells was determined as reported [5]. The experimental protocol was approved by the Animal Research Committee of the Hiroshima Prefectural Institute of Industrial Science and Technology.

Ploidy Measurement. Hepatocytes were fixed in 70% ethanol, washed with PBS, treated with 100 μg/ml RNase A, and incubated with 50 μg/ml propidium iodide [8]. Ploidy of these hepatocytes was determined with FACS using LYSIS II 2.0 software (Becton Dickinson). Statistical significance of differences between 2 samples was calculated with Student’s *t*-test. *P* value of less than 0.05 was considered significant.

cDNA Microarray. cDNA microarray analysis was carried out on R3Hs and R2Hs using Atlas Rat 1.2 Array membranes on which 1,176 genes are spotted (Clontech, Palo Alto, CA). cDNA labeling, hybridization, and analysis of the membrane were carried out according to the manufacturer's protocol. The intensity of the background hybridization signal was subtracted from the measured intensity for each of genes that were preferentially expressed in R2Hs ("R2H-genes" whose precise definition is described in Results section), and the subtracted value was used as the intensity of hybridization for R2H-gene. The measured intensity of each "R3H-gene", a gene that was preferentially expressed in R3Hs, was subtracted by the background intensity as in R2H-genes. The subtracted value was normalized by being multiplied with the global normalization coefficient (1.45), the ratio of the sum of the subtracted values of all R3H-genes to that of all R2H-genes. The normalized value was used as the intensity of hybridization for R3H-gene.

cDNA representational difference analysis (RDA). We collected fractions of R3Hs and R2Hs from 12 and 15 rats, respectively, as above. mRNA was purified from each of them by a mRNA separation kit (Clontech). cDNAs were synthesized from each of poly A^+ RNA (0.48 μg) of R3Hs and R2Hs using a cDNA synthesis kit (Life Technologies, Rockville, MD) and were used as testers and drivers for the cDNA RDA, respectively [9]. The subtractive hybridization was repeated 3 times.

Quantification of mRNA. cDNAs were synthesized by PowerScript reverse transcriptase (Clontech) and were amplified with a set of gene specific primers and SYBR Green PCR mix in a PRISM 7700 Sequence Detector (Applied Biosystems, Tokyo, Japan). PCR primers used in real-time RT-PCR were listed in Table 1. A series of diluted plasmid cDNAs containing each gene was used to make the standard amplification curves [10]. The mRNA copy numbers in cDNA samples were calculated using the standard amplification curves.

Immunohistochemistry. Cryosections (5 μm thick) were prepared from adult male rat liver, fixed by acetone at 4^{o}C for 10 min, and hydrated by PBS. After blocking with 1.5 % normal goat sera for 15 min at room temperature, sections were incubated for 1 hr with the 200-fold diluted antibodies against rabbit anti-connexin 26 antibodies (a gift from Dr. Enomoto, Akita University). The sections were washed 3 times with PBS for 5 min and incubated again for 30 min with 200-fold diluted FITC-labeled anti-rabbit IgG (Vector laboratories, CA).

In Situ Hybridization. cDNAs were amplified from adult rat liver RNA by RT-PCR using primers listed in Table 1. The PCR products were subcloned into pGEM-T vectors (Promega, Tokyo, Japan). Digoxigenin-labeled cRNA probes were synthesized with a DIG RNA labeling kit (Roche Molecular Biochemicals, Tokyo, Japan). Adult male rat livers were perfused with 4% paraformaldehyde in PBS and excised. Cryosections (5 μm)

were rehydrated and digested with 1 μg/ml proteinase K in PBS at 37 °C for 4 min. Hybridization and wash procedures were described elsewhere [10].

Results

Isolation of R3Hs and R2Hs from adult rat liver

Hepatocytes were obtained from the adult liver by collagenase perfusion method, pelleted, and centrifuged on 45% Percoll as outlined in Fig. 1. The obtained pellets containing both SHs and PHs were sequentially separated by centrifugal elutriation into 7 fractions, A (16-20 ml/min), B (20-24), C (24-28), D (28-32), E (32-36), F (36-40), and G (40-44). The mean cell diameter of hepatocytes in fractions A to G was 17.2, 17.8, 18.8, 20.7, 21.5, 22.5, and 23.2 μm, respectively, indicating that hepatocytes were fractionated according to size-difference. Each of these 7 fractions was analyzed with FACS using parameters of SSC, a measure of cytoplasmic complexity, and FL1 that measured autofluorescence of cells (Fig. 2). Previously we identified R3Hs as hepatocytes with lower FL1 and SSC [5]. Such R3Hs were mainly seen in fractions from 16 to 28 ml/minutes (Fig. 2A-C, rectangles). The three fractions, fraction A-C, were collected, mixed together, and separated into R2Hs and R3Hs again by FACS as above. The separated R3Hs were used as the purified R3Hs. Its purity was 99.3%, which was obtained by FACS analysis (Fig. 2H). Similarly, “large-sized hepatocytes” (R2Hs) were isolated as a reference cell. R2Hs were previously defined as hepatocytes with higher FL1 and SSC [5]. Hepatocytes with higher FL1 and SSC were mainly seen in the fractions from 24-44 ml/minutes (Fig.

2C-G). R2Hs were sorted from fraction G with FACS and used as the purified R2Hs. Its purify was 98.2% (Fig. 2I). These purified R3Hs and R2Hs were used for further analysis.

Characterization of R3Hs

We measured the cell diameter, nuclearity, and ploidy of the purified R3Hs and R2Hs. These parameters were also determined for the original hepatocytes isolated from liver (the hepatocytes before fractionation with centrifugal elutriation shown in Fig. 1), PHs prepared as previously [2], and SHs prepared as previously [2]. Original hepatocytes had the mean diameter of 23.0 ± 0.9 μm (Table 2). Approximately 26% of them was binuclear, and most (approximately 86%) were of binuclear diploid (2 x 2n) and tetraploid (4n). The size (20.2 ± 1.3 μm) of SHs was smaller than that (24.7 ± 1.5 μm) of PHs. The rate of the binucleation and the ploidy (2 x 2n, 4n, 2 x 4n, and 8n) of SHs was 12.3 ± 5.2 and 76.3 ± 2.7%, respectively, which was lower than those of PHs, 25.1 ± 2.5% and 95.1 ± 2.8%, respectively. The size (23.2 ± 0.5 μm) of R2Hs was similar to that of PHs. The ploidy rate of R2Hs was 96.8 ± 9.7%, which was similar to that of PHs. In contrast, R3Hs had the smallest diameter (17.4 ± 0.0 μm) and the lowest ploidy (17.6 ± 13.6%) among these hepatocyte preparations. No binucleated hepatocytes were seen in the R3Hs. These data show that R3Hs in the adult rat liver are small-sized mononuclear hepatocytes with lower ploidy.

cDNA microarray analysis and real-time RT-PCR of R3Hs and R2Hs

To characterize R3Hs at the gene expression level, we identified differentially expressed genes between R3Hs and R2Hs utilizing cDNA microarray method. Among 1,176 spotted genes on the microarray, 690 and 591 genes were hybridized with cDNA probes prepared from R3Hs and R2Hs, respectively. From 690 genes we selected 24 genes as R3H-genes (genes that are preferentially expressed in R3Hs) that meet the following two criteria: (1) the intensity of a gene in R3Hs is more than 10; (2) the ratio of the intensity of a gene in R3Hs to that in R2Hs is more than 2. Similarly, from the 591 genes, we selected 38 genes as R2H-genes. These R3H- and R2H-genes are listed in Table 3. We selected five genes from each of these R3H- and R2H-genes, that are apparently involved in liver functions, and made real-time RT-PCR on the selected genes to examine whether the genes are actually expressed in R3Hs or R2Hs as expected: pancreatic secretory trypsin inhibitor (Psti), prostaglandin E2 receptor EP3 subtype (Pge2r), hydroxysteroid sulfotransferase (Sta), cytochrome P450 (Cyp) 17, and p55cdc from R3H-genes and Cyp7a1, 1a2, 2e1, 3a1, and 2c22 from R2H-genes. As a result, all these genes were found to meet the requirement for the R3H- (the expression ratio in R3Hs to that in R2Hs > 2) or R2H-genes (the expression ratio in R2Hs to that in R3Hs > 2) (Table 4).

Identification of R3H-Genes by cDNA RDA

To further screen R3H-genes that might not be covered on the microarray, we also performed cDNA RDA using mRNAs of R3Hs as testers and those of R2Hs as drivers. We cloned 91 genes as candidates for R3H-genes, and determined mRNA expression of

61 genes in R3Hs and R2Hs by real-time RT-PCR. As a result, we could select 5 genes whose expression was more than 3-fold higher in R3Hs than in R2Hs, mitotic centromere-associated kinesin (Mcak, 9.70-fold), Cdc2 (7.16-fold), connexin 26 (Cx26, 5.32-fold), unknown gene (GenBank Accession number: AB088476, 4.93-fold), and EST207254 (3.70-fold) (Table 5). Genes of albumin and transferrin were expressed in R3Hs at 2.47-and 2.26-fold higher than in R2Hs (data not shown).

Lobular localization of R3Hs

Little has been known where R3Hs are in the liver lobules [2, 3, 5, 6]. The R3H- and R2H-genes identified in this study are considered to be useful molecular markers to obtain insights into histological distributions of R3Hs and R2Hs, respectively. From the above identified genes we selected Cx26, Psti, and Sta for R3H-markers, and Cyp7a1 and Cyp2c22 for R2H-markers, because these genes/proteins have been well characterized. The expression profiles of these genes/proteins were examined on adult liver histological sections (Fig. 3). Cx26 was more abundantly located on the membrane of periportal (PP) hepatocytes (PP-hepatocytes) than that of perivenous (PV) hepatocytes (PV-hepatocytes) (Fig. 3A). In situ hybridization revealed that Psti mRNAs were expressed in PP-hepatocytes, but not in PV-hepatocytes (Fig. 3B). No signals were detected in hepatocytes using sense Psti probes (data not shown). Expression of Sta mRNAs was also restricted to PP-hepatocytes (Fig. 3C). In contrast, Cyp7a1 and Cyp2c22 were preferentially expressed in PV-hepatocytes (Fig. 3D, E). These results strongly suggest

that R3Hs and R2Hs dominate the PP- and PV-zone of liver lobules, respectively.

Discussion

In the present study we isolated a R2H-free pure population of R3Hs from adult rat liver by a method consisting of centrifugal elutriation and FACS sorting. Its purity was as high as 99.3%. This method enabled us a large amount of R3Hs at once for various analytical examinations. We determined cytological parameters of fresh preparations of R3Hs and compared them with those of other preparations of hepatocytes. R3Hs had a smaller diameter (17.4 μm), no binucleation, and lower ploidy (diploid, 82.4%). Size (23.2 μm) of R2Hs was similar to that of PHs. It is known that the growth potential of hepatocytes decreases with the binucleation and as ploidy increases [11]. Indeed, R2Hs showed a very high ploidy, the rate of polyploidy being 96.8%, which was similar to that of PHs. Thus, our present and previous [5] data indicate that R3Hs in adult rat liver are proliferative and small-sized mononuclear hepatocytes with lower ploidy.

Utilizing R2Hs as a reference cell, we attempted to comprehensively identify preferentially expressed genes in R3Hs by cDNA microarray analysis and cDNA RDA. Cx26 mRNA was expressed 5.3-fold higher in R3Hs than in R2Hs. PP-hepatocytes express Cx26 at a high level as previously reported [12, 13]. In situ hybridization revealed that Psti mRNA was exclusively expressed in PP-hepatocytes. In rats, two different Psti genes, Psti-I and Psti-II, were reported [14]. The sequence similarity between Psti-I and Psti-II mRNAs is 91%. Although the primers for RT-PCR and probes for in situ

hybridization used in this study hybridize both Psti genes, hepatocytes are known to express only the Psti-II gene [14]. Thus, the level of Psti mRNA expression measured in this study represents that of Psti-II. We identified Sta as a R3H-gene. Its expression was restricted to PP-hepatocytes as previously shown by immunohistochemistry [15]. These results strongly suggest that R3Hs dominate the PP-regions of liver lobules. Our conclusion supports a report that smaller hepatocytes show a less ploidy and a high mitogenicity, and are localized in PP-zones of the adult rat liver [16].

We found that R3Hs express many hepatocyte-related genes such as albumin and transferrin. No expression of c-kit, a marker for oval cells [17], was detected in both R3Hs and R2Hs on our microarray analysis and real-time RT-PCR (data not shown). Thus, we currently consider that the phenotype of R3Hs is largely similar to that of adult liver hepatocytes. However, the expression of many CYP enzymes including Cyp1a2, 2c22, 2e1, 3a1, and 7a1 is significantly low in R3Hs, indicating that R3Hs are not fully differentiated. We propose that R3Hs differentiate further and become matured hepatocytes, i.e. R2Hs.

It was reported that small hepatocyte like progenitor cells (SHPCs) appear in regenerating liver after partial hepatectomy in rats treated with retrorsine [18]. An experiment using genetically labeled hepatocytes indicated that mature hepatocytes are the source of SHPCs in the injured liver [19]. SHPCs express albumin and transferrin [18]. They are featured by the low expression of Cyp2e1 and 3a1, and the high expression of α-fetoprotein and WT1. In this study, we found that the expression of mRNAs of CYP

enzymes including Cyp1a2, 2c22, 2e1, 3a1, and 7a1 was lower in R3Hs than in R2Hs. However, the expression of mRNAs of α-fetoprotein and WT1 was equally low in both R3Hs and R2Hs (data not shown). Thus, R3Hs are different population from SHPCs. The expression of mRNAs of many CYP enzymes was low in R3Hs, implying that R3Hs may be a source of SHPCs. Annexin III has recently been identified as a marker for SHs by proteome analysis [20]. However, we failed to detect its mRNA in both R3Hs and R2Hs by cDNA microarray analysis and real-time RT-PCR (data not shown), strongly suggesting that annexin III-expressing cells are a different population from R3Hs.

Hepatocytes around PP-zone (zone 1) enter in S phase between 12 and 18 hr after partial hepatectomy, and subsequently hepatocytes in other regions of the parenchyma enter in S phase [21]. Studies on cell kinetics revealed that PP-hepatocytes have a shorter G1 phase than PV-hepatocytes and some hepatocytes in PP-zones cycle 2 or 3 times after partial hepatectomy [22]. Cdc2 mRNA is up-regulated in S, G2, and M phases in the regenerating liver [23]. We showed that Cdc2 gene was 7.16-fold higher in R3Hs than in R2Hs. We consider that R3Hs are the cells that were previously identified as highly proliferative hepatocytes around PP-zone and the hepatocytes that were reported to first respond to the stimuli of partial hepatectomy [21, 22].

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Figure legends

Fig. 1. A flow chart of the isolation of R3Hs and R2Hs. Hepatocytes were isolated from adult rat liver by the collagenase perfusion method, centrifuged, and purified on Percoll isodensity medium. The purified fraction (original hepatocytes) that contained SHs and PHs was sequentially separated by centrifugal elutriation into 7 fractions, A (16-20 ml/min), B (20-24), C (24-28), D (28-32), E (32-36), F (36-40), and G (40-44). The mean diameter of cells in each of these fractions was determined. Each of the fractions was analyzed with FACS using parameters of SSC and FL1, which revealed that these fractions contained R2Hs and R3Hs with different ratios as shown in Fig. 2. The fractions of A-C (16 to 28 ml/min) and G (40 to 44 ml/min) were collected, sorted with FACS. The R3Hs and R2Hs thus obtained were used as the purified R3Hs and R2Hs for further analyses in this study, respectively. The purity of the purified R3Hs and R2Hs was determined by FACS as shown in Fig. 2H and I, respectively.

Fig. 2. FACS analysis of hepatocytes fractionated by centrifugal elutriation. Hepatocytes were isolated from adult rat liver and were sequentially separated by centrifugal elutriation into 7 fractions, A (16-20 ml/min), B (20-24), C (24-28), D (28-32), E (32-36), F (36-40), and G (40-44). Each of these fractions was subjected to FACS analysis. R3Hs and R2Hs were identified as hepatocytes with a lower and higher FL1 and SSC, respectively, and are indicated by rectangles in which the percentage of R3Hs or R2Hs is also shown. The centrifugal elutriates from 16 to 28 ml/min fractions (A-C) were collected and sorted with FACS as R3Hs. Similarly, elutriates of 40 to 44 ml/min (G) were sorted as R2Hs. These R3Hs and R2Hs were subjected to further FACS analysis, which is shown in H and I, respectively. The FACS profiles show that these R3Hs and R2Hs were nearly pure.

Fig. 3. Localization of R3H- or R2H-gene-expressing hepatocytes in the adult liver. A. Immunohistochemistry of Cx26. Cx26 is seen on the membrane of hepatocytes as green spots, which are more abundant in PP hepatocytes than in PV hepatocytes. c, central vein; p, portal vein. B-E. In situ hybridization of mRNAs of Psti (B), Sta (C), Cyp7a1 (D), and Cyp2c22 (E). Psti mRNAs are observed as blue in PP-hepatocytes, but not in PV-hepatocytes (B). Sta mRNAs are observed in PP-hepatocytes, but not in PV-hepatocytes (C). mRNAs of Cyp7a1 (D) and Cyp2c22 (E) are observed in PV-hepatocytes, but not in PP-hepatocytes. Bars, 50 μm.

Table 1.

Oligonucleotide primers used in real-time RT-PCR and in situ hybridization

Genes	**Forward primers (5′–3′)**	**Reverse primers (5′–3′)**
Real-time RT-PCR		
Cdc2	CACGTCAAGAACCTGGATGAAA	GAGATTCGTTTGGCTGGATCATAG
Cx26	GGAGATGAGCAAGCCGATTTT	TAGTGGTCGTGGCACACATCCT
Cyp1a2	GATGAGAAGCAGTGGAAAGACC	GGCCGTGTTGTCATTGGTAA
Cyp2c22	TGCTCAGTAAAGTCAGCCAAGG	GCCGTGTTTGTTTCCATATTTC
Cyp2e1	TGACTTTGGCCGACCTGTT	TGAGGATCAGGAGCCCATATCT
Cyp3a1	TTCACCGTGATCCACAGCA	TGCTGCCCTTGTTCTCCTT
Cyp7a1	GAGCCAAGTCAAGTGTCCCC	AGTGGTGGCAAAATTCCCA
Cyp17	TTTCTGATCGACCCTTTCAAAG	AGCCTGTGCATCCATCCAT
Mcak	TCCCCTCCCCAGAAAACTT	CGCCCTGTACTTGACATTTACC
Psti	CACAGTTCTTCTGAGTTTTGGACC	GCTGGAGGGTTACCTGCTAAA
p55cdc	TCGGAAGGCTTGCAGATACAT	AGTCATTCCGGATTTCAGGG
Pge2r	AAGGAGAAGGAGTGCAATTCCT	TCCAAGATCTGGTTCAGCGA
Rat EST 207254	TGGACTGTGCAGCAAATGG	CAGAGGTTTCTCCTTTGGCA
Sta	ACGAACTGGCTGATTGAAATTG	GATGGTCACAGATTGGATCCA
Unknown (AB088476)	AAAAGCCACGCCCACAG	TCCGCCATCTGAGGTCGG
In situ hybridization		
Cyp2c22	ATCAGCACCATGTCAGAAGGA	CAAAACGGTGCTGGAAAATG
Cyp7a1	GCTTTCCAGTGCATCCTTGA	GACTCTCAGCCGCCAAGTG
Psti	CCCTGCACAGTTCTTCTGAGTT	CAGCAAACCTTCTTGCTAAACA
Sta	ACGAACTGGCTGATTGAAATTG	CAAATCCAGCTCATCTGGCT

Table 2.

Characterization of hepatocytes in adult rats

	Mean diameter (μm)	Binucleated (%)	Ploidy (%)		
			2*n*[a]	2 × 2*n*,[b] 4*n*[a]	2 × 4*n*,[b] 8*n*[a]
Hepatocytes[c]	23.0 ± 0.9	26.4 ± 2.5	10.5 ± 1.3	85.6 ± 1.7	3.9 ± 1.0
PHs	24.7 ± 1.5	25.1 ± 2.5	4.8 ± 0.2	88.4 ± 1.3	6.7 ± 1.5
SHs	20.2 ± 1.3[d]	12.3 ± 5.2[d]	23.8 ± 1.2[d]	74.3 ± 1.7[d]	2.0 ± 1.0[d]
R2Hs	23.2 ± 0.5	40.5 ± 5.5	3.2 ± 0.3	91.2 ± 5.0	5.6 ± 4.7
R3Hs	17.4 ± 0.0[d]	0[d]	82.4 ± 13.7[d]	17.0 ± 13.2[d]	0.6 ± 0.4

Each value represents the mean ± SD of triplicate experiments.

[a] 2*n*, 4*n*, and 8*n* represent di-, tetra-, and octa-ploidy, respectively.
[b] 2 × 2*n* and 2 × 4*n* represent binuclear diploid and binuclear tetraploid cells, respectively.
[c] Original hepatocytes, hepatocytes before fractionation with centrifugal elutriation.
[d] $P < 0.05$ for PHs vs. SHs and R2Hs vs. R3Hs. *P* values were determined by Student's *t* test.

Table 3.

Gene expression profiles of R3Hs and R2Hs determined by cDNA microarray analysis

Accession No.	Genes	Intensity[a]		Ratio[b]
		R3H	R2H	
R3H-genes				
M27882	Psti	72	5	14.4
M21208	Cyp17	21	2	10.5
D14869	Pge2r	39	5	7.8
X63410	Sta	26	4	6.5

Accession No.	Genes	Intensity[a]		Ratio[b]
		R3H	R2H	
U05341	p55cdc	17	4	4.3
M60921	B-cell translocation gene 2	179	46	3.9
L19181	PTP-PS	86	23	3.7
L12380	ADP-ribosylation factor 1	14	4	3.5
M18335	Cyp2c7	2400	722	3.3
U11681	Rapamycin target protein	13	4	3.3
M20035	Prothymosin-⁎	34	13	2.6
U13253	Epidermal fatty acid-binding protein	307	131	2.3
U22424	11-β-Hydroxysteroid dehydrogenase 2	23	10	2.3
J04791	Ornithine decarboxylase	44	20	2.2
U17035	Interferon inducible protein 10	330	151	2.2
M91808	Sodium channel, β1 subunit	11	5	2.2
D63834	Monocarboxylate transporter	46	22	2.1
X17163	c-jun	585	286	2.0
M37828	Cyp4a8	110	54	2.0
S81448	Steroid 5-⁎-reductase 1	65	32	2.0
L26288	CaM-like protein kinase	36	18	2.0
M96601	Taurine transporter	30	15	2.0
X57523	Antigen peptide transporter 1	14	7	2.0
M35992	Intestinal fatty acid-binding protein	10	5	2.0
Accession No.	Genes	Intensity		Ratio[c]

Accession No.	Genes	Intensity[a]		Ratio[b]
		R3H	**R2H**	
		R2H	R3H	
R2H-genes				
J05509	Cyp7a1	42	1	42.0
X70871	Cyclin G	13	1	13.0
J02627	Cyp2e1	1497	185	8.1
U60085	Cyp3a9	116	15	7.7
M10161	Cyp3a1	2059	282	7.3
Y08172	2-Arylpropionyl-CoA epimerase	441	75	5.9
K02422	Cyp1a2	232	39	5.9
M27156	Probasin	10	2	5.0
D38222	Tyrosine phosphatase-like protein	10	2	5.0
U88036	Brain digoxin carrier protein	257	65	4.0
M22413	Carbonic anhydrase III	1237	376	3.3
D14014	Cyclin D1	23	7	3.3
J02592	Glutathione *S*-transferase Yb subunit	3201	1016	3.2
J05030	Short chain acyl-CoA dehydrogenase	15	5	3.0
M61219	Prohibitin	188	68	2.8
X78997	Cadherin 17	37	13	2.8
K01931	Glutathione *S*-transferase Y-b subunit	481	187	2.6
X54080	Cytochrome *c* oxidase, subunit VIIa	103	40	2.6
U23769	LIM domain protein CLP36	47	18	2.6
U72350	Bcl-x*	21	8	2.6

Accession No.	Genes	Intensity[a]		Ratio[b]
		R3H	R2H	
M38566	Cyp27	123	50	2.5
M38178	3-β-Hydroxysteroid dehydrogenase	112	44	2.5
L22294	Pyruvate dehydrogenase kinase	28	11	2.5
X57986	cAMP-dependent protein kinase	10	4	2.5
L19031	Organic anion transporter	233	98	2.4
U76379	Organic cation transporter 1A	86	36	2.4
U29701	GABA transaminase	25	11	2.3
M83298	Protein phosphatase 2A, regulatory subunit B	18	8	2.3
U65007	c-Met	40	18	2.2
M95738	β-Alanine-sensitive neuronal GABA transporter	24	11	2.2
U06069	Syntaxin-binding protein 1	11	5	2.2
M58041	Cyp2c22	990	466	2.1
U18293	Protein tyrosine phosphatase 2E1	42	20	2.1
J02657	Cyp2c11	7184	3654	2.0
M58587	IL-6R	102	52	2.0
M62388	17-kDa ubiquitin-conjugating enzyme E2	45	23	2.0
X17621	K^+ channel RCK2	10	5	2.0
X63675	Pim-1	20	10	2.0

Note. The list shows genes identified as preferentially expressed in R3Hs or R2Hs on the microarray.

[a] The intensity of hybridization for R3H- and R2H-genes.

[b] The ratio of the intensity of R3Hs to that of R2Hs.

[c] The ratio of the intensity of R2Hs to that of R3Hs.

Table 4.

Quantification of gene expression in R3Hs and R2Hs by real-time RT-PCR

Genes[a]	**mRNA copies/pg total RNA**[b]		**Ratio**[c]	
	R3Hs	**R2Hs**	**R3Hs/R2Hs**	**R2Hs/R3Hs**
R3H genes				
Psti	49.17 ± 28.21	6.87 ± 1.45	7.16	
Pge2r	4.79 ± 1.88	1.17 ± 0.51	4.08	
Sta	95.25 ± 56.65	29.22 ± 15.02	3.26	
Cyp17	0.29 ± 0.15	0.09 ± 0.01	3.06	
p55cdc	0.95 ± 0.18	0.35 ± 0.34	2.71	
R2H genes				
Cyp7a1	1.39 ± 0.39	15.72 ± 7.44		11.34
Cyp1a2	3.87 ± 0.44	15.07 ± 0.89		3.89
Cyp2e1	15.00 ± 0.77	58.11 ± 10.80		3.88
Cyp3a1	21.71 ± 5.00	64.15 ± 36.67		2.95
Cyp2c22	10.84 ± 3.75	28.15 ± 21.42		2.60

Note. Real-time RT-PCR was carried out 3 times for each gene. Each value represents the mean ± SD of six independent experiments.

[a] Differentially expressed genes in R3Hs and R2Hs identified by cDNA microarray analysis.
[b] Differentially expressed genes identified by the microarray were subjected to quantification by real-time RT-PCR to validate the difference observed by microarray.
[c] The ratio of mRNA expression between R3Hs and R2Hs.

Table 5.

Quantification of expression of R3H-genes in R3Hs and R2Hs by real-time RT-PCR

Genes[a]	**mRNA copies/pg total RNA**[b]		**Ratio**[c]
	R3Hs	**R2Hs**	**R3Hs/R2Hs**
Mcak	0.21 ± 0.09	0.02 ± 0.01	9.70
Cdc2	0.58 ± 0.08	0.08 ± 0.06	7.16
Cx26	5.42 ± 1.12	1.02 ± 0.23	5.32
Unknown (AB088476)	2.45 ± 1.12	0.50 ± 0.14	4.93
Rat EST 207254	0.47 ± 0.20	0.13 ± 0.04	3.70

Note. Real-time RT-PCR for R3Hs and R2Hs was carried out 3 times for each gene. Each value represents the mean ± SD of six independent experiments.

[a] Genes isolated from cDNA RDA of R3Hs against R2Hs.
[b] Differentially expressed genes identified by cDNA RDA were subjected to quantification by real-time RT-PCR to validate the difference observed by cDNA RDA.
[c] The ratio of mRNA expression in R3Hs to that in R2Hs.

Fig.1

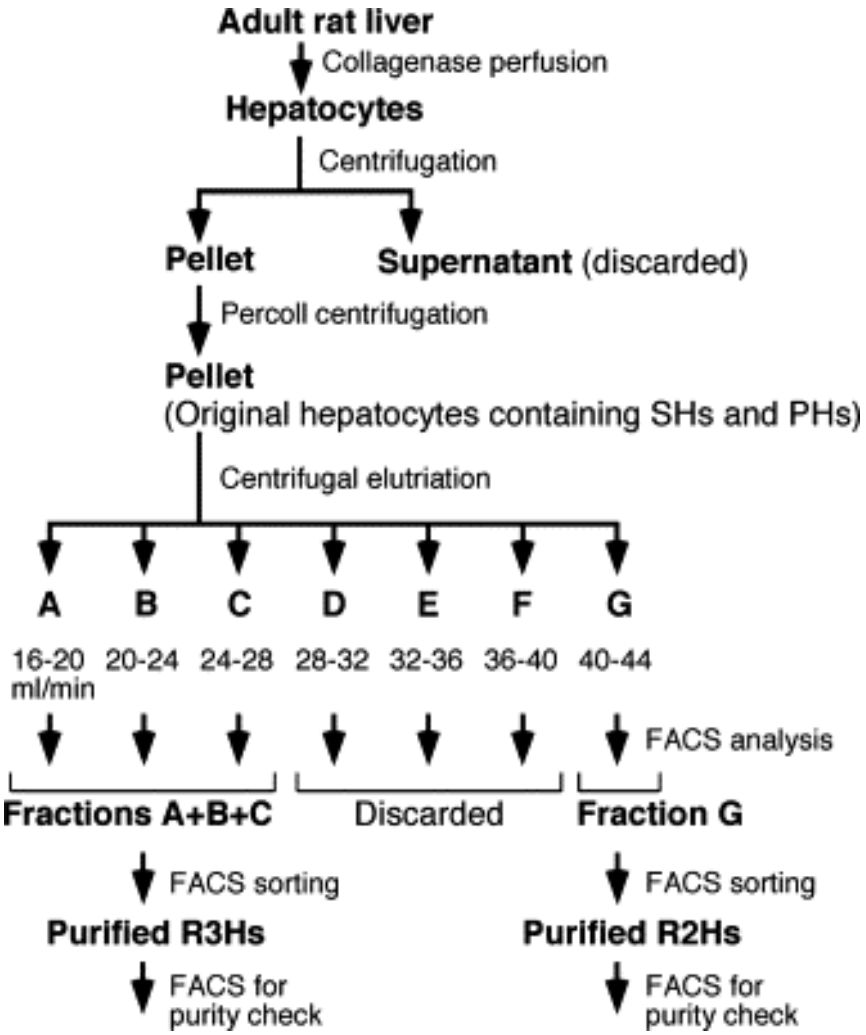
Adult rat liver
Collagenase perfusion
Hepatocytes
Centrifugation
Pellet
Supernatant (discarded)
Percoll centrifugation
Pellet
(Original hepatocytes containing SHs and PHs)
Centrifugal elutriation
A
B
C
D
E
F
G
16-20 ml/min
20-24
24-28
28-32
32-36
36-40
40-44
FACS analysis
Fractions A+B+C
Discarded
Fraction G
FACS sorting
FACS sorting
Purified R3Hs
Purified R2Hs
FACS for purity check
FACS for purity check

Fig.2

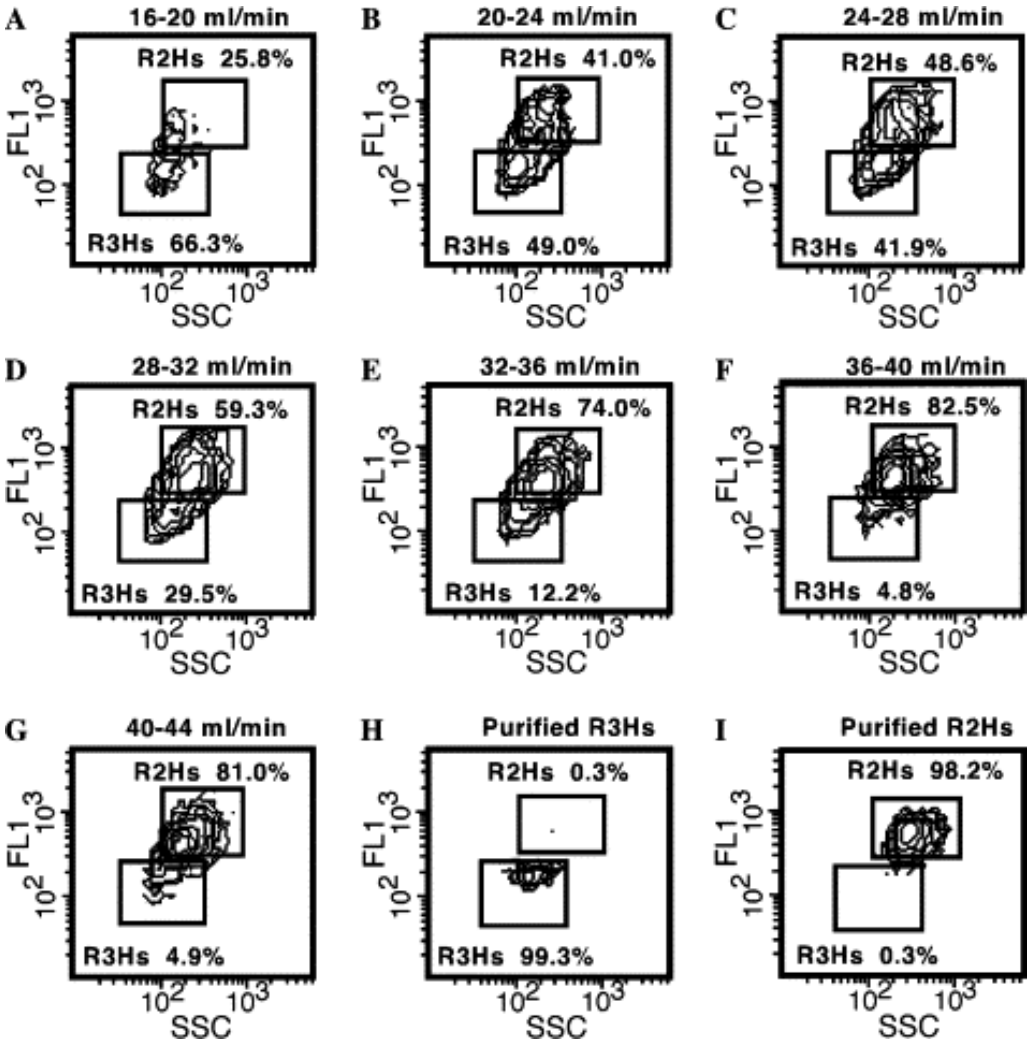
A
16-20 ml/min
R2Hs 25.8%
R3Hs 66.3%
B
20-24 ml/min
R2Hs 41.0%
R3Hs 49.0%
C
24-28 ml/min
R2Hs 48.6%
R3Hs 41.9%
D
28-32 ml/min
R2Hs 59.3%
R3Hs 29.5%
E
32-36 ml/min
R2Hs 74.0%
R3Hs 12.2%
F
36-40 ml/min
R2Hs 82.5%
R3Hs 4.8%
G
40-44 ml/min
R2Hs 81.0%
R3Hs 4.9%
H
Purified R3Hs
R2Hs 0.3%
R3Hs 99.3%
I
Purified R2Hs
R2Hs 98.2%
R3Hs 0.3%
FL1
SSC
10^2
10^3

Fig.3