Elevation and characteristics of *Rab30* and *S100a8/S100a9* expression in an early phase of liver regeneration in the mouse

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Abstract. Recent studies have revealed that cytokines, including TNFα and IL-6 play key roles in the priming phase of liver regeneration. However, further knowledge of molecular events in the priming phase is needed for more comprehensively understanding the initiation of liver regeneration. In the present study, we attempted to identify additional genes involved in an early phase (2-6 h post partial hepatectomy, PH). The expression of 71 genes was shown to be up-regulated more than 3-fold in the liver at 2 h and 6 h post PH, as compared to 0 h (normal livers) using microarray analysis. Among them, *Rab30* and *S100a8/S100a9*, were identified as novel genes up-regulated over 20-fold at 2 h post PH as compared to normal liver, and were further examined by RT-qPCR to confirm microarray results. *Rab30* showed no significant up-regulation in organs other than the liver, whereas *S100a8/S100a9* showed significant up-regulation in other organs, such as the lung and spleen at 6 h post PH as compared to those of sham-operated mice, indicating the existence of a different up-regulation machinery between *Rab30* and *S100a8/S100a9*. Their expression was further investigated in the liver at various developmental stages. *Rab30* was shown to be expressed only in newborn liver, whereas *S100a8/S100a9* was highly expressed in embryo stages, and exhibited the highest levels in newborn liver. These findings imply that *Rab30* and *S100a8/S100a9* are possibly involved in the functional switch from hematopoiesis support to metabolism in the newborn stage, but might play different roles in liver development. In conclusion, *Rab30* and *S100a8/S100a9* were indicated to play roles in the initiation of liver regeneration as well as possibly in the functional switch of the liver in the newborn stage.

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Introduction

The liver is an important organ that plays a central role in the metabolic homeostasis of the body, which consists of metabolism, synthesis, storage and redistribution of carbohydrates, fat and vitamins. In addition, the liver produces a large number of proteins, including serum albumin, enzymes, and cofactors (1). From the developmental point of view, the liver supports hematopoiesis at the embryonic stages, and undergoes functional switches to gain major functions in metabolic homeostasis at the postnatal stages (2). Most of these functions at the postnatal stages are carried out by the hepatocytes, a parenchymal cell type that comprises about 80% of the hepatic cells. The remaining 20% of the hepatic cells are non-parenchymal cells including Kupffer, stellate, endothelial cells, and lymphocytes. These cells have various functions such as phagocytosis and cytokine production (1).

The occurrence of liver regeneration has been clearly demonstrated by Higgins *et al* (3) in rodents that underwent removal of two-thirds of this organ (partial hepatectomy, PH). Rodents that underwent PH regained the original mass of the liver by enlargement of the remaining liver one week post PH (1,3). This regeneration of the liver has been found not to be a genuine regeneration, such as that observed in amphibians; however, it provides the basis for clinical liver treatment, such as surgery to eliminate hepatic cancer and for performing transplantation. The mechanisms of liver regeneration have been the focus of many investigations over the years. Recently, cytokines including IL-6 and TNFα have been demonstrated to be involved in the initiation of the liver regeneration (1). However, the study on *Il6* knock-out mice has shown that IL-6 is not indispensable in liver regeneration (4). Likewise, the analysis of *Tnfα* knock-out mice has shown that *TNFα* is not indispensable in liver regeneration (5). Hence, currently, the overlapping multiple pathways related to the cytokines are considered to be involved in the initiation

of liver regeneration post PH (6-8). In addition, peptides/proteins released from organs other than the liver were shown to participate in liver regeneration. For example, insulin from the pancreas, epidermal growth factor from the duodenum and salivary glands, norepinephrine from the adrenal glands, and triiodothyronine (T3) from the thyroid gland have been demonstrated to be implicated in liver regeneration (7).

Although many factors have been shown to be involved in liver regeneration as mentioned above, further knowledge of the molecular events involved in this process is essential to more comprehensively understand the regeneration. In the present study, we have attempted to identify novel up-regulated genes in the priming and in the extended phase of liver regeneration using microarray and RT-qPCR analyses, and then determine the expression sites of those genes using *in situ* hybridization in order to further understand their function.

Materials and methods

Partial hepatectomy. Eight-week-old (8W) SPF (specific pathogen-free) male BALB/c mice weighing 24-28 g were purchased from Clea (Tokyo, Japan). The mice were maintained in a temperature-controlled room on a 12 h light-dark cycle, with free access to water and standard chow. PH (70%) was performed on anesthetized mice, essentially following the procedure described by Higgins *et al* (3). The mice were sacrificed at five time-points: 0, 2, 6, 12, and 24 h post PH (n=3 in each time-point). Sham-operation was performed on mice as a control (n=3 for each time-point).

Tissue samples. For RNA analysis, hepatectomized and sham-operated 8W BALB/c mice were sacrificed at the time-points indicated above, and the organs/tissues (liver, brain, heart, lung, spleen, kidney, and testis) of the mice were collected, immediately frozen in liquid nitrogen and stored at -80˚C until use. Livers at different developmental stages, i.e., embryonic day (E) 14, E17, newborn (NB), one-week-old (1W), and 8W, were obtained from C57BL/6J mice at the RIKEN BioResource Center (Ibaraki, Japan). The livers were frozen as described above and stored at -80˚C until use.

For *in situ* hybridization, the organs/tissues of postnatal mice, except for NB mice, were first fixed *in situ* by perfusion of 4% (w/v in phosphate-buffered saline) ice-cold paraformaldehyde solution into the mice, and then the resulting organs/tissues were excised to be further fixed overnight in the paraformaldehyde solution. The organs/tissues of NB mice and embryos were excised to be fixed overnight in the paraformaldehyde solution. The organs/tissues thus fixed were embedded in paraffin blocks, and then sliced into 4 μm sections to make a tissue array on glass slides.

Animal care and experiments. All animal experiments in this study were carried out according to the Guidelines of the University of Tsukuba for the Care of Laboratory Animals and the Regulation for Animal Experiments or according to the standards established by RIKEN under the Guidelines for the care and use of experimental animals.

Total RNA extraction. Total RNAs were isolated from frozen tissues using Isogen (Nippon Gene, Tokyo, Japan) according to the manufacturer's instruction. The quality and concentration of the RNA were assessed with the NanoDrop Spectrophotometer (NanoDrop Technologies, WI, USA) according to the manufacturer's instructions. The 260/280 nm absorbance ratios of all RNA samples were 1.8-2.0. The Agilent 2100 Bioanalyzer (Agilent Technologies, CA, USA) and the RNA 6000 Nano LabChip kit (Agilent Technologies) were used to evaluate the integrity of the RNA. Total RNAs examined were shown to be evaluated as RNA integrity numbers (RIN) over 8.0. Based on the instructions of the Bioanalyzer, total RNAs thus obtained were judged to be suitable for further analysis; i.e., microarray analysis and RT-qPCR.

Microarray analysis. An equal amount of total RNAs prepared from 3 liver samples at each time-point were mixed to make a total RNA mixed sample of each time-point. Cyanine 3 (Cy3)-CTP-labeled cRNA was synthesized from 400 ng of the 0 h total RNA mixed sample (total RNA mixed sample of normal livers) using a Low RNA Input Linear Amplification kit (Agilent Technologies); and cyanine 5 (Cy5)-CTP-labeled cRNAs were synthesized from 400 ng of 2, 6, 12, and 24 h total RNA mixed samples following the manufacturer's instructions.

Agilent 44K x 4 mouse oligo microarray slides (Agilent Technologies) were hybridized with a mixture of Cy3- and Cy5-labeled cRNAs (825 ng each) in a hybridization solution prepared with the *in situ* Hybridization kit Plus (Agilent Technologies), following the manufacturer's instructions. The Cy3 and Cy5 fluorescence signal images on the slides were obtained by a DNA microarray scanner (Agilent Technologies), and processed using the Feature Extraction software version 8.1, based on the manufacturer's instructions. The signals of the genes thus obtained were subjected to quantile normalization in order to examine the differences of gene expression between the 0 h RNA and the other RNA mixed samples.

RT-qPCR. An aliquot of each RNA sample was mixed with an RNA fragment (218 nucleotides) synthesized from the pEGFP-C1 vector (Invitrogen, CA, USA) to attain a final amount of $5x10^{-5}$ pmol/10 μg total RNA (9). The resulting mixtures were subjected to first-strand cDNA synthesis using random hexamer primers (Takara, Shiga, Japan) and Reverse Transcriptase (Promega, Tokyo, Japan), according to the procedure recommended by Promega. The first-strand cDNAs were then subjected to quantitative PCR (qPCR) using SYBR-Green Real-time PCR Master Mix Plus (Toyobo, Osaka, Japan) and the primer pairs described below. The qPCRs were conducted using the Applied Biosystem 7500 Real-Time PCR system (Applied Biosystems, CA, USA) under the conditions of 1 min at 95˚C followed by 40 cycles each at 95˚C for 15 sec and 60˚C for 60 sec, following the procedure recommended by the manufacturer. Results of qPCR are presented as the means ± standard errors (SE) of the samples.

Since the copy numbers of gene transcripts were shown to be different depending on the region of the genes (10), sequences for the primer pairs of qPCR and probes for *in situ* hybridization were selected in a region as close as possible to the probe sequences of the microarray using Genetyx software

Table I. Primer sequences for RT-qPCR.

Primer name	Sequence (5'→3')	Size (mer)	PCR products (bp)
Igfbp1-forward	ATCTGCCAAACTGCAACAAG	20	121
Igfbp1-reverse	GACCCAGGGATTTTCTTTC	19	
Ccnd1-forward	CTGTTAGGTTCTAGTGTTCCGTC	23	120
Ccnd1-reverse	CAGCTTGCTAGGGAACTTGG	20	
Rab30-forward	GGTTGCGGGAGATAGAACAG	20	121
Rab30-reverse	GCCTCTGAGAACTCTTCTGCT	21	
S100a9-forward	CACAGTTGGCAACCTTTATGAA	22	69
S100a9-reverse	GGTCCTCCATGATGTCATTTATG	23	
S100a8-forward	CTGAGTGTCCTCAGTTTGTG	20	78
S100a8-reverse	TTGCATTGTCACTATTGATGTCC	23	

Table II. cDNA probe sequences for *in situ* hybridization.

Probe name	Sequence (5'→3')	Size (mer)
Prm1 antisense cRNA probe (positive control)	UUUUCAACAUUUAUUGACAGGUGGCAUUGUUCCUUAGCAGGCUCCUG UUUUUCAUCGGACGGUGGCAUUUUUCAAGAUGUGGCGAGAUGCUCUU GAAGUCUGGUAAAAUUCUCACGCAGG	120
LNE cRNA probe (negative control)	UGCCUGCAAAGAUGAGGAGGGAUUGCAGCGUGUUUUUAAUGAGGUCA UCACGGGAUCCCAUGUGCGUGACGGACAUCGGGAAACGCCAAAGGAG AUUAUGUACCGAGGAAGAAUGUCGCU	120
Rab30 antisense cRNA probe	GCCUCUGAGAACUCUUCUGCUCUCUGCUGGGAGACCUCUCGCCUUUCA GCCAGGUCAAUCUUGUUGCCUACUAACACAGUGAUGACUUUAUUGCU AGCAUACUGUUCUAUCUCCCGCAACC	121
S100a9 antisense cRNA probe	AGCUUCUCAUGACAGGCAAAGAUCAACUUUGCCAUCAGCAUCAUACA CUCCUCAAAGCUCAGCUGAUUGUCCUGGUUUGUGUCCAGGUCCUCCA UGAUGUCAUUUAUGAGGGCUUCAUUU	120

Table III. Top 10 genes up-regulated more than 3-fold in the liver at 2 and 6 h post PH, compared to that at 0 h post PH.

Gene symbol	Accession no.	Expression (fold-change)				Gene name
		2 h	6 h	12 h	24 h	
Mt2	NM_008630	101.27	130.82	106.96	50.27	Metallothionein 2
Igfbp1	NM_008341	85.37	82.62	29.83	6.57	Insulin-like growth factor binding protein 1
S100a9	NM_009114	48.55	38.74	16.98	23.18	S100 calcium binding protein A9
Saa1	NM_009117	32.47	69.51	61.27	62.57	Serum amyloid A1
Saa2	NM_011314	28.08	34.78	32.42	40.24	Serum amyloid A2
2310016C08Rik	NM_023516	26.24	43.88	23.96	7.95	Unknown
Fabp4	NM_024406	21.14	34.34	33.42	40.57	Fatty acid binding protein 4
Mt1	NM_013602	20.97	38.58	38.89	11.35	Metallothionein 1
Saa3	NM_011315	20.69	41.23	46.75	42.08	Serum amyloid A3
Rab30	NM_029494	20.05	52.09	6.48	<3.00	Member of the RAS oncogene family

(Genetyx, Tokyo, Japan) (Tables I and II). To confirm whether the fragments amplified in the qPCR were derived from the target sequences, the qPCR-amplified fragments were purified though 2% agarose gel electrophoresis and subjected to direct sequence analysis using the ABI PRISM 3100 Genetic Analyzer (Applied Biosystems).

Histological study. Tissue sections on glass-slides were stained with hematoxylin and eosin (H&E) and photographed through a MIRAX desk (Carl Zeiss, Tokyo, Japan).

In situ hybridization. *In situ* hybridization was performed as described earlier (11), with the following exception: the hybridization was carried out in a solution containing 50% formamide, 2X SSC, 1.0 mg/ml transfer RNA, 1.0 mg/ml salmon sperm DNA, 1.0 mg/ml BSA, 1.0 mg/ml yeast RNA, 2.0% SDS, and 3.0 μg/ml digoxigenin (DIG)-labeled cRNA probe at 42˚C for 60 h. Hybridization signals were detected with the NBT/BCIP system (Roche Diagnostics, Tokyo, Japan), and photographed through the MIRAX desk (Carl Zeiss) under the same conditions to perform the comparison of signal intensities.

For the cRNA probes, regions corresponding to those examined by qPCR were selected to make ca.120 nucleotide-sequences (Table II). As a negative control cRNA probe, a LNE cRNA probe that did not give a signal in any of the tissues was used for *in situ* hybridization (12). In addition, a mouse *Prm1* antisense cRNA probe was used as a positive control by detecting *Prm1* mRNA in 8W testis sections for all the *in situ* hybridization experiments. These DIG-labeled cRNA probes were obtained from Tsukuba GeneTech Laboratories (Ibaraki, Japan).

Results

Identification of novel genes participating in liver regeneration. Cytokines including TNFα and IL-6 have been demonstrated to play key roles in the priming phase (0-4 h post PH) of liver regeneration. In the present study, we screened up-regulated genes during liver regeneration at the early phase (until 6 h post PH) using microarray analysis in order to obtain novel genes involved in liver regeneration. The microarray analysis revealed that the expression of 71 genes was elevated more than 3-fold at 2 and 6 h post PH, as compared to 0 h (data not shown). The top 10 overexpressed genes are listed in Table III. *Mt2*, *Igfbp1*, *Saa1*, *Saa2* and *Mt1*, listed in Table III, were reported as up-regulated genes during the priming phase of liver regeneration in earlier studies (13-15). In addition, a 5.6-fold elevation of *Ccnd1*, which is involved in DNA replication, was observed at 24 h post PH (data not shown). This result was also consistent with the findings obtained in earlier studies (16-18). Furthermore, H&E staining revealed that hepatocytes followed the process of liver regeneration with the passage of time after PH (data not shown). These histological observations indicated that the liver regeneration post PH in the present study followed the same process as reported in earlier studies.

Based on these findings, *Rab30* and *S100a9* were selected for precise analysis as candidates of novel genes to be involved in the initiation of liver regeneration, though *S100a9* had been listed as an up-regulated gene in the SAGE (serial analysis of gene expression) analysis (18). In view of the fact that S100a9 was shown to form a heterodimer with S100a8 in neutrophils and monocytes (19-21), it is possible that *S100a8* is also up-regulated together with *S100a9*. However, *S100a8* data were eliminated in the Feature Extraction process because at least one value at the time-points showed no significant differ-

Table IV. Expression of *Rab* family genes in liver regeneration using microarray analysis.

Gene symbol	Accession no.	Expression fold			
		2 h	6 h	12 h	24 h
Rab1	NM_008996	1.24	1.75	2.01	1.71
Rab1b	NM_029576	0.62	0.54	0.63	0.63
Rab2	NM_021518	1.00	1.07	1.32	1.32
Rab2b	NM_172601	0.90	1.05	1.14	0.83
Rab3a	NM_009001	0.36	0.15	0.33	0.47
[a]*Rab3b*	NM_023537	0.99	0.94	0.91	0.84
Rab3d	NM_031874	0.60	0.32	0.41	1.14
Rab4a	NM_009003	1.04	0.77	1.21	1.49
Rab4b	NM_029391	1.14	1.56	1.63	1.03
Rab5a	NM_025887	1.19	1.48	1.25	1.02
[a]*Rab5b*	NM_011229	0.58	0.89	1.07	0.86
Rab5c	NM_024456	1.83	1.95	1.35	1.70
Rab6	NM_024287	0.99	1.32	1.75	1.25
[a]*Rab6b*	NM_173781	0.98	0.93	0.90	0.82
Rab7	NM_009005	1.11	1.34	1.36	1.15
Rab8a	NM_023126	0.72	0.71	0.86	1.12
[a]*Rab8b*	NM_173413	1.01	1.26	2.79	4.19
Rab9	NM_019773	1.17	2.00	1.25	1.56
[a]*Rab9b*	NM_176971	1.00	0.96	0.73	0.98
Rab10	NM_016676	0.89	0.95	0.80	0.78
Rab11a	NM_017382	0.94	1.01	1.04	1.53
Rab11b	NM_008997	0.94	0.96	1.33	0.64
Rab12	NM_024448	1.39	2.25	1.20	1.48
Rab13	NM_026677	1.16	0.83	0.91	2.32
Rab14	NM_026697	1.03	0.93	0.98	0.85
[a]*Rab15*	NM_134050	1.83	8.06	1.28	8.19
Rab17	NM_008998	0.71	0.65	0.54	0.98
Rab18	NM_181070	1.67	2.79	2.34	1.89
[a]*Rab19*	NM_011226	1.28	1.28	0.87	1.44
Rab20	NM_011227	1.68	1.15	0.85	0.63
Rab21	NM_024454	1.13	1.08	1.07	1.21
Rab22a	NM_024436	0.71	0.94	0.60	0.61
Rab23	NM_008999	0.45	0.88	1.81	1.64
Rab24	NM_009000	0.93	0.74	0.93	0.80
[a]*Rab25*	NM_016899	1.48	1.15	0.92	1.21
[a]*Rab26*	AK080607	0.99	0.94	0.90	1.05
[a]*Rab27a*	NM_023635	1.01	0.62	0.94	1.43
[a]*Rab27b*	AK161136	0.97	0.92	0.88	0.82
Rab28	NM_027295	1.42	1.66	1.78	2.24
Rab30	NM_029494	20.05	52.09	6.48	1.42
Rab31	NM_133685	2.31	3.45	2.59	4.17
Rab32	NM_026405	0.59	0.54	0.59	1.36
Rab33a	NM_011228	0.97	0.86	1.11	1.15
Rab33b	NM_016858	0.90	2.18	2.03	1.13
Rab34	NM_033475	1.15	0.80	1.22	1.53
Rab35	NM_198163	0.80	1.18	1.53	1.15
[a]*Rab36*	NM_029781	1.00	0.95	0.93	0.87
[a]*Rab37*	NM_021411	1.00	1.29	0.91	0.85
[a]*Rab38*	NM_028238	0.98	0.94	1.68	1.41
[a]*Rab39*	NM_175562	0.66	0.78	3.29	2.44
[a]*Rab39b*	NM_175122	1.17	0.92	1.26	1.87
[a]*Rab40b*	NM_139147	0.99	0.95	0.91	0.85
Rab40c	NM_139154	0.55	0.66	1.00	0.72

[a]Data were not found in the processed data, but existed in the row data. They were eliminated in the process of the row data with the Feature Extraction due to fact that at least one value at the time points did not show significant difference between the values and the background.

Table V. Expression of *S100* family genes in liver regeneration using microarray analysis.

Gene symbol	Accession no.	Expression fold			
		2 h	6 h	12 h	24 h
S100a1	NM_011309	1.45	1.62	1.01	1.20
[a]*S100a3*	NM_011310	0.69	1.44	1.30	1.35
S100a4	NM_011311	3.82	9.38	2.39	19.72
[a]*S100a5*	NM_011312	1.00	0.86	0.89	0.81
S100a6	NM_011313	3.46	5.44	2.20	11.27
[a]*S100a8*	NM_013650	60.14	116.69	38.96	80.17
S100a9	NM_009114	48.55	38.74	16.98	23.18
S100a10	NM_009112	2.87	6.30	7.68	13.77
S100a11	NM_016740	2.87	4.17	5.55	7.47
S100a13	NM_009113	1.29	1.31	0.76	1.29
[a]*S100a14*	NM_025393	0.69	2.82	0.36	4.27
[a]*S100a15*	NM_199422	1.01	0.95	0.98	0.86
S100a16	NM_026416	0.84	0.87	1.12	1.96
[a]*S100b*	NM_009115	1.74	1.32	0.91	1.18
[a]*S100g*	NM_009789	1.00	94.79	0.92	0.86

[a]Data were not found in the processed data, but existed in the row data. They were eliminated in the process of the row data with the Feature Extraction due to fact that at least one value at the time-points did not show significant difference between the values and the background.

ence from the background value in the microarray analysis. Therefore, the *Rab* and *S100* family members including *S100a8* eliminated in the Feature Extraction process were re-examined (Tables IV and V). As shown in Table V, *S100a8* was up-regulated in the eliminated family members and its up-regulation pattern was similar to that of *S100a9*. Consequently, *Rab30, S100a8*, and *S100a9* were the only members up-regulated in the gene families examined, and it was indicated that *Rab30* and *S100a8/S100a9* had unique functions among the respective family members, and *S100a9* functioned together with *S100a8* in liver regeneration.

In order to confirm the up-regulation of *Rab30*, *S100a8*, and *S100a9* at 2 h and 6 h post PH, RT-qPCR was performed independently for all the total RNA samples (n=3 at each time-point). In addition, the expression levels of *Igfbp1* and *Ccnd1* were examined as authentic controls in liver regeneration. The expression of these genes was normalized based on the value of EGFP RNA (22) and were calculated to obtain means ± SE at each time-point. The expression of *Igfbp1* showed a significant 44.3-fold increase at 2 h post PH as compared to that at 0 h, and gradually decreased to 7.1-fold; and that of *Ccnd1* was elevated 3.1-fold at 24 h post PH as compared to that of 0 h (data not shown). These results were essentially consistent with those of the microarray analysis and with those reported by others (13, 16-18). In the case of *Rab30*, the highest expression, a 26.4-fold increase over the expression at 0 h post PH, was observed at 2 h post PH and decreased gradually in the following time-points examined (Fig. 1A). The expression of *S100a9* showed a 37.9-fold increase at 2 h post PH, gradually decreased until 12 h post PH to 14.6-fold, and increased to 30.0-fold at 24 h post PH (Fig. 1B). For *S100a8*, its expression was elevated 71.8-fold at 6 h post PH, decreased to 26.9-fold at 12 h post PH, and increased again to 120.0-fold at 24 h post PH, following a similar pattern with that of the *S100a9* expression (Fig. 1). These findings were essentially consistent with those obtained from the microarray analysis.

In the RT-qPCR, total liver RNAs prepared from the sham-operated mice at each time-point were studied for the expression levels of the genes, in order to examine whether the elevated expression levels of the genes were the result of PH. As shown in Fig. 1, it has been revealed that all or a major part of the elevated expression levels of *Rab30*, *S100a8* and *S100a9* were attributed to PH. These results, taken together, indicate that *Rab30* plays a role in the priming phase of liver regeneration, and that *S100a8/S100a9* play a role in the priming and in a later phase starting from 24 h post PH. These results also indicate that *Rab30* and *S100a8/S100a9* have different functions in liver regeneration.

Rab30 and S100a8/S100a9 expression in other organs of mice at 6 h post PH, and in livers at various developmental stages. To obtain information about the functions of *Rab30*

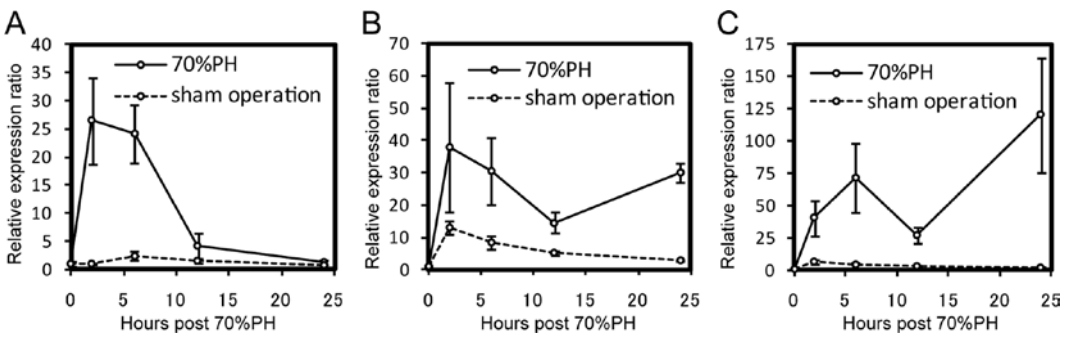

Figure 1. Relative amount of (A) *Rab30*, (B) *S100a9* and (C) *S100a8* mRNAs in the early phase of liver regeneration. Total RNAs were isolated from regenerating livers of PH mice, and from livers of sham-operated mice (n=3 at 0, 2, 6, 12, and 24 h post PH or sham-operation), and cDNAs from these total RNAs were synthesized as described in Materials and methods. The amounts of *Rab30*, *S100a9* and *S100a8* mRNAs in the total RNA samples were determined as described in Materials and Methods by RT-qPCR using the primer pairs listed in Table I. Means ± SE were obtained using the standard-curve method according to the procedure recommended by Applied Biosystems, and calculated taking the values of qPCR at 0 h post PH (normal livers) as 1.0. Solid lines represent the relative mRNA amounts of respective genes in the regenerating livers. Dashed lines represent the relative mRNA amounts of respective genes in the livers of sham-operated mice. The ordinates represent relative mRNA amounts and the abscissas the periods of time in hours post PH or sham-operation.

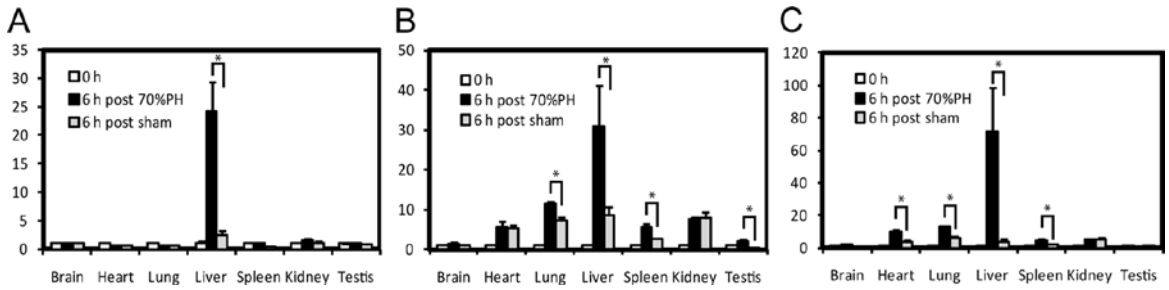

Figure 2. Expression of (A) *Rab30*, (B) *S100a9* and (C) *S100a8* mRNA in various organs at 6 h post PH. The relative expression of *Rab30*, *S100a9* and *S100a8* mRNA in the brain, heart, lung, liver, spleen, kidney, and testis at 6 h post PH were determined by RT-qPCR using the primer pairs listed in Table I. Means ± SE of the organs/tissues were obtained using a standard curve method, and calculated taking the values of qPCR for corresponding normal organs/tissues as 1.0. The ordinate represents relative mRNA amounts.

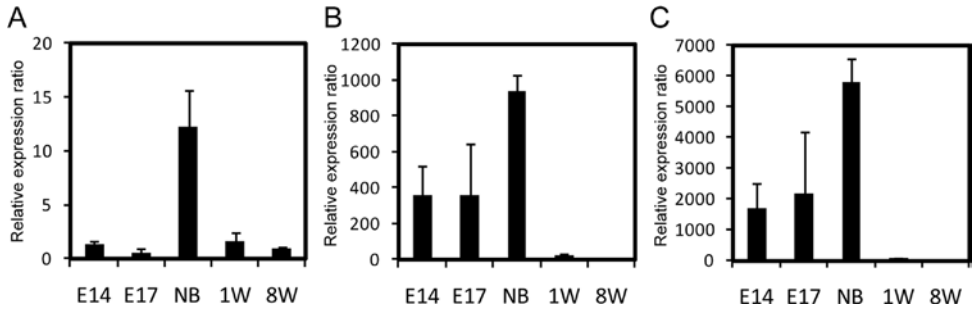

Figure 3. Expression of (A) *Rab30*, (B) *S100a9* and (C) *S100a8* mRNA in livers at various developmental stages. The relative expression levels of *Rab30, S100a9* and *S100a8* mRNA in livers of E14, E17, NB, 1W, and 8W mice were determined by RT-qPCR as described in Materials and methods. Means ± SE in the livers at various developmental stages were calculated taking the mean values of 8W as 1.0.

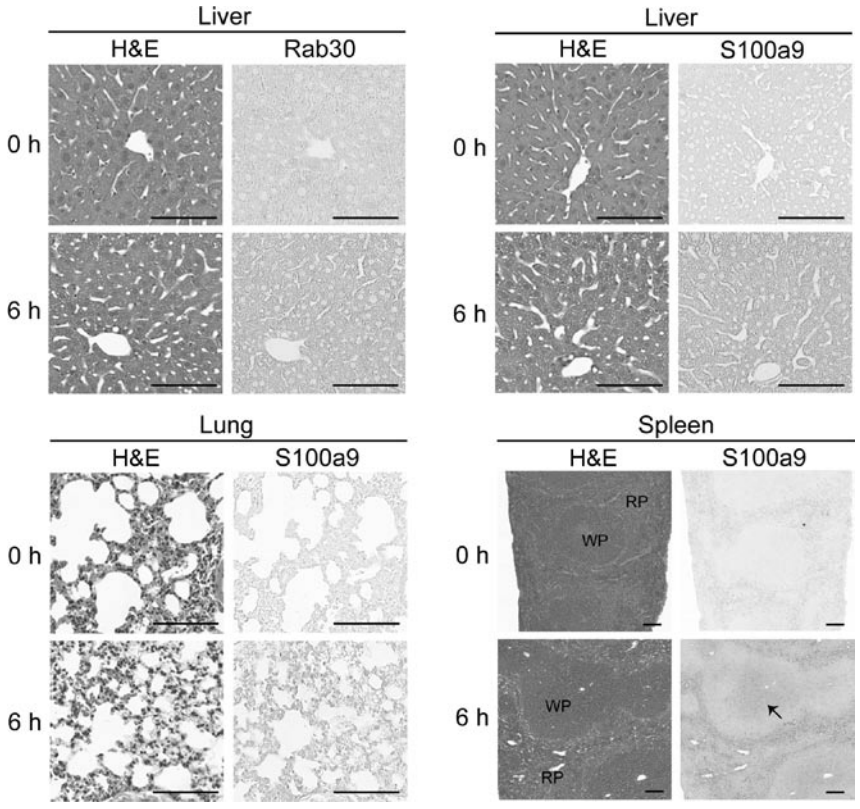

Figure 4. Localization of *Rab30* and *S100a9* mRNA in organs at 6 h post PH. At 6 h post PH significant up-regulation of *Rab30* as compared to that of sham-operated mice was observed in lvers subjected to *in situ* hybridization. Likewise, significant up-regulation of *S100a9* is observed in the liver, lung, and spleen at 6 h post PH as determined by *in situ* hybridization. *In situ* hybridization was performed using DIG-labeled *Rab30* or *S100a9* antisense cRNA probes, according to the procedure described in Materials and methods. The cRNA probe sequences are indicated in Table II. Serial sections were subjected to H&E staining and *in situ* hybridization, followed by photographing of the corresponding regions. RP, red pulp; WP, white pulp. Scale bar, 100 μm.

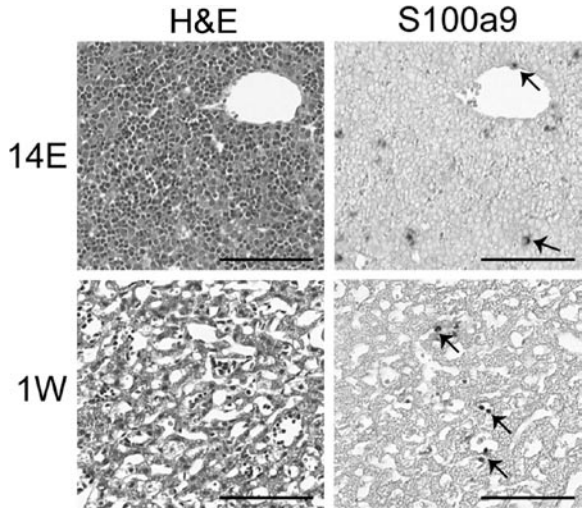

Figure 5. Localization of *S100a9* mRNA in livers at various developmental stages. Localization of *S100a9* mRNA in livers at the developmental stages was detected by *in situ* hybridization using the DIG-labeled cRNA probe as described in Table II. Serial sections of the liver at each stage were used for H&E staining, and the detection of *S100a9* mRNAs. Arrows indicate non-parenchymal cells expressing *S100a9* mRNA. 14E, embryonic day 14; 1W, one-week-old. Scale bar, 100 μm.

and *S100a8*/*S100a9*, the expression levels of *Rab30*, *S100a8*, and *S100a9* were measured in the brain, heart, lung, spleen, kidney and testis of mice at 6 h post PH in comparison with those of sham-operated and normal mice. As shown in Fig. 2, *Rab30* demonstrated no significant difference between PH mice and sham-operated/normal mice, whereas *S100a8* and *S100a9* showed significant differences in their expression in organs other than the liver between PH mice and sham-operated/normal mice. The fact that *Rab30* expression was elevated only in liver led us to speculate that the signal for *Rab30* activation was conveyed directly to hepatocytes after PH. As for *S100a8* and *S100a9*, since their elevated expression levels were observed in the liver as well as in other organs, the signal for *S100a8* and *S100a9* activation was inferred to be hematogenously conveyed to hepatocytes.

We further examined the expression levels of *Rab30* and *S100a8*/*S100a9* in the process of liver formation and maintenance using the livers of mice at E14, E17, NB, 1W and 8W. *Rab30* showed a marked expression only in NB livers, whereas *S100a8* and *S100a9* showed relatively higher expression in embryonic livers, the highest in NB livers like *Rab30*, and very low expression levels in 1W and 8W livers (Fig. 3). These observations indicate that in addition to the involvement in liver regeneration, *Rab30* may be involved in the functional switch from hematopoietic support to metabolic homeostasis; and *S100a8*/*S100a9* may be involved in hematopoietic support as well as in the functional switch.

Expression sites of Rab30 and S100a9 in the organs of mice at 6 h post PH and in livers at various developmental stages. For analysis of the gene expression sites in organs, *in situ* hybridization was performed using DIG-labeled cRNA probes as described in Materials and methods. Due to the fact that the expression profiles of *S100a8* and *S100a9* were essentially the same (see above) and the fact that *S100a8* and *S100a9* form a heterodimer to function in cells (19-21), the *S100a9* DIG-labeled cRNA probe was used as a representative probe for *S100a8*/*S100a9* analysis. The organs at 6 h post PH showing significant elevation of *Rab30* and *S100a8*/*S100a9* (the liver for *Rab30*, and the liver, lung, and spleen for *S100a9*) were subjected to *in situ* hybridization. *Rab30* and *S100a9* mRNAs were uniformly detected in hepatocytes (Fig. 4). In addition, *S100a9* mRNA was detected uniformly in alveolar cells in lung and in the central region of white pulp populated with B and T cells and in red pulp populated with monocytes and macrophages (23) in the spleen (Fig. 4).

When the liver was examined at various developmental stages, E14, E17, NB, 1W and 8W, *Rab30* expression was observed to be uniform in fetal and postnatal hepatocytes (data not shown), and *S100a9* expression was observed to be more specific in a portion of non-parenchymal cells, possibly in myeloid cells (23) in E14 and 1W livers (Fig. 5), and more uniform in E17 and NB livers (data not shown).

Discussion

In order to understand the liver regeneration process post PH, numerous studies have been conducted since Higgins *et al* first described the liver regeneration of rodents that underwent a two-third removal of the liver in 1931 (3). Recently, a number of genes have been shown to participate in the initiation of liver regeneration (1,7,8). In the present study, *Rab30* and *S100a8*/*S100a9* genes have been newly identified to be up-regulated at 2 and 6 h post PH using microarray analysis and RT-qPCR. The *Rab30* and *S100a8*/*S100a9* expression amounts and sites were investigated in various organs of the mice at 6 h post PH and in the livers at various developmental stages. According to our results, *Rab30* and *S100a8*/*S100a9* were shown to participate in the initiation of liver regeneration in possibly different ways. In addition, *Rab30* appeared be involved mainly in the functional switch from hematopoietic support to metabolic homeostasis; and *S100a8*/*S100a9*, in the hematopoietic support as well as in the functional switch.

Rab30 is a member of the *Rab* family belonging to the *Ras* superfamily, which is found in species ranging from yeast to human and encode the proteins having GTPase activity. *Rab* family members are shown to function as regulators of particular steps in membrane trafficking pathways (24). *S100a8* and *S100a9* are members of the *S100* family encoding the proteins bearing a Ca^{2+} binding EF-hand motif. *S100a8* and *S100a9* have been shown to form a heterodimer in neutrophils and monocytes, and was detected extracellulary on the vasculature at inflammatory sites, indicating that *S100a8*/*S100a9* may influence neutrophil chemotaxis and adhesion (19-21).

In order to examine the possible similarity of the signal transduction pathway of *Rab30* with the pathways of *Hgf*, *Tnfα*, *Tnfrsf1α* (*Tnfr1*), *Il6*, *Tgfα*, *Egf*, *Egfr*, *Met*, *Igfbp1*, *Tgfb1*, *Tgfbr1*, *Mmp9*, which had been demonstrated to be up-regulated during the priming phase of liver regeneration in earlier studies (1,7,8), the 2 kb upstream regions of *Rab30* were compared with those of the above-mentioned genes. The *Rab30* upstream region was shown to have no notable similarity with that of any other genes examined. To the best of our knowledge, the role of *Rab30* has been studied in *Drosophila* using microarray analysis and an RNA interference system

(25). It was demonstrated that *Rab30* is involved in dorsal closure in embryogenesis. The amino acid sequence of mouse Rab30 is 63% identical to that of *Drosophila* Rab30, and 100% identical to that of humans and dogs (25). These findings indicate that *Rab30* is also involved in the early stages of development in mammals, including mice, in addition to its involvement in liver regeneration and the functional switch in NB from hematopoiesis to metabolic homeostasis.

When the upstream regions of *S100a8* and *S100a9* were examined, no responsive elements were found in *S100a8*; and the NF-κB and TATA box were found, in *S100a9*. Since no significant similarity was found between the two regions, it was indicated that *S100a8* and *S100a9* might be differently controlled. However, based on the fact that S100a8 and S100a9 have been found to form a heterodimer in neutrophils and monocytes, and have been detected extracellulary on the vasculature at inflammatory sites (19-21), in addition to the fact that administration of the immunosuppressant FK778 impaired liver regeneration in PH rats (26), the heterodimer of S100a8 and S100a9 has been inferred to play a role in the inflammatory process of the priming phase in the liver regeneration.

The present study indentified *Rab30* and *S100a8/S100a9* as novel genes, which were shown to participate in the initiation of liver regeneration. The genes were indicated to be under different signal transduction pathways and might play different roles in liver regeneration as well as in liver formation. Future studies including the investigation of knock-out mice of these genes will provide additional information to understand their impact on liver regeneration.

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