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Serial analysis of gene expression in sinusoidal endothelial cells from normal and injured mouse liver

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Abstract

Here we describe gene expression profiles of mouse liver sinusoidal endothelial cells (LSECs) revealed by serial analysis of gene expression (SAGE). We prepared SAGE libraries of LSECs from normal and injured liver by CCl_4 administration, and we obtained 32,867 tags from normal and 37,493 tags from injured liver, representing 6011 unique transcripts. CCl_4 administration upregulated several genes related to cell growth and differentiation (Cdkn1a, Irf1, Il4ra, etc.), whereas it downregulated genes related to cell growth or protein transport (Kdr, Igfbp4, Ap1b1, etc.). To identify genes preferentially expressed in LSEC, we compared our SAGE libraries with 77 publicly available libraries generated from various mouse tissues and cell lines. We identified 23 genes, including Stab2 and uncharacterized genes, as possible markers for LSEC, which will be useful to analyze the specific role for LSECs in normal as well as regenerating liver.

Keywords: Liver sinusoidal endothelial cell; Serial analysis of gene expression; Transcriptome; Liver regeneration; Carbon tetrachloride

The liver is a unique organ, with the capability to regenerate from injury caused by various hepatotoxins or partial hepatectomy. Hepatocytes, liver parenchymal cells, are quiescent in normal adult liver and enter into the cell cycle upon liver injury. This regeneration process is tightly regulated [1,2] and has been used as a useful model to study the molecular mechanism of cell growth and differentiation as well as organogenesis. Studies using knockout mice have identified several key molecules involved in liver regeneration, including tumor necrosis factor (TNF) receptor type 1 [3], interleukin 6 (IL-6) [4], inducible nitric oxide synthase [5], complement component C5 [6], peroxisome proliferator activated receptor-α [7], oncostatin M (OSM) receptor [8], and so on. Interestingly, some of these factors work in a paracrine manner to mediate intercellular communication among different types of cells that constitute the liver lobule, i.e., hepatocytes and various nonparenchymal cells (NPCs), including sinusoidal endothelial cells, Kupffer cells, and stellate cells [9]. For example, OSM produced by Kupffer cells regulates the cell cycle of hepatocytes and protects hepatocytes from cell death [8]. Furthermore, OSM acts on NPCs and induces the tissue inhibitor of metalloprotease-1 to regulate degradation of extracellular matrices [8]. As various types of liver cells orchestrate liver regeneration, it is necessary to understand the nature and function of each cell population.

The liver sinusoidal endothelial cell (LSEC) is a unique class of endothelial cells distinct from the other classes of endothelial cells in the body. LSECs have no regular basement membrane, but exhibit a cluster of fenestrae in their sieve plate and possess a high endocytotic capacity [10,11]. These features are thought to be essential for

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liver functions. Furthermore, recent studies have indicated that LSECs play an active role in liver development and regeneration [12,13]. However, the nature and function of the LSEC remain to be established. To clarify the characteristics of the LSEC at the molecular level, we examined the gene expression profile of LSEC.

Serial analysis of gene expression (SAGE) is a sequence-based technique that relies on two major principles. First, a short DNA tag (10–15 bp) is sufficient to identify individual gene product. Second, concatenation of short DNA tags allows the efficient analysis of transcripts by sequencing of multiple tags within a single clone [14]. SAGE has several advantages over other procedures. It does not depend on prior information of transcripts [14]. The experimental results are directly comparable to existing libraries from different sources in the public SAGEmap expression database [15]. A SAGE library can be generated from a small amount of samples [16]. Therefore, we applied SAGE to analyze the molecular nature of LSECs from normal and injured livers. To our knowledge, this is the first study to show the transcriptome of a fractionated LSEC population in normal and regenerating livers. We show several genes that are up- or down-regulated by liver injury and also new marker genes of LSECs.

Materials and methods

Animals and induction of liver injury. Eight to ten-week-old C57BL/6J male mice (Nihon Clea, Tokyo, Japan) were used in all experiments. Acute hepatic injury was induced by the intraperitoneal injection of a 10% (v/v) solution (200 μl/body) of carbon tetrachloride (CCl_4, Wako) diluted with corn oil (Wako).

Cell preparation. Single cell suspensions of liver cells were prepared by the 2-step collagenase perfusion method [17] and centrifuged twice at 45*g* for 1 min. The supernatant was removed and centrifuged 3 times at 80*g* for 2 min to precipitate damaged hepatocytes and cell debris. The resulting supernatant, which contains nonparenchymal cells (NPCs) containing LSECs, Kupffer cells, stellate cells, and blood cells, was removed and precipitated by centrifugation at 300*g* for 5 min. The cells were subjected to hemolysis with hypotonic buffer and filtered through a cell strainer to remove cell debris. NPCs resuspended in 2.5 mL PBS were layered on a 1.037 g/mL solution of Percoll (Pharmacia) and centrifuged at 400*g* for 20 min [17,18]. The resulting upper layer and interface, containing stellate cells, dead cells, and debris, were removed. The lower layer, the high density (hd) NPC fraction, was diluted with PBS and centrifuged at 300*g* for 5 min to pellet the cells.

Cell staining and sorting. hdNPCs were sequentially incubated with fluorescein isothiocyanate (FITC)-conjugated anti-mouse CD16/CD32 antibody, followed by phycoerythrin (PE)-conjugated anti-mouse PECAM-1 and either biotinylated anti-mouse CD45 or biotinylated anti-mouse CD11b. All antibodies were purchased from Becton–Dickinson. After washing with the staining medium (PBS containing 5% fetal bovine serum and 2 mM EDTA), NPCs were incubated with allophycocyanin-labeled streptavidin (Molecular Probes). Finally, cells were washed 2 times and resuspended in staining medium containing propidium iodide (5 μg/mL). Labeled cells were analyzed and sorted by FACS-vantage (Becton–Dickinson).

SAGE libraries. SAGE libraries were constructed from purified LSECs (450,000 cells from normal liver and 480,000 cells from injured liver) using the I-SAGE Kit (Invitrogen) according to the manufacturer's protocol. Samples were sequenced by an ABI 377 XL automated sequencer (ABI). Sequence files were analyzed using SAGE 2000 software (www.invitrogen.com/sage/) and the National Center for Biotechnology Information (NCBI) SAGE database (http://www.ncbi.nlm.nih.gov/SAGE/). Linker sequences and the repeated ditags were eliminated before analysis.

Quantitative real-time RT-PCR. Total RNA was isolated using the RNeasy mini kit (Qiagen). First-strand cDNA was synthesized from 600 ng total RNA using the First-strand cDNA synthesis kit (Amersham pharmacia biotech). The primers were designed using the Web

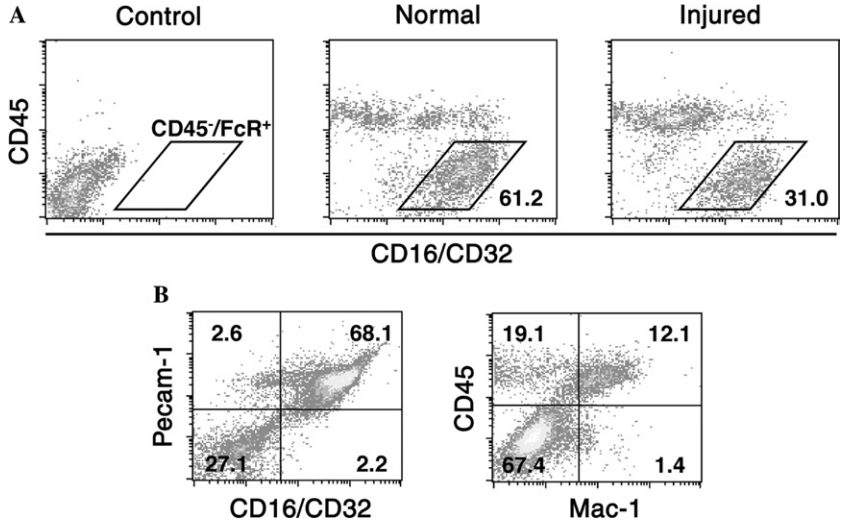

Fig. 1. Flow-cytometric analysis of mouse NPCs and isolation of $CD45^{-}CD16/32^{+}$ cells. (A) hdNPCs obtained from normal or injured (24 h after CCl_4 administration) liver were stained with anti-CD45 mAb and anti-CD16/32 mAb, and were then analyzed by FACS. LSECs were isolated as $CD45^{-}CD16/32^{+}$ cells (box) by FACS-vantage. This population accounted for 61.2% of normal hdNPCs and 31.0% of injured hdNPCs. (B) hdNPCs were further analyzed by FACS to examine the expression of PECAM-1 and Mac-1. hdNPCs obtained from normal liver were stained with mAbs as indicated. The numbers are percentages of cells in each fraction. Note that CD16/32+ (FcR^{+}) hdNPCs also expressed the pan-endothelial marker, PECAM-1, and $CD45^{-}$ hdNPCs did not express the macrophage/monocyte marker, Mac-1.

Primer software (http://www.yeastgenome.org/) and synthesized by Hokkaido System Science. Real-time PCR using the LightCycler instrument (Roche Diagnostics) was performed as described in the LightCycler—FastStart DNA Master SYBR Green 1 (Roche Diagnostics). The primers used were as follows: IL4 receptor α: sense 5′-ACTTTCGGACTAGACACGGA-3′, antisense 5′-CTTCTGGTGGT ATTCCCTCA-3′; transglutaminase 2: sense 5′-GTTAGGGTGAG GAGTTGAGGGA-3′, antisense 5′-GGGCTAACTTGGGATGAT GACC-3′; phosphoglycerate kinase 1: sense 5′-CCAAAGGATCA AGGCTGCTG-3′, antisense 5′-TTAGCGCCTCCCAAGATAGC-3′; growth arrest specific 6: sense 5′-CTTGCATCAGACGGCCAGAC-3′, antisense 5′-GAACGGCCTCCAGCGAAGAA-3′; latent TGF-β binding protein 4: sense 5′-GGACTTTGAGGATGATGGTG-3′, antisense 5′-ACACATGCAGACTAGAGGGA-3′; cysteine rich protein 2: sense 5′-CAGCAAAGCCTCTAGTGTCA-3′, antisense 5′-ATCAGGGACACAGGAACACA-3′; and vascular endothelial zinc finger-1: sense 5′-AGAAAGAAGCTGCCAACCTG-3′, antisense 5′-AGGCATACTTTCTACAGGCC-3′.

Table 1
Top 50 of the most abundantly expressed genes in normal LECs

Tag sequence	Count	UniGene cluster	Symbol	Description
GTGGCTCACA	446	21758	—	Multiple matches
TATCTGTGCA	360	22699	Sepp1	Selenoprotein P, plasma, 1
GCTGCCCTCC	358	104368	Rpl32	Ribosomal protein L32
GGATATGTGG	231	181959	Egr1	Early growth response 1
ATACTGACAT	221	353721	—	Transcribed sequence
TGGGTTGTCT	219	297482	—	Multiple matches
TAGCTTTAAA	204	233470	Igfbp7	Insulin-like growth factor binding protein 7
TTGCTGCCTT	171	291551	—	Multiple matches
GTCAATGACG	156	18625	Aqp1	Aquaporin 1
TGTAACCTGA	145	272258	Dnase1 l3	Deoxyribonuclease 1-like 3
GATTGAGAAT	126	16771	—	Multiple matches
AGCAGTCCCC	124	1011	Ncoa3	Nuclear receptor coactivator 3
AGGCAGACAG	124	335315	Eef1a1	Eukaryotic translation elongation factor 1 α1
CATCGCCAGT	123	305152	Apoe	Apolipoprotein E
CAGTGCAACT	122	22699	Sepp1	Selenoprotein P, plasma, 1
ATAATACATA	118	200362	Cybb	Cytochrome *b*-245, β polypeptide
TTGGTGAAGG	116	142729	Tmsb4x	Thymosin, β4, X chromosome
TGGAAAGTGA	112	246513	Fos	FBJ osteosarcoma oncogene
TTGTTGCTAC	112	—	—	No match
CTTTAGTGAC	107	109183	—	Multiple matches
ATGACTGATA	96	261851	—	Transcribed sequence
GGCTTCGGTC	94	3158	Rplp1	Ribosomal protein, large, P1
GGGAGTGGGG	82	268933	Egfl7	EGF-like domain 7
CACAAACGGT	79	270283	Rps27	Ribosomal protein S27
GCCTTCCAAT	78	220038	Ddx5	DEAD (Asp-Glu-Ala-Asp) box polypeptide 5
CTAGTCTTTG	76	354387	Rps29	Ribosomal protein S29
ATGTCTCAAA	72	209290	Tuba2	Tubulin, α2
TGGTTGCTGG	64	2296	—	Multiple matches
AGGACAAATA	63	261750	—	RIKEN cDNA B430306D02 gene
CAGATCTTTG	63	331	Ubc	Ubiquitin C
TAACTGACAA	63	147226	Mt2	Metallothionein 2
GCCTTTATGA	61	16775	Rps24	Ribosomal protein S24
GCACAACTTG	60	329243	Calm2	Calmodulin 2
CCCCAGCCAG	56	236868	Rps3	Ribosomal protein S3
CGCGTATGAG	56	239041	Dusp1	Dual specificity phosphatase 1
TGGTGGACCT	56	105218	Irf1	Interferon regulatory factor 1
TTTTCAAAAA	56	163	B2m	β-2 microglobulin
ATTCTCCAGT	55	140380	Rpl23	Ribosomal protein L23
CAAACTCTCA	54	291442	Sparc	Secreted acidic cysteine rich glycoprotein
TGGTCTGGTC	54	199381	Ifitm2	Interferon induced transmembrane protein 2
GCCCCCTTCC	53	1167	Junb	Jun-B oncogene
AACGGCTAAA	52	—	—	No match
GGATTTGGCT	52	341719	Rplp2	Ribosomal protein, large P2
TCAGTTTATT	52	218473	Tde1	Tumor differentially expressed 1
TAACTGCATT	51	264709	Serpin a3g	Serine (or cysteine) proteinase inhibitor, clade A, member 3G
AGAGCGAAGT	50	290786	Rpl41	Ribosomal protein L41
AGGTCGGGTG	49	180458	Rpl13a	Ribosomal protein L13a
GAGCGTTTTG	49	5246	Ppia	Peptidylprolyl isomerase A
TGGATCAGTC	49	323894	Rpl19	Ribosomal protein L19
TGGTGTAGGA	49	330160	Hspa5	Heat shock 70 kDa protein 5 (glucose-regulated protein)

The top 50 transcripts expressed in normal LECs are listed. The tag sequence represents the 10-bp SAGE tag. The most probable UniGene cluster matches are listed. Count indicates the number of times the tag was identified.

Northern blot analysis. Total RNA was extracted using Trizol reagent (Invitrogen). A 10 μg aliquot of total RNA was separated on a 1.2% agarose gel containing 2% formaldehyde and transferred to a positively charged nylon membrane (Roche Diagnostics). After UV irradiation, the membrane was hybridized with digoxigenin (DIG)-labeled cDNA probes at 42 °C overnight. Blots were then treated with alkaline phosphatase-conjugated anti-DIG antibody (Roche Diagnostics) and developed with CDP-star (New England Biolabs) according to the manufacture's instructions.

Results

Flow cytometric isolation of mouse LSECs

To obtain highly purified LSECs, we applied the flow cytometric method. hdNPCs prepared by centrifugation through Percoll were further fractionated by flow cytom-

Table 2
Top 50 of the most abundantly expressed genes in injured LECs

Tag sequence	Count	UniGene cluster	Symbol	Description
GTGGCTCACA	663	—	—	Multiple matches
GCTGCCCTCC	392	104368	Rpl32	Ribosomal protein L32
TTGCTGCCTT	359	—	—	Multiple matches
ATACTGACAT	330	353721	—	Transcribed sequence
ATAATACATA	259	200362	Cybb	Cytochrome *b*-245, β polypeptide
TATCTGTGCA	230	22699	Sepp1	Selenoprotein P, plasma, 1 tumor protein,
TGGGTTGTCT	227	297482	Tpt1	Translationally controlled 1
TTGTTGCTAC	213	—	—	No match
GGATATGTGG	201	181959	Egr1	Early growth response 1
AGCAGTCCCC	189	1011	Ncoa3	Nuclear receptor coactivator 3
TAGCTTTAAA	165	233470	Igfbp7	Insulin-like growth factor binding protein 7
ATGACTGATA	143	261851	—	Transcribed sequence
TAACTGACAA	140	147226	Mt2	Metallothionein 2
AGGCAGACAG	139	335315	Eef1a1	Eukaryotic translation elongation factor 1 α1
TGGTGGACCT	129	105218	Irf1	Interferon regulatory factor 1
GGCTTCGGTC	124	3158	Rplp1	Ribosomal protein, large, P1
TGGAAAGTGA	111	246513	Fos	FBJ osteosarcoma oncogene
CAGATCTTTG	105	331	Ubc	Ubiquitin C
GAATAATAAA	104	290774	Hspa8	Heat shock protein 8
GATTGAGAAT	103	—	—	Multiple matches
TGGTGTAGGA	103	330160	Hspa5	Heat shock 70 kDa protein 5 (glucose-regulated protein)
CAGTGCAACT	98	22699	Sepp1	Selenoprotein P, plasma, 1
GCCTTCCAAT	95	220038	Ddx5	DEAD (Asp-Glu-Ala-Asp) box polypeptide 5
TGTAACCTGA	90	272258	Dnase1 l3	Deoxyribonuclease 1-like 3
CTTTAGTGAC	89	—	—	Multiple matches
GTCAATGACG	89	18625	Aqp1	Aquaporin 1
TAACTGCATT	88	264709	Serpin a3g	Serine (or cysteine) proteinase inhibitor, clade A, member 3G
CCCCAGCCAG	85	236868	Rps3	Ribosomal protein S3
GATTGTCAGA	85	25613	Ier3	Immediate early response 3
GGGAGTGGGG	85	268933	Egfl7	EGF-like domain 7
CACAAACGGT	81	270283	Rps27	Ribosomal protein S27
CAAACTCTCA	78	291442	Sparc	Secreted acidic cysteine rich glycoprotein
TGGTTGCTGG	78	—	—	Multiple matches
GGATTTGGCT	77	341719	Rplp2	Ribosomal protein, large P2
TGGATCAGTC	76	323894	Rpl19	Ribosomal protein L19
AGGACAAATA	75	261750	—	RIKEN cDNA B430306D02 gene
GCCCCCTTCC	73	1167	Junb	Jun-B oncogene
CTAGTCTTTG	72	354387	Rps29	Ribosomal protein S29
GTTTGCTGTG	71	236553	Ctsb	Cathepsin B
AACAATTTGG	70	289868	Rpl9	Ribosomal protein L9
ATTCTCCAGT	70	140380	Rpl23	Ribosomal protein L23
GACCTGGAGC	68	43778	Rps14	Ribosomal protein S14
GCCCGGGAAT	67	276337	Rpl17	Ribosomal protein L17
CCCTGGGTTC	65	—	—	Multiple matches
GATTCCGTGA	65	10474	Rpl37	Ribosomal protein L37
GTAAGCATAA	65	282093	Ubb	Ubiquitin B
TCAGTTTATT	65	218473	Tde1	Tumor differentially expressed 1
TGTAGTGTAA	65	260904	Rps8	Ribosomal protein S8
TTGGTGAAGG	63	142729	Tmsb4x	Thymosin, β4, X chromosome
ATGTCTCAAA	62	209290	Tuba2	Tubulin, α2

The top 50 transcripts expressed in injured LECs are listed. The tag sequence represents the 10-bp SAGE tag. The most probable UniGene cluster matches are listed. Count indicates the number of times the tag was identified.

etry using antibodies against CD45 (leukocyte common antigen) and CD16/32 (receptors for the Fc fragment of IgG). LSECs were isolated as the $CD45^-$ $CD16/32^+$ cells; $61.2 \pm 6.8\%$ (mean $\pm$ SD) and $31.0 \pm 2.3\%$ of hdNPCs were $CD45^-$ $CD16/32^+$ in normal and injured liver, respectively (Fig. 1A). The purity of isolated LSECs was further assessed by the expression of marker proteins and by uptake of DiI-labeled acetylated LDL (DiI-AcLDL). $CD45^-$ hdNPCs did not express CD11b (Mac-1; monocyte/macrophage marker), indicating that Kupffer cells were completely eliminated (Fig. 1B). Almost all $CD16/32^+$ hdNPCs expressed CD31 (PECAM-1; endothelial marker) (Fig. 1B), consistent with the previously described phenotypic characteristics of murine LSECs [19–21]. Furthermore, most $CD45^-$ $CD16/32^+$ cells incorporated DiI-AcLDL (>97%) and contained few desmin-positive stellate cells (<1%) (data not shown). These results indicated that the $CD45^-$ $CD16/32^+$ cells we prepared were a highly pure population of LSECs.

Abundant SAGE tags in normal and injured LSECs

We obtained 32,867 and 37,493 tags from normal LSECs (nLSECs) and injured LSECs (iLSECs), respectively, allowing identification of 6011 unique transcripts that appeared more than two times. The GenBank database was used to identify individual genes. Tables 1 and 2 list the 50 most abundant tags expressed in normal and injured LSECs, respectively. As expected, a large fraction of these abundant tags matched to housekeeping genes such as ribosomal proteins. There were some abundantly expressed transcripts encoding secretory proteins: Sepp1 (selenoprotein P, plasma, 1), Igfbp7 (insulin-like growth factor binding protein 7), Egfl7 (EGF-like domain 7), Sparc (secreted acidic cysteine rich glycoprotein), and transcription factors: Egr-1 (early growth response 1), Irf1 (interferon regulatory factor 1), Fos (FBJ osteosarcoma oncogene), and Jun-B (Jun-B oncogene).

Comparison of expression profiles of normal and injured LSECs

The 32,867 and 37,493 tags from the nLSEC and iLSEC libraries, respectively, were normalized to 32,867, and then compared with each other. Although the expression levels of most transcripts in these cells were similar (Fig. 2), 75 transcripts were expressed at significantly different levels ($P < 0.01$), i.e., 35 and 40 transcripts among them were expressed at a higher level in iLSECs and nLSECs, respectively. Table 3 shows the list of differentially expressed genes ($\geqslant$ twofold and $P < 0.01$ differences) excluding tags with multiple matches. The transcripts increased in iLSECs included secretory proteins: Cxcl10 (chemokine ligand 10), Inhbb (inhibin β-B), and Tpbpb (trophoblast specific protein β), a membrane receptor: Il4ra (interleukin 4 receptor, α),

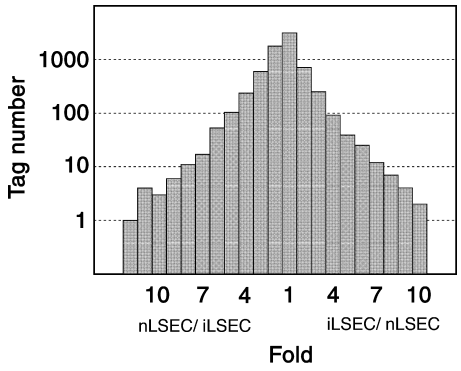

Fig. 2. Comparison of gene expression levels in normal and injured LSECs. The distribution of all SAGE tags was plotted on a semilogarithmic plot. The total number of tags in each library was normalized to 32,867. The fold induction in nLSECs or iLSECs was determined by dividing the number of tags in nLSECs by that in iLSECs and vice versa. To avoid division by 0, we used a tag value of 1 for any tag that was not detectable in one of the samples. We then rounded off these ratios to the nearest integer; their distribution is plotted on the abscissa. The number of tags displaying each ratio is plotted on the ordinate.

enzymes: Npl (*N*-acetylneuraminate pyruvate lyase), Tgm2 (transglutaminase 2, C polypeptide), and Pgk1 (phosphoglycerate kinase 1), and a transcription factor: Irf1, which might play a role in the regenerating processes. The transcripts decreased in iLSECs included membrane proteins: KDR (kinase insert domain protein receptor), Nrp (neuropilin), and Esam1 (endothelial cell-specific adhesion molecule), secretory proteins: Gas6 (growth arrest specific 6), Sepp1, Igfbp4 (insulin-like growth factor binding protein 4), and Ltbp4 (latent TGF-β binding protein 4), and transport-related proteins: Vps35 (vacuolar protein sorting 35), Ap1b1 (adaptor protein complex AP-1, β1 subunit), and Ehd3 (EH-domain containing 3).

To validate the SAGE results, quantitative real-time PCR (Q-PCR) assays for selected transcripts were performed on total RNA isolated from normal and injured LSECs (Fig. 3). Results obtained by SAGE and Q-PCR were consistent, though the levels of differences detected by the two methods were not exactly the same. To examine the expression patterns during liver regeneration, Northern blot analyses for selected transcripts were performed on total RNA isolated from whole liver, hepatocytes, and NPCs (Fig. 4). Even though the NPCs contain some cells other than LSECs, the results were consistent with the SAGE data. Interestingly, there were some genes, Npl, Tpbpb, and Inhbb, that were transiently induced during liver regeneration only in NPCs and not hepatocytes.

Identification of markers for LSEC

To identify genes preferentially expressed in LSEC, we compared our SAGE libraries with other libraries

Table 3
Transcripts differentially expressed in normal and injured LECs

Tag sequence	Count		UniGene cluster	Symbol	Description
	Normal	Injured			
Upregulated in injured LECs					
Membrane and secretory proteins					
TACAGTATAA	0	9	3092	Inhbb	Inhibin β-B
ATGTCTTCAA	0	9	30144	Tpbpb	Trophoblast specific protein β
CAATAAAACT	2	21	30144	Tpbpb	Trophoblast specific protein β
TAAATGATAA	2	13	1421	Adamts1	A disintegrin-like and metalloprotease with thrombospondin type 1 motif, 1
GAGCTTTATG	4	23	233802	Il4ra	Interleukin 4 receptor, α
TTTATGTTGA	5	19	281642	Maea	Macrophage erythroblast attacher
CCCAGGCTGC	19	43	877	Cxcl10	Chemokine (C-X-C motif) ligand 10
Cytoplasmic proteins					
AAGATTTCTC	0	9	168	Ifrd1	Interferon-related developmental regulator 1
GATGTACTAG	1	11	153684	Sertad1	SERTA domain containing 1
ATTCCTACCC	1	11	6718	Ifit1	Interferon-induced protein with tetratricopeptide repeats 1
AACAAAATCT	1	10	3903	Rasd1	RAS, dexamethasone-induced 1
CTCTGAAAAG	2	13	260618	Txn1	Thioredoxin 1
AGAGAGCGTT	4	17	257765	Clic4	Chloride intracellular channel 4 (mitochondrial)
TATTGTGGCT	6	25	195663	Cdkn1a	Cyclin-dependent kinase inhibitor 1A (P21)
GACCTGGAGC	22	59	43778	Rps14	Ribosomal protein S14
AGGAAGGCGG	16	42	11376	Rpl36	Ribosomal protein L36
GAATAATAAA	36	90	290774	Hspa8	Heat shock protein 8
TGAAGGAACA	15	37	13886	Sui1-rs1	Suppressor of initiator codon mutations, related sequence 1
TGGTGGACCT	55	112	105218	Irf1	Interferon regulatory factor 1
Enzymes					
ACGCTGGTCT	0	9	24887	Npl	*N*-Acetylneuraminate pyruvate lyase
TGAACACTGA	5	50	330731	Tgm2	Transglutaminase 2, C polypeptide
GCAATCTGAT	3	18	336204	Pgk1	Phosphoglycerate kinase 1
TTCTGGGCTC	7	23	17932	Pnp	Purine-nucleoside phosphorylase
GCCTCCAAGG	11	29	333399	Gapd	Glyceraldehyde-3-phosphate dehydrogenase
Downregulated in injured LECs					
Membrane proteins					
TTTAGTTTCT	7	0	27363	Ppap2b	Phosphatidic acid phosphatase type 2B
ACCCAAGAAT	7	0	285	Kdr	Kinase insert domain protein receptor
CAAATGCGAT	10	1	41751	Esam1	Endothelial cell-specific adhesion molecule
TTTTGTACCC	11	2	271745	Nrp	Neuropilin
CACAGCCCAC	30	6	266885	—	DNA segment, Chr 11, ERATO Doi 736, expressed
TGACCTGTAG	23	6	271745	Nrp	Neuropilin
GTGTAGATGC	21	8	27363	Ppap2b	Phosphatidic acid phosphatase type 2B
GAATATGCAG	29	13	285	Kdr	Kinase insert domain protein receptor
Secretory protein					
CTTCTTTCTG	9	1	3982	Gas6	Growth arrest specific 6
CATCGCCAGT	121	40	305152	Apoe	Apolipoprotein E
GAAGCCAACC	37	13	22699	Sepp1	Selenoprotein P, plasma, 1
AAGTCACCAG	24	9	233799	Igfbp4	Insulin-like growth factor binding protein 4
CCCAGTCCCT	32	14	272251	Ltbp4	Latent transforming growth factor β binding protein 4
Protein transport related					
TCAGCTGAAT	11	1	195451	Gosr2	Golgi SNAP receptor complex member 2
GTCAGAAGCA	9	1	296520	Vps35	Vacuolar protein sorting 35
CTTCTGGTGT	18	4	274816	Ap1b1	Adaptor protein complex AP-1, β1 subunit
TTAATACAAC	29	10	18526	Ehd3	EH-domain containing 3
Miscellaneous					
ATTTGCACAC	10	0	292510	Rac1	RAS-related C3 botulinum substrate 1
GCTACGATGT	8	0	286863	—	RIKEN cDNA 2810025M15 gene
TTTGTTGTGG	9	1	—	—	No match
CCTGTGTGAA	30	7	255909	Sdpr	Serum deprivation response
GCGACAGAGG	23	6	2121	—	DNA segment, Chr 12, ERATO Doi 647, expressed
GCTATGGAGA	26	7	133825	Crip2	Cysteine rich protein 2
GTTAAAGGAA	29	12	33450	—	Transcribed sequences
CCAGAGTCAG	27	12	34379	—	cDNA sequence BC054438
GGCAGCCCCC	35	16	260878	—	RIKEN cDNA 2410026K10 gene
TTGGTGAAGG	114	54	142729	Tmsb4x	Thymosin, β4, X chromosome

Only tags with $\geqslant$twofold and $P < 0.01$ differences are shown. Not shown are tags with multiple matches. The tag sequence represents the 10-bp SAGE tag. The most probable UniGene cluster are listed. Count indicates the number of times the tag was identified. In this table, each tag count was normalized using SAGE software to 32,867.

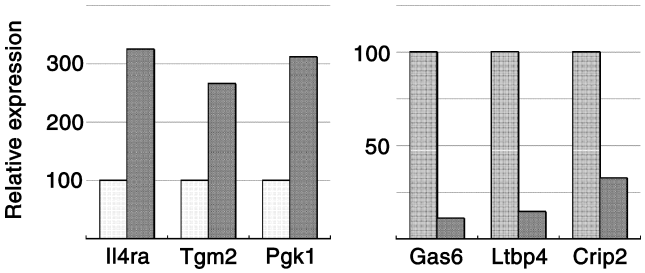

Fig. 3. Real-time quantitative PCR analyses. cDNA was synthesized from total RNA of LSECs isolated from normal or injured liver by FACS-vantage. Quantitative PCR was performed with a LightCycler. Because the expression level of Gapd (glyceraldehyde-3-phosphate dehydrogenase), which is generally used as an internal control, was different between normal and injured liver (Table 3), we normalized the signals with the Vezf1 level, which is a zinc finger-containing protein expressed specifically within endothelial cells [39], which was expressed at a similar level in normal and injured LSECs (data not shown). It is already known that the expression of housekeeping genes is altered by CCl_4 treatment [40]. Relative expression levels (the expression level in normal LECs = 100) are shown.

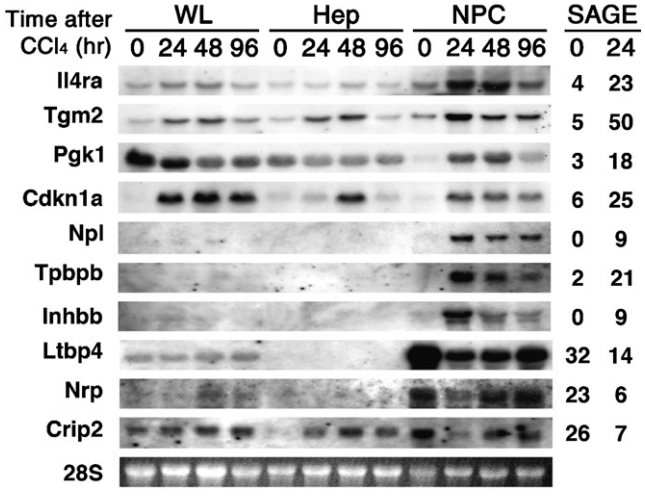

Fig. 4. Northern blot analysis of selected transcripts that were up- or down-regulated during liver regeneration. Total RNA was extracted from the whole liver (WL), hepatocytes (Hep), and nonparenchymal cells (NPCs) at various time points after injury as indicated. Each lane was loaded with 10 μg of total RNA from each sample. Corresponding SAGE data are as shown; numbers in left and right lanes show normal and injured LSECs, respectively.

generated from various mouse tissues and cell lines that have been publicly available to date (77 libraries/August, 2004) [22]. By normalizing the total tags in each library to one million, we selected the tags that numbered more than 300 in the nLSEC library, i.e., the transcripts abundantly expressed in nLSEC. To sort out the transcripts preferentially or specifically expressed in nLSEC, we calculated three kinds of values, as follows: (1) the expression ratio between nLSEC and each of other libraries, ratio X_{TagY} = count of tagY in library X/count of tagY in nLSEC, (2) specificity index (SI), $\mathrm{SI}_{\mathrm{TagY}} = \Sigma_{x=\mathrm{all}}$ ratio X_{TagY}, and (3) the number of libraries (NL), $\mathrm{NL}_{\mathrm{TagY}}$ = the number of libraries, except nLSEC and iLSEC, which contained TagY. If the values of a tag of interest are low, its LSEC specificity is high. We found that 24 transcripts in Table 4 met the following criteria: (1) ratio $X_{\mathrm{TagY}} < 0.5$, (2) $\mathrm{SI}_{\mathrm{TagY}} < 1$, and (3) $\mathrm{NL}_{\mathrm{TagY}} < 10$. Eleven of them had been previously shown to be preferentially expressed in endothelium (footnote a in Table 4), indicating that this simple comparison actually allows the identification of markers for endothelium. Among the rest of the transcripts, there were four transcripts that had not been well characterized (footnote b in Table 4), suggesting that these genes are possibly new markers for LSEC.

Discussion

In this study we applied SAGE, a powerful and comprehensive method, to examine the transcriptome of LSECs, a unique class of endothelial cells which have been poorly characterized at the molecular level. While various kinds of gene expression analyses were performed in the liver, most of these analyses were performed using whole liver tissue [23–26]. As the liver consists of many distinct cell populations [9], the expression profiles of whole liver provide little information as to the nature and function of each cell population. Thus, it is crucial to use a pure cell population for gene expression analysis in order to describe the characteristics of the cell type of interest. In this study, we purified LSECs for gene expression analyses by using a combination of centrifugation and expression of cell surface antigens, CD45 and CD16/32.

By comparing our SAGE data with 77 public libraries generated from various mouse tissues and cell lines, we found several candidates for an LSEC marker (Table 4). Among them, stabilin 2 was recently reported to be expressed in sinusoidal endothelia in liver, lymph node, spleen, and bone marrow, but not in other endothelia [27]. Those genes specifically expressed in a subset of endothelial cells will be useful to study their specific properties and the differentiation pathway from their progenitors. As monoclonal antibodies against such proteins will be necessary to distinguish LSECs from other types of endothelial cells and for isolation of LSECs, we are currently making such antibodies.

Recently, it has been reported that LSEC produces a series of mitogenic/survival factors that can protect parenchymal cells from injury and initiate regeneration [13], but the detailed molecular mechanisms remain unknown. We attempted to identify genes differentially expressed in LSECs in normal and injured liver. In the CCl_4-induced acute liver injury model, liver damage and DNA replication of hepatocytes peaked at around 48 h after CCl_4 treatment [8]. If LSECs release some mitogenic/survival factors for parenchymal cells, they might be expressed prior to the peak of liver damage

Table 4
High abundance transcripts in normal and injured LECs relative to other SAGE catalogs

Tag sequence	Count		Specificity index	Number of libraries	UniGene cluster	Symbol	Description
	Normal	Injured					
Membrane proteins							
CTGCACTGTG	1187	743	0.118	5	2857	Admr	Adrenomedullin receptor [a]
TGGTGTACTG	1034	690	0.503	8	46367	Gpihbp1	GPI-anchored HDL-binding protein 1[a]
TTTCCAGGAC	548	398	0.471	3	279611	Stab2	Stabilin 2[a]
GCCAGGTCAG	487	238	0.374	7	280563	—	RIKEN cDNA 1200003C23 gene [b]
AAGGAATACT	396	159	0.934	5	1151	Dpp4	Dipeptidylpeptidase 4[a]
GTTGAATAAA	335	106	0.066	2	12862	Pcdh12	Protocadherin 12[a]
CAAATGCGAT	335	53	0.257	5	41751	Esam1	Endothelial cell-specific adhesion molecule[a]
CAGAAAGGTC	335	238	0.704	7	272673	Mmrn2	Multimerin 2[a]
GCGATTCACA	335	371	0.681	6	23845	C1qtnf1	C1q and tumor necrosis factor related protein 1
Secretory proteins							
GAAGGTTGCT	639	663	0.532	9	235230	Bmp2	Bone morphogenetic protein 2
CCCAGGCTGC	609	1327	0.138	5	877	Cxcl10	Chemokine (C-X-C motif) ligand 10[a]
GCAACTCTTA	578	451	0.129	3	1805	F8	Coagulation factor VIII[a]
TAACTATCAA	487	371	0.970	5	15534	Il1a	Interleukin 1 α
ATCTCCTAGA	304	212	0.110	1	123013	Oit3	Oncoprotein induced transcript 3
Nucleus proteins							
GAACAGTTAT	365	451	0.329	6	42162	Sox7	SRY-box containing gene 7[a]
CGGCCTTCTC	335	212	0.666	9	292415	Ets1	E26 avian leukemia oncogene 1, 5′ domain[a]
TGACCTTTAT	304	371	0.290	2	11	Myf6	Myogenic factor 6
Cytoplasmic proteins							
TGTAACCTGA	4412	2388	0.106	4	272258	Dnase1l3	Deoxyribonuclease 1-like 3
CCCTGTGGGG	309	610	0.360	8	24433	Sumo3	SMT3 suppressor of mif two 3 homolog 3 (yeast)
Miscellaneous							
TAATTAGACA	304	371	0.036	1	34874	Ushbp1	Usher syndrome 1C binding protein 1
GTACAGTGTA	1034	796	0.830	7	89848	—	Transcribed sequences[b]
GTTAAAGGAA	913	371	0.492	7	33450	—	Transcribed sequences [b]
CAGATAGCTT	1278	928	0.610	4	—	—	No match [b]

Tags expressed at higher levels in normal LSEC compared with other libraries are listed. Not shown are tags with multiple matches. The tag sequence represents the 10-bp SAGE tag. Count indicates the number of times the tag was identified. In this table, each tag count was normalized to 1,000,000 using SAGE software. The most probable UniGene cluster matches are listed.

[a] Characterized genes that have already been shown to be expressed predominantly in endothelium.

[b] Transcripts which had not been well characterized.

and hepatocyte proliferation. We therefore examined the gene expression profile of LSEC at 24 h after CCl_4 administration.

We obtained SAGE data of LSECs from normal and injured liver, and found that several genes are up- or down-regulated by liver injury, including those encoding secretory proteins. Although we did not find up-regulation of secretory proteins that are expected to positively regulate the proliferation or survival of parenchymal cells, we observed decreased expression of Igfbp4 [28] and Ltbp4 [29], which might be implicated in the negative regulation of parenchymal cell proliferation. These results suggest that LSECs indirectly regulate the proliferation of parenchymal cells by reducing the inhibitory effects on proliferation.

At 24 h after CCl_4 administration, we observed increased expression of transcripts that are known to inhibit endothelial cell proliferation, such as Cdkn1a (cyclin-dependent kinase inhibitor 1A (P21)) [30], Pgk1 [31], and Irf1 [32]. Moreover, we also observed decreased expression of transcripts encoding growth factor receptors, such as Kdr and Nrp. These results suggest that proliferation of LSECs is restricted in this period, in agreement with the fact that Brd-U-labeled NPCs reach a peak around 72 h after CCl_4 treatment [33].

Among the differentially expressed genes during liver regeneration, 40 transcripts were downregulated in injured LSECs. Interestingly, we noted that some of them, including Gosr2 (Golgi SNAP receptor complex member 2) [34], Vps35 [35], Ap1b1 [36], and Ehd3 [37] (Table 3), are related to protein transport, such as endosomal and lysosomal protein sorting. LSECs are known to have a high endocytic capacity to clear a variety of macromolecular wastes from the bloodstream [10]. Our SAGE results suggest that such endocytic capacity in LSECs is lower in injured liver, consistent with the increased serum levels of connective tissue macromolecules, such as hyaluronan and *N*-terminal propeptide of collagen type III, in liver disease [38].

Gene expression profiles provide insight into the nature and function of cells. In particular, much information can be obtained when a pure cell population is used for the analysis. In this study, we show the gene expression profile of LSEC in normal and CCl_4-injured liver. By extending this approach to different time points and different liver injury models as well as other liver cell types such as Kupffer cells and stellate cells, it will be possible to understand the liver regeneration process. The SAGE data in this study have been deposited in the SAGEmap/Gene Expression Omnibus database (http://www.ncbi.nlm.nih.gov/geo/info/linking/, GEO Accession No.: GSE1689, GSM29039; normal LSEC, GSM29040; and injured LSEC).

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