Supporting Information

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SI Text

Web-Based siRNA Design Protocols Targeting Claudin-5. Sequences of the claudin-5 siRNA used in this study were from Dharmacon and were as follows (1). Sense sequence: CGUUGGAAAUUCUGGGUCUUUdTdT. Antisense sequence: AGACCCAGAAUUUCCAACGUUdTdT. NT control siRNA targeting luciferase was used as an NT control. Sense sequence: cuuacgcugaguacgucgadTdT. Antisense sequence: ucgaaguacucagcguaagdTdT.

In Vivo Delivery of siRNA to Retinal Capillary Endothelial Cells. Mice weighing 20–30 g were individually restrained inside a 60-mL volume plastic tube. The protruding tail was warmed for 5 min before injection under a 60-W lamp, and the tail vein was clearly visualized by illumination from below. A total of 20 μg of targeting siRNA, NT siRNA made up with PBS to a volume in milliliters of 10% of the body weight in grams, or PBS alone was injected into the tail vein at a rate of 1 mL/sec by using a 26-gauge (26G 3/8) needle. After 24, 48, and 72 h, protein was isolated from total retinal tissue by homogenization in lysis buffer containing 62.5 mM Tris, 2% SDS, 10 mM DTT, and 10 μL of protease inhibitor mixture per 100 mL (Sigma–Aldrich). The homogenate was centrifuged at 10,000 $\times$ *g* for 20 min at 4 °C, and the supernatant was removed for claudin-5 analysis (2).

Indirect Immunostaining of Retinal Flatmounts and Retinal Cryosections. After fixation of eyes with 4% paraformaldehyde (pH 7.4) for 4 h at room temperature and three subsequent washes with PBS, retinal cryosections were blocked with 5% normal goat serum (NGS) in PBS for 20 min at room temperature. Rabbit anti-claudin-5 (Zymed) was incubated on sections overnight at 4 °C. After incubation, sections were washed three times in PBS and subsequently blocked again with 5% NGS for 20 min at room temperature. Secondary rabbit IgG-Cy3 (Jackson Immunoresearch) antibodies were incubated with the sections at 37 °C for 2 h, followed by three washes with PBS. All sections were counterstained with DAPI for 30 sec at a dilution of 1:5,000 of a stock 1 mg/mL solution. Analysis of stained sections was performed at room temperature with an epifluorescence microscope (Zeiss Axioplan 2) or confocal microscopy (Olympus FluoView TM FV1000).

For flatmount analysis, neural retinas were dissected from the eye and permeabilized for 6 h with PBS/0.5% Triton X-100. Retinas were blocked overnight in 5% NGS in PBS/0.5% Triton X-100. Retinas were subsequently incubated with rabbit anti-claudin-5 (Zymed), 1:100 dilution, for 48 h, followed by six washes with PBS/0.5% Triton X-100. Retinas were incubated for 6 h at 37 °C with secondary rabbit IgG-Cy3 (Jackson Immunoresearch), and after 10 washes with PBS/0.5% Triton X-100, retinas were flatmounted and analyzed by using an Olympus FluoView TM FV1000 confocal microscope.

ERG Analysis of C57/Bl6 and IMPDH1$^{-/-}$ Mice. IMPDH1$^{-/-}$ mice on a C57/Bl6 background were dark-adapted overnight and prepared for ERG under dim red light. Pupillary dilation was carried out by instillation of 1% cyclopentolate and 2.5% phenylephrine. Animals were anesthetized by i.p. injection of ketamine (2.08 mg per 15 g body weight) and xylazine (0.21 mg per 15 g body weight). Standardized flashes of light were presented to the mouse in a Ganzfeld bowl to ensure uniform retinal illumination. The ERG responses were recorded simultaneously from both eyes by means of gold wire electrodes (Roland Consulting) using Vidisic (Dr. Mann Pharma) as a conducting agent and to maintain corneal hydration. Reference and ground electrodes were positioned s.c. at $\approx$1 mm from the temporal canthus and anterior to the tail, respectively. Body temperature was maintained at 37 °C by using a heating device controlled by a rectal temperature probe. Responses were analyzed by using a RetiScan RetiPort electrophysiology unit (Roland Consulting). The protocol was based on that approved by the International Clinical Standards Committee for human ERG. Cone-isolated responses were recorded by using a white flash of intensity 3 candelas/m^{-2} per sec presented against a rod-suppressing background light of 30 candelas/m^{-2}, to which the previously dark-adapted animal had been exposed for 10 min before stimulation. The responses to 48 individual flashes, presented at a frequency of 0.5 Hz, were computer-averaged. Following the standard convention, a waves were measured from the baseline to a-wave trough, and b waves from the a-wave trough to the b-wave peak. On the day of GTP injection, mice were anesthetized and administered an i.p. injection of a solution containing 3.3 mg of GTP (BioChemika 51123; Sigma–Aldrich), and retinal function subsequently analyzed by ERG.

Assessment of iBRB Integrity by Perfusion of Tracer Molecules. After perfusion with the tracer molecule, eyes were placed in 4% paraformaldehyde (pH 7.4) for 4 h at room temperature and subsequently washed three times with PBS. After cryoprotection using a 10%, 20%, and 30% sucrose gradient, 12-μm frozen sections were cut on a cryostat at −20 °C, and microperoxidase was visualized by incubation with AEC developing solution (Sigma–Aldrich) for 10 min. This allowed for the assessment of paracellular diffusion across the iBRB of a compound with a molecular mass of $\approx$1,900 Da.

TUNEL Analysis. Eyes from mice were fixed in 3.5% formaldehyde for 4 h, followed by three washes in PBS. Eyes were cryoprotected by using a sucrose gradient (10%, 20%, and 30%), and subsequently, 12-μm sections were cut by using a cryostat. To detect cell death, sections were then incubated with staining mix (in situ cell death detection kit, TMR red; Roche) according to the manufacturer's instructions for 1 h at 37 °C in the dark, followed by two washes in PBS for 5 min each in the dark. Nuclei were counterstained with DAPI (1:10,000 in PBS) and mounted by using Aqua-Polymount mounting medium (Polysciences). Sections were visualized at room temperature by using a fluorescent microscope (Zeiss Axioplan 2) with integrated software. Three to five mice in each experimental group were analyzed, with data being expressed as mean TUNEL-positive cells per section $\pm$ SEM, as determined by an individual blinded to the treatment. Results were analyzed by using a two-tailed Student's *t* test, with $P < 0.05$ considered significant.

Light Ablation of BALB/c Mice. All animals were previously kept in cyclic light (12 h of light and 12 h of dark; 60 lux) before all experimentation. Three-month-old BALB/c mice were injected with either targeting or NT siRNA by hydrodynamic tail vein injection as described above and subsequently with ALLM i.p. (20 mg/kg; Calbiochem). Mice were dark-adapted for 24 h before being exposed to constant light. Immediately before light exposure, their pupils were dilated with 1% cyclopentolate and 2.5% phenylephrine. Mice then were placed in cages with reflective sides and exposed to constant white light of 7,900 lux for 2 h. The

mice were returned to the dark room and killed by CO_2 at 12, 24, and 48 h and at 4 and 7 days after the initial 2 h of light exposure.

MRI. Mice were anesthetized with isofluorane and were physiologically monitored (ECG, respiration, and temperature) and placed on an MRI-compatible support cradle, which has a built-in system for maintaining the animal's body temperature at 37 °C. The cradle was then positioned within the MRI scanner. Accurate positioning was ensured by acquiring an initial rapid pilot image, which was then used to ensure the correct geometry was scanned in all subsequent MRI experiments. Upon insertion into the MRI scanner, T2-weighted images were acquired (resolution, $0.141 \times 0.141 \times 5$ mm^3; matrix, $128 \times 128 \times 36$; TR/TE, 4,179.3/36 ms; flip angle, 90°; acquisition time, 1 min 6 sec). iBRB integrity was then visualized in high-resolution T1-weighted MR images (resolution, $0.156 \times 0.156 \times 5$ mm^3; field of view = $20 \times 20 \times 17.9$ mm^3; matrix, $128 \times 128 \times 30$; TR/TE, 500/2.7 ms; flip angle, 30°; number of averages, 3; acquisition time, 2 min 24 sec; repetitions, 10) before and after injection of contrast agent Gd-DTPA. A 0.1 mM/L per kg bolus of Gd-DTPA was administered via the tail vein. After injection of Gd-DTPA, repeated T1-weighted scans were performed over a period of 24 min, and images shown are representative of the final scans of this 24-min period. Statistical analysis of densitometric results after MRI were from combined regions of the whole eye and were performed by using ANOVA, with significance represented by $P \leq 0.05$. All MRI scans were performed on at least three mice from each experimental treatment.

Microarray Analysis of Transcriptional Changes in Mouse Brain After Transient Suppression of Claudin-5. Isolated brain samples were analyzed for changes in global gene expression after transient suppression of claudin-5. Analysis was performed on Affymetrix GeneChips, which represent the full complement of mouse genes. Analysis was undertaken 48 h after injection of siRNA. Uninjected mouse brains were used as a baseline for comparison, and an NT siRNA-injected group was used as an additional comparison control. Each analysis was carried out by using five replicates. RNA was extracted from brains, converted to labeled cRNA, and stringently assessed for quality. Fragmented cRNA samples were then hybridized to the arrays for 16 h and were stained and scanned by using a GeneChip Scanner 3000. Expression data were generated by using Affymetrix GCOS software. After normalization, expression data were analyzed by using a variety of bioinformatics approaches, including fold change analysis, ANOVAS, and clustering.

Statistical Analyses. Statistical analysis was performed by using Student's t test, with significance represented by $P \leq 0.05$ (n = 5 mice per treatment). For multiple comparisons, ANOVA was used with a Tukey–Kramer posttest, and significance was represented by $P \leq 0.05$.

1. Reynolds A, et al. (2004) Rational siRNA design for RNA interference. *Nat Biotechnol* 22:326–330.

2. Campbell M, et al. (2008) RNAi-mediated reversible opening of the blood-brain barrier. *J Gene Med* 10:930–947.

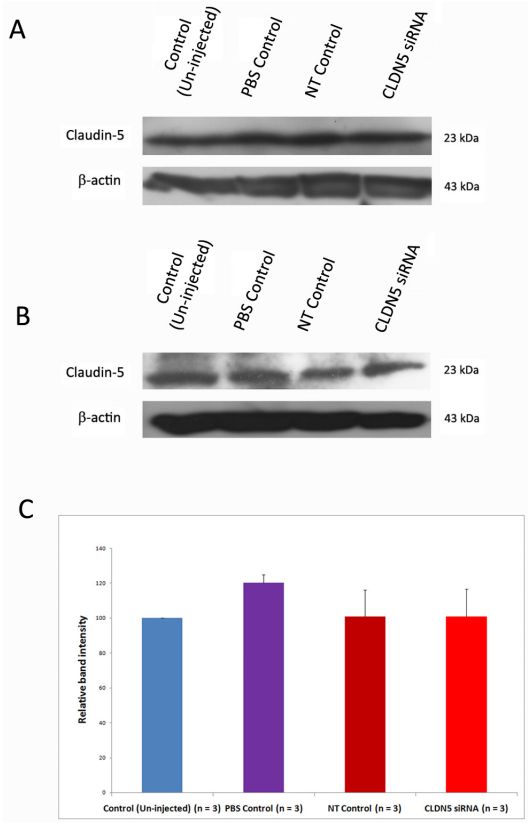

Fig. S1. Western blot analysis of claudin-5 levels in the neural retinas of mice 24 h after injection of siRNA showed no significant changes in levels of expression (*A* and *B*). (*C*) Densitometric analysis of Western blots is representative of three independent experiments.

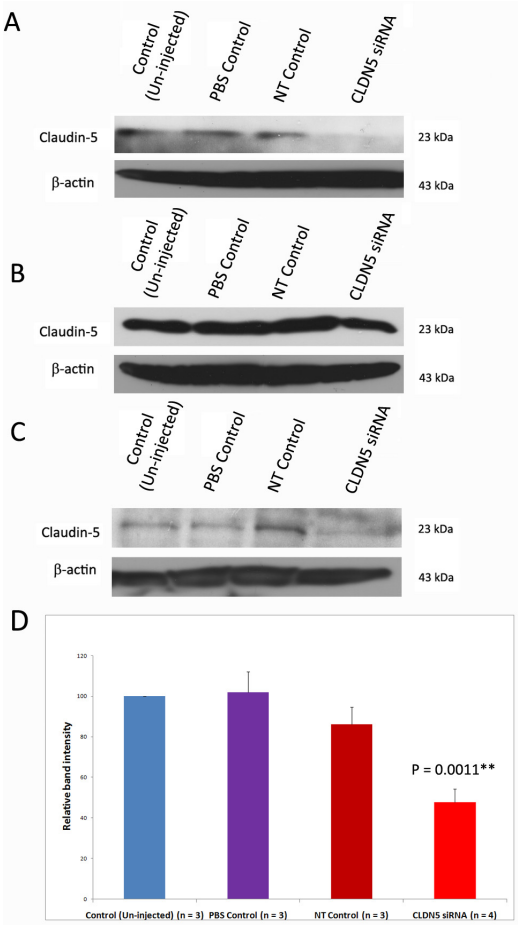

Fig. S2. Western blot analysis of claudin-5 levels in the neural retinas of mice 48 h after injection of siRNA showed a significant decrease in levels of expression of claudin-5 (*A*–*C*). **, $P = 0.0011$. (*D*) Densitometric analysis of Western blots is representative of four independent experiments, and on average there was ≈52.2% suppression in the claudin-5 siRNA-injected groups when band intensities were taken as a ratio of the corresponding β-actin bands.

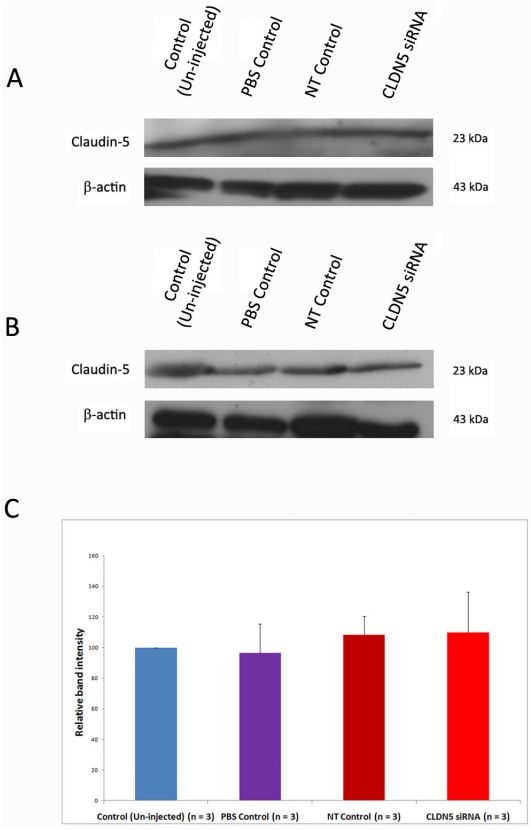

Fig. S3. Western blot analysis of claudin-5 levels in the neural retinas of mice 72 h after injection of siRNA showed no significant changes in levels of expression (*A* and *B*). (*C*) Densitometric analysis of Western blots is representative of 3 independent experiments.

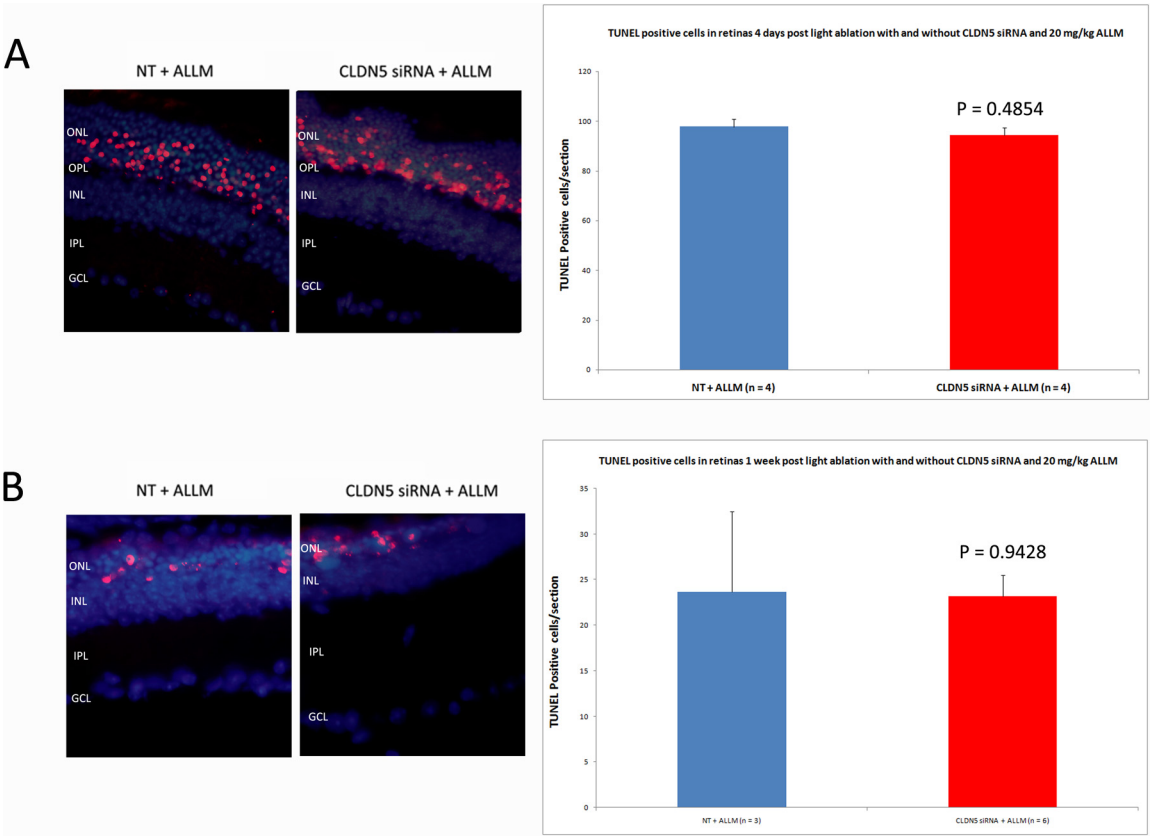

Fig. S4. Enhanced delivery of a calpain inhibitor to retinas of light-exposed BalB/c mice. Effects of either an NT or claudin-5 siRNA together with a potent calpain inhibitor, ALLM, on light-induced retinal degeneration were assessed in BalB/c mice. Claudin-5 or NT siRNA was administered to BALB/c mice 48 h before injection of 20 mg/kg ALLM. Mice were dark-adapted for 24 h before being exposed to white fluorescent light of 7,900 lux for 2 h. Subsequently, retinas were analyzed for TUNEL positivity 4 days and 1 week after light exposure. (*A*) At the 4-day time point after light exposure, there were no differences in the number of TUNEL-positive cells observed in mice receiving NT siRNA plus ALLM and mice receiving CLDN5 siRNA plus ALLM ($P = 0.4854$). (*B*) At the 1-week time point after light exposure, there were no differences in the number of TUNEL-positive cells observed in mice receiving NT siRNA plus ALLM and mice receiving CLDN5 siRNA plus ALLM ($P = 0.9428$).

Table S1. Microarray analysis of differentially regulated genes in neuronal tissue 48 h after injection of claudin-5 siRNA

Affymetrix probe IDs	Control intensity	Claudin-5 siRNA intensity	Log ratio	Log error	Fold change	*P*	Accession no.	Primary sequence name	Sequence description
1456993_at	0.16	0.06	−0.43	0.13	−2.72	0.0049	BB390674	D2Ertd640e	DNA segment, Chr 2, ERATO Doi 640, expressed
1440014_at	0.06	0.02	−0.40	0.12	−2.53	0.0046	AW553510	—	—
1419175_a_at	0.04	0.02	−0.35	0.10	−2.22	0.0012	NM_013483	Btn1a1	Butyrophilin, subfamily 1, member A1
1458568_at	0.05	0.02	−0.31	0.10	−2.05	0.0006	BB427942	—	—
1440628_at	0.16	0.08	−0.31	0.06	−2.03	0.0010	BB309880	—	Transcribed locus, weakly similar to NP_001041402.1 hypothetical protein LOC499136 (*Rattus norvegicus*)
1446366_at	0.08	0.04	−0.29	0.10	−1.94	0.0004	BB426020	Trpc4	Transient receptor potential cation channel, subfamily C, member 4
1444722_at	0.04	0.02	−0.28	0.08	−1.89	0.0042	BG075584	—	Transcribed locus
1429344_at	0.17	0.09	−0.27	0.06	−1.88	0.0005	BB357317	9.13002E+15	Hypothetical 9130022E09
1443489_at	0.06	0.03	−0.27	0.08	−1.85	0.0001	BB158810	—	—
1429902_at	0.10	0.06	−0.26	0.10	−1.82	0.0018	BB165850	5830443J22Rik	RIKEN cDNA 5830443J22 gene
1425831_at	0.10	0.06	−0.24	0.06	−1.73	0.0021	BC002058	Zfp101	Zinc finger protein 101
1420230_at	0.08	0.05	−0.24	0.08	−1.72	0.0011	AA414993	AA414993	Expressed sequence AA414993
1442223_at	0.13	0.08	−0.23	0.07	−1.69	0.0023	AV329519	Enah	Enabled homolog (*Drosophila*)
1457167_at	0.10	0.06	−0.22	0.06	−1.65	0.0019	BB140799	Med14	Mediator complex subunit 14
1446364_at	0.18	0.11	−0.21	0.07	−1.64	0.0030	BB385572	—	—
1446117_at	0.15	0.09	−0.21	0.06	−1.63	0.0021	BB540135	Spire1	Spire homolog 1 (*Drosophila*)
1439619_at	0.22	0.14	−0.21	0.06	−1.62	0.0008	BG143445	Tcf12	Transcription factor 12
1429951_at	0.12	0.07	−0.21	0.05	−1.62	0.0017	AK005150	Ssbp2	Single-stranded DNA-binding protein 2
1458296_at	0.08	0.05	−0.20	0.06	−1.59	0.0045	BI465650	—	Transcribed locus
1436767_at	1.00	0.64	−0.20	0.04	−1.58	0.0046	BB475271	Luc7l2	LUC7-like 2 (*Saccharomyces cerevisiae*)
1444749_at	0.11	0.07	−0.19	0.05	−1.54	0.0038	BB427399	—	Transcribed locus
1439125_at	0.18	0.12	−0.18	0.07	−1.53	0.0005	AV330236	—	Transcribed locus
1436713_s_at	0.19	0.12	−0.18	0.06	−1.51	0.0043	BM119226	Meg3	Maternally expressed 3
1454864_at	0.07	0.10	0.18	0.06	1.52	0.0014	BB478535	Zfp592	Ainc finger protein 592
1441419_at	0.05	0.08	0.19	0.06	1.54	0.0001	BB027107	—	Transcribed locus, weakly similar to XP_001479449.1 PREDICTED: similar to pORF1 (*Mus musculus*)
1427130_x_at	0.04	0.07	0.23	0.07	1.69	0.0034	AJ249392	1700021K02Rik	RIKEN cDNA 1700021K02 gene
1417618_at	0.30	0.52	0.23	0.06	1.70	0.0017	NM_010582	Itih2	Interalpha trypsin inhibitor, heavy chain 2
1423100_at	1.58	2.80	0.25	0.04	1.77	0.0003	AV026617	Fos	FBJ osteosarcoma oncogene
1427682_a_at	0.47	0.90	0.28	0.06	1.91	0.0041	X06746	Egr2	Early growth response 2
1442350_at	0.01	0.03	0.34	0.11	2.17	0.0010	AV233462	—	Transcribed locus
1459372_at	0.02	0.04	0.38	0.13	2.42	0.0019	AV348246	Npas4	Neuronal PAS domain protein 4
1431590_at	0.01	0.03	0.41	0.16	2.56	0.0042	AK015859	4930521C21Rik	RIKEN cDNA 4930521C21 gene

Microarray analysis of neuronal tissue from the brains of mice 48 h after injection of claudin-5 siRNA compared with control (uninjected) mice showed 32 differentially regulated genes. When an NT siRNA was compared to claudin-5 siRNA, 22 differentially regulated genes were observed. Five mice were analyzed per experimental group.

Table S2. Microarray analysis of differentially regulated genes in neuronal tissue 48 h after injection of claudin-5 siRNA

Affymetrix probe IDs	NT siRNA intensity	Claudin-5 siRNA intensity	Log ratio	Log error	Fold change	*P*	Accession no.	Primary sequence name	Sequence description
1457945_at	0.03	0.01	−0.69	0.14	−4.94	0.0000	BM121819	—	Transcribed locus
1432806_at	0.03	0.01	−0.60	0.19	−4.02	0.0002	AK020529	9430099H24Rik	RIKEN cDNA 9430099H24 gene
1458110_at	0.03	0.01	−0.47	0.15	−2.94	0.0008	BB485193	D430030G11Rik	RIKEN cDNA D430030G11 gene
1444100_at	0.18	0.07	−0.43	0.14	−2.68	0.0001	AI480718	—	Transcribed locus
1447670_at	0.08	0.03	−0.38	0.10	−2.43	0.0004	AV030235	Psmd9	Proteasome (prosome, macropain) 26S subunit, non-ATPase, 9
1442185_at	0.05	0.02	−0.38	0.11	−2.38	0.0006	AV382148	—	—
1442153_at	0.06	0.03	−0.33	0.09	−2.16	0.0001	BB206087	—	Transcribed locus
1437219_at	0.16	0.08	−0.32	0.09	−2.07	0.0005	AW553541	—	Transcribed locus
1452757_s_at	4.37	2.12	−0.31	0.04	−2.06	0.0000	AK011116	Hba-a1	Hemoglobin α, adult chain 1
1429344_at	0.19	0.09	−0.31	0.05	−2.05	0.0003	BB357317	9.13002E+15	Hypothetical 9130022E09
1430362_at	0.03	0.01	−0.31	0.12	−2.05	0.0009	AI606154	5730409N24Rik	RIKEN cDNA 5730409N24 gene
1428361_x_at	3.54	1.89	−0.27	0.04	−1.88	0.0002	AK011116	Hba-a1	Hemoglobin α, adult chain 1
1437320_s_at	0.12	0.07	−0.26	0.06	−1.81	0.0009	BM117916	Xpa	Xeroderma pigmentosum, complementation group A
1430089_at	0.14	0.08	−0.25	0.08	−1.76	0.0009	BB032195	5830469G19Rik	RIKEN cDNA 5830469G19 gene
1444749_at	0.13	0.07	−0.24	0.05	−1.74	0.0004	BB427399	—	Transcribed locus
1456223_at	0.46	0.27	−0.24	0.04	−1.72	0.0003	BF322016	—	Transcribed locus
1417184_s_at	13.79	8.97	−0.19	0.03	−1.54	0.0000	BC027434	Hbb-b1	Hemoglobin, β adult major chain
1427865_at	3.34	2.21	−0.18	0.03	−1.51	0.0003	AF071431	—	β globin
1439869_at	0.12	0.18	0.18	0.06	1.51	0.0006	BB326106	2900054J07Rik	RIKEN cDNA 2900054J07 gene
1426866_at	0.12	0.21	0.25	0.04	1.78	0.0001	AK011230	Chst14	carbohydrate (*N*-acetylgalactosamine 4–0) sulfotransferase 14
1438660_at	0.04	0.06	0.25	0.08	1.78	0.0003	BM239196	Gcnt2	glucosaminyl (*N*-acetyl) transferase 2, I-branching enzyme
1429286_at	0.16	0.42	0.43	0.07	2.67	0.0004	AK004474	1190003M12Rik	RIKEN cDNA 1190003M12 gene

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Other Supporting Information Files

Dataset S1
Dataset S2