Molecular Biology Reports

Acute phosphate-salt exposure and expression of iron- and heme-related genes in HepaRG cells

Takeshi Honda, Tatsuya Sakaguchi, Yuka Kondo, Yuma Yabune, Yoshihiro Motomiya, Yuichiro Higashimoto
Corresponding author: Yuichiro Higashimoto, Department of Chemistry, Kurume University School of Medicine, 67 Asahi-machi, Kurume, Fukuoka 830-0011, Japan; higashiy@med.kurume-u.ac.jp

Online Resource 1 Primer sequences, reference-mix Ct values, and HAMP records for quantitative PCR

A. Targets and primer sequences

Target	Role	Data basis	Cultures (n)	Ct range, V and P wells	Forward primer (5′–3′)	Reverse primer (5′–3′)
HJV	Principal	Raw Ct, duplicate wells	6 (1–6)	26.27–29.64	GGAGCTTGGCCTCTACTGGA	ATGGTGAGCTTCCGGGTG
HPX	Principal	Raw Ct, duplicate wells	6 (1–6)	21.39–23.38	CGTGACTGAACGCTGCTCA	CTCCCGGTCCCATTTGTGAC
HMOX1	Principal	Raw Ct, duplicate wells	5 (1, 2, 3, 5, 6)	26.05–28.70	CCAGGCAGAGAATGCTGAGTTC	AAGACTGGGCTCTCCTTGTTGC
TF	Principal	Recorded relative quantities, duplicates	4 (identities not recorded)	Not recorded	GGTGGCAGAGTTCTATGGGTC	ACAGTAAAGTAAGCCTATGGGGA
TMPRSS6	Principal	Recorded relative quantities, duplicates	4 (identities not recorded)	Not recorded	ATCATGGCGGTCGTCTGGAAGA	CTGTTGTCCAGCGTCAGGTTCA
FTH1	Principal	Recorded relative quantities, duplicates	4 (identities not recorded)	Not recorded	CGCCAGAACTACCACCAG	TTCAAAGCCACATCATCG
TFR2	Principal	Raw Ct, duplicate wells	4 (1, 2, 3, 6)	28.40–30.27	TATACTCCTGGAGCTGGTGC	GCACGCTGAGGTAGCCCTCTA
ALAD	Principal	Raw Ct, duplicate wells	4 (1, 2, 3, 6)	25.03–27.03	GACATACAGCCTATCACCAGCC	CGCCAAAGATCAAGACACAGCG
BLVRA	Principal	Raw Ct, duplicate wells	4 (1, 2, 3, 6)	23.56–24.19	AGTGGCAGCCACCTCTGAAT	CTGGCTGCTCCACTCCTACA
HAMP	Quality assessment only	Raw Ct, duplicate wells	5 (1, 2, 3, 5, 6)	31.88–37.78	CTCTGTTTTCCCACAACAGAC	ATGGGGAAGTGGGTGTCTC
TTR	Exploratory	Recorded relative quantities, duplicates	4 (identities not recorded)	Not recorded	CCCTGCTGAGCCCCTACTC	TCCCTCATTCCTTGGGATTG
HIF1A	Exploratory	Recorded relative quantities, duplicates	4 (identities not recorded)	Not recorded	AGTGTACCCTAACTAGCCGA	CACAAATCAGCACCAAGC
GDF15	Exploratory	Recorded relative quantities, duplicates	4 (identities not recorded)	Not recorded	CCCTGCAGTCCGGATACTC	GAACAGAGCCCGGTGAAG
APOA1	Exploratory	Raw Ct, duplicate wells	4 (1, 2, 3, 6)	20.30–22.30	GTGGATGTGCTCAAAGACAGCG	GCTTGCTGAAGGTGGAGGTCAC
HPRT1	Reference gene	Contributes to the reference-mix Ct	6	Individual Ct not recorded	TGACACTGGCAAAACAATGCA	GGTCCTTTTCACCAGCAAGCT
TBP	Reference gene	Contributes to the reference-mix Ct	6	Individual Ct not recorded	TGTATCCACAGTGAATCTTGGTTG	GGTTCGTGGCTCTCTTATCCTC
RPLP0	Reference gene	Contributes to the reference-mix Ct	6	Individual Ct not recorded	TGGTCATCCAGCAGGTGTTCGA	ACAGACACTGGCAACATTGCGG
18S rRNA	Reference gene	Contributes to the reference-mix Ct	6	Individual Ct not recorded	CTCAACACGGGAAACCTCAC	CGCTCCACCAACTAAGAACG

Cultures 1–3 correspond to the three RNA-seq samples per condition (same cell lots and samples); cultures 4–6, where analyzed, were not sequenced. Culture identities were not recorded for the targets analyzed from recorded relative quantities (TF, TMPRSS6, FTH1, TTR, HIF1A, GDF15).

B. Reagents and normalization

Item	Description
RNA isolation	ReliaPrep RNA Cell Miniprep System (Promega; Z6012); concentration and purity by NanoDrop 2000 (Thermo Fisher Scientific)
Reverse transcription	1 µg total RNA, ReverTra Ace qPCR RT Master Mix (TOYOBO; FSQ-201)

qPCR reagent and instrument	SYBR Green qPCR Master Mix (Thermo Fisher Scientific; A66732); QuantStudio 1 Real-Time PCR System (Thermo Fisher Scientific)
Technical replicates	Duplicate wells per sample; averaged (raw Ct targets) or combined by geometric mean (recorded relative quantities) before analysis
Normalization	Reference-mix Ct = arithmetic mean Ct of HPRT1, TBP, RPLP0, and 18S rRNA (equivalent to the geometric mean of their relative expression when efficiencies are equal); ΔΔCt against the matched vehicle sample

C. Reference-mix Ct by culture

Culture	Reference-mix Ct, V	Reference-mix Ct, P	Difference (P − V)
1	25.22	24.83	-0.39
2	25.35	24.91	-0.45
3	25.54	25.07	-0.47
4	25.13	24.21	-0.92
5	25.29	24.88	-0.42
6	25.08	24.90	-0.18
Mean ± SD			-0.47 ± 0.24

The reference-mix Ct was lower in P than in V in all six cultures (one-sample t-test of the paired differences, p = 0.005), corresponding to an approximately 1.4-fold higher apparent abundance of the reference genes in P. Individual Ct values of the four reference genes were not recorded, so gene-wise stability measures (for example, geNorm or NormFinder) could not be calculated. In the RNA-seq data, mean TPM ratios (P versus V) were 1.29 for HPRT1, 1.28 for TBP, and 1.08 for RPLP0; 18S rRNA was not available in the RNA-seq data.

D. HAMP Ct records (V and P wells)

Culture	V well 1	V well 2	|Difference|	P well 1	P well 2	|Difference|
1	37.78	32.83	4.95	35.55	33.93	1.62
2	33.39	33.57	0.17	35.30	33.95	1.35
3	32.46	32.31	0.15	32.65	32.85	0.20
5	33.76	34.42	0.66	35.80	34.91	0.88
6	32.11	31.88	0.23	33.70	34.34	0.64

Ct values are for technical duplicate wells. Three of the ten duplicate pairs differed by more than one cycle (V, culture 1; P, cultures 1 and 2). HAMP fold changes were not used for inference; see the main text.

Molecular Biology Reports

Acute phosphate-salt exposure and expression of iron- and heme-related genes in HepaRG cells

Takeshi Honda, Tatsuya Sakaguchi, Yuka Kondo, Yuma Yabune, Yoshihiro Motomiya, Yuichiro Higashimoto
Corresponding author: Yuichiro Higashimoto, Department of Chemistry, Kurume University School of Medicine, 67 Asahi-machi, Kurume, Fukuoka 830-0011, Japan; higashiy@med.kurume-u.ac.jp

Online Resource 2 Differential expression of iron- and heme-related genes and hepatic comparators in the RNA-seq data under three analyses

Gene	TCC M-value (P vs V)	TCC FDR	limma-voom ~ P log2 FC	limma-voom ~ P FDR	limma-voom ~ lot + P log2 FC	limma-voom ~ lot + P FDR	Within-lot log2 P/V, lot 1	Within-lot log2 P/V, lot 2	Within-lot log2 P/V, lot 3
HJV	−1.459	**0.00019**	−1.44	**1.7×10^{-4}**	−1.45	**6.8×10^{-7}**	−1.89	−1.52	−0.94
TF	−0.831	**0.0039**	−0.80	**0.0032**	−0.80	**1.7×10^{-7}**	−0.91	−0.66	−0.83
HPX	−0.623	**0.0010**	−0.60	**7.8×10^{-4}**	−0.60	**6.2×10^{-6}**	−0.66	−0.47	−0.67
ALAD	−0.653	**0.00020**	−0.63	**1.8×10^{-4}**	−0.63	**5.9×10^{-6}**	−0.73	−0.59	−0.58
TMPRSS6	−0.573	0.075	−0.54	0.093	−0.55	**0.0033**	−0.86	−0.47	−0.21
TFR2	−0.659	0.052	−0.63	0.058	−0.66	**0.024**	−0.95	−0.76	−0.25
BLVRA	0.317	0.083	0.32	0.079	0.34	**0.037**	0.35	0.52	0.13
HMOX1	0.304	0.175	0.29	0.18	0.32	0.10	0.27	0.42	0.27
FTH1	0.655	0.285	0.90	0.11	0.90	**4.6×10^{-4}**	−0.04	1.37	1.35
TFRC	0.049	0.739	0.07	0.63	0.07	0.65	−0.04	0.12	0.12
SLC40A1	−0.215	0.097	−0.19	0.13	−0.19	0.11	−0.14	−0.13	−0.32
ALB	−0.786	**0.00057**	−0.77	**1.2×10^{-4}**	−0.77	**2.3×10^{-7}**	−0.73	−0.76	−0.81
TTR	−0.615	**0.011**	−0.58	**0.010**	−0.58	**9.9×10^{-6}**	−0.72	−0.50	−0.53

P, phosphate; V, vehicle. Positive values indicate higher expression in phosphate-treated samples. FDR values below 0.05 are in bold.

TCC, the original analysis (TMM normalization, edgeR) of the P versus V comparison; M-values and q-values (shown as FDR) were taken from the original TCC result table.

limma-voom ~ P, the limma-voom analysis of the six V and P samples with condition as the only factor; limma-voom ~ lot + P, the same analysis with lot as a blocking factor. Each of the three cell lots supplied one V and one P sample (V1 and P1, lot 1; V2 and P2, lot 2; V3 and P3, lot 3). Both analyses used 13,463 genes after low-expression filtering, TMM normalization, voom precision weights, empirical-Bayes moderation, and Benjamini–Hochberg adjustment.

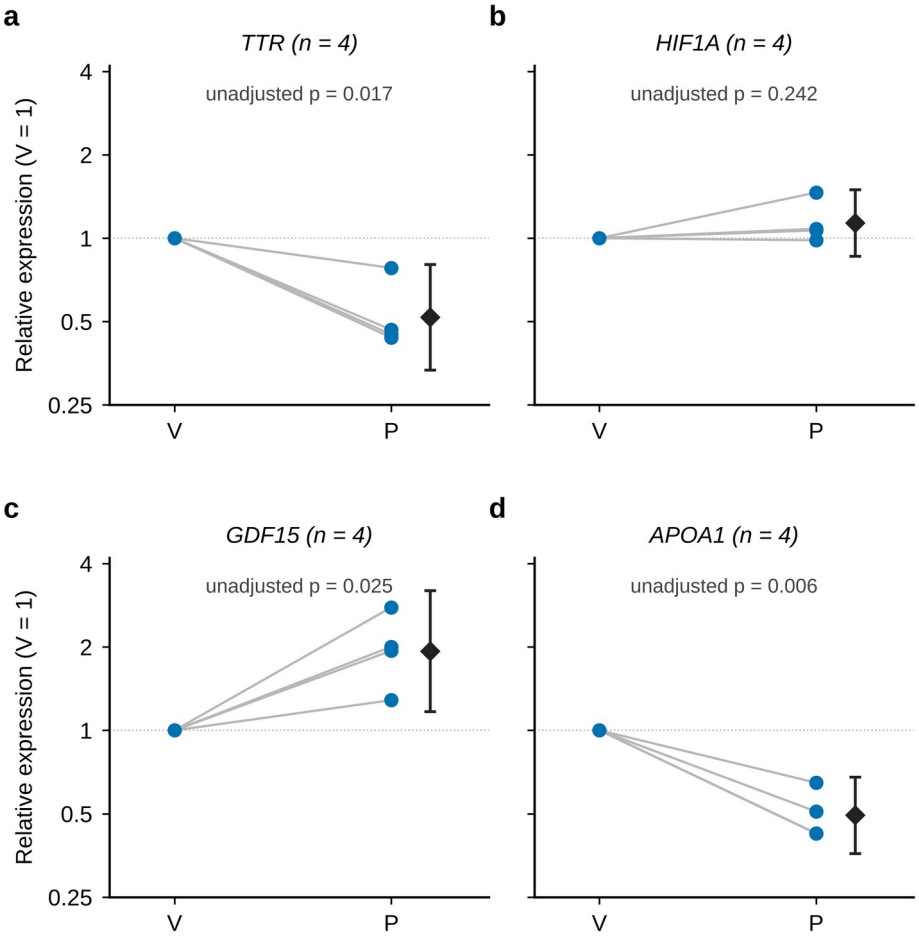
Within-lot log2 P/V is the difference in TMM-normalized log2 counts per million between the P and V samples of each lot, without model-based moderation. The FDR values of the ~ lot + P model were smaller than those of the ~ P model for all genes except TFRC, and whether TFR2, TMPRSS6, BLVRA, and FTH1 met the FDR threshold depended on the model. The within-lot values for FTH1 (−0.04, 1.37, and 1.35) differ among lots, whereas those for TFR2, TMPRSS6, and BLVRA have the same sign in all three lots.

Analyses were implemented in Python (NumPy 2.5.3, SciPy 1.18.1, statsmodels 0.15.0, and inmoose 0.9.1). The code, the count matrix of the six samples, and the sample-to-lot table are available from the corresponding author on reasonable request.

Molecular Biology Reports

Acute phosphate-salt exposure and expression of iron- and heme-related genes in HepaRG cells

Takeshi Honda, Tatsuya Sakaguchi, Yuka Kondo, Yuma Yabune, Yoshihiro Motomiya, Yuichiro Higashimoto
Corresponding author: Yuichiro Higashimoto, Department of Chemistry, Kurume University School of Medicine, 67 Asahi-machi, Kurume, Fukuoka 830-0011, Japan; higashiy@med.kurume-u.ac.jp

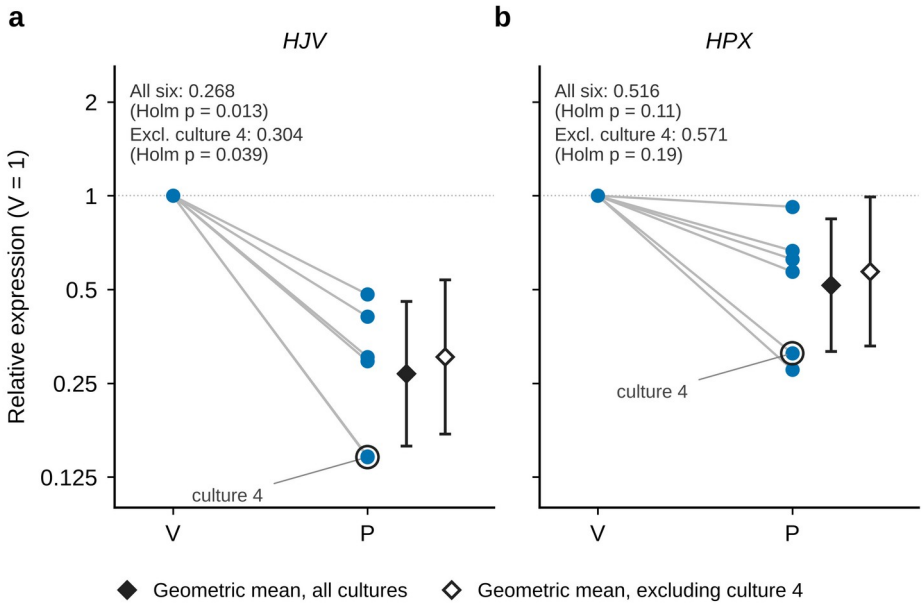


Online Resource 3 Exploratory qPCR targets. a TTR, b HIF1A, c GDF15, d APOA1 (phosphate versus vehicle). Gray lines join matched cultures (four cultures per target; identities were not recorded for a–c, and d shows cultures 1, 2, 3, and 6); black diamonds and bars show the geometric mean with unadjusted 95% confidence intervals. For a–c, recorded relative quantities of the two technical duplicates were combined by geometric mean before analysis; for d, duplicate raw Ct values were averaged and normalized to the reference-mix Ct. Displayed p-values are unadjusted paired log-scale tests and are not corrected for multiplicity.

Molecular Biology Reports

Acute phosphate-salt exposure and expression of iron- and heme-related genes in HepaRG cells

Takeshi Honda, Tatsuya Sakaguchi, Yuka Kondo, Yuma Yabune, Yoshihiro Motomiya, Yuichiro Higashimoto
Corresponding author: Yuichiro Higashimoto, Department of Chemistry, Kurume University School of Medicine, 67 Asahi-machi, Kurume, Fukuoka 830-0011, Japan; higashiy@med.kurume-u.ac.jp



Online Resource 4 Sensitivity analysis excluding culture 4. a HJV, b HPX (phosphate versus vehicle). Gray lines join matched cultures; the circled point marks culture 4. Filled diamonds show the geometric mean of all six cultures (primary analysis) and open diamonds the geometric mean after excluding culture 4, each with the unadjusted 95% confidence interval. Holm-adjusted p-values are from paired log-scale tests; for the analysis excluding culture 4 the Holm family of nine tests was recomputed. The multiplicity-adjusted conclusions were unchanged after excluding culture 4: HJV remained significant, whereas HPX was not significant in either analysis (Holm-adjusted p = 0.11 with all six cultures and 0.19 without culture 4). Culture 4 did, however, influence the HPX effect size and unadjusted p-value (ratio 0.516 versus 0.571; unadjusted p = 0.018 versus 0.048). These results show how the estimates depend on sample inclusion and do not establish that the exclusion was technically justified.