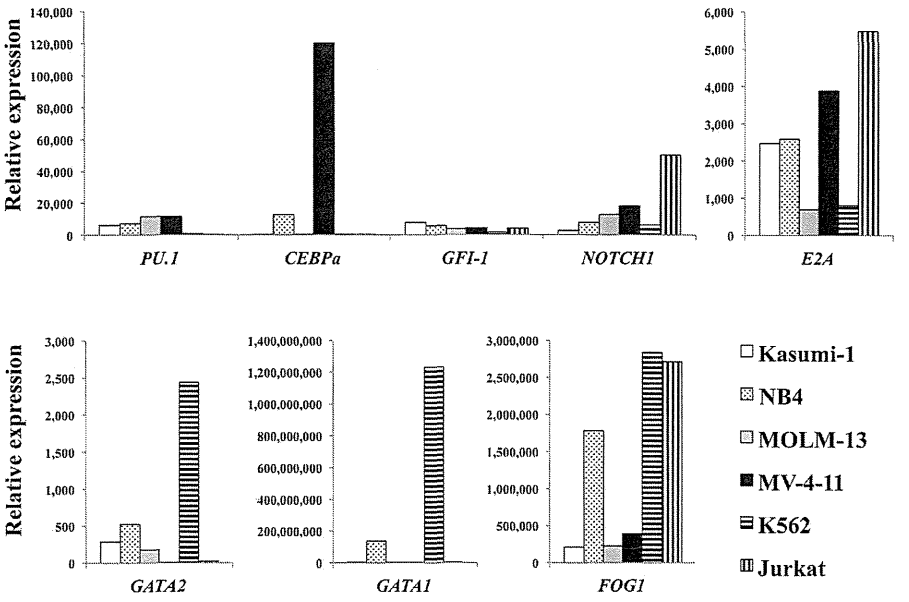

Figure 4. *Comparative expression of genes for hematopoietic transcription factors. RNA was extracted from each cell line and gene expressions of hematopoietic transcription factors, such as Transcription factor PU.1 (PU.1), CCAAT/enhancer-binding protein alpha (CEBPα), Growth factor independent 1(GFI1), Neurogenic locus notch1 homolog protein 1 (NOTCH1), Transcription factor E2-alpha (E2A), GATA-binding factor 2 (GATA2), GATA1, and Friend of gata1 (FOG1) were investigated by real-time reverse transcription–polymerase chain reaction. All data were analyzed with the delta-delta Ct method.*

Kasumi-1 cells that were established from acute myeloblastic leukemia (M2), reportedly express CD33, CD34, CD38, CD13, and CD15 (7), and the present study confirms their expression. However, we also found expression of CD135, HLA-DR, CD45, CD14 and CD16 with flow cytometric analysis, suggesting that we maintain this cell line as it is and our additional results offer robust evidence for defining these cells. As Kasumi-1 cells express Acute myeloid leukemia 1 (AML1)–Eight twenty one (ETO) fusion gene product (13) and also have mutation in the *c-KIT* gene, they were utilized in research of regulation of the promoter region of the AML1–ETO fusion gene (14), of inhibitors against c-KIT proteins (15) and of mechanisms of apoptosis (16) (17). Zapotocky *et al.* also focused on hematopoietic differentiation and showed that valproic acid triggers differentiation defined by c-Kit, CD33, CD34, CD11b expression in Kasumi-1 cells. They also found the positive effect of valproic acid on up-regulation of *CEBPα* and PU.1 expression. Our results also reveal hematopoietic transcription factors *CEBPα*, *PU.1* and *GFI1* were expressed in Kasumi-1 cell in that order and their expressions are the lowest among the six cell lines we examined (Figure 4), suggesting that Kasumi-1 cells could potentially be a useful tool for examining the effect of increasing the expression of these transcription factors. Using the surface markers that we examined here, we can expect to gain more evidence for differentiation.

NB4 cells that were established from acute promyelocytic leukemia (M3) expressed CD38, CD33, CD13, CD15 and CD11b, but not CD11c, CD34, or CD14 (8). Except for CD11c and CD14, our results shown in Figure 2 and 3B demonstrated the same phenotypic pattern as the previous report. It is likely that this discrepancy was caused by difference in the antibody clones of CD11c and CD14 between our study and previous reports. In addition to the antibodies listed above, we also investigated the expression

Table II. *Summary of flow cytometric data.*

Kasumi-1									
Markers	CD135$^-$ CD38$^+$	CD135$^+$ CD38$^+$	CD135$^+$ CD38$^-$	c-KIT$^-$ CD34$^+$	c-KIT$^+$ CD34$^+$	c-KIT$^+$ CD34$^-$			
%	0.31±0.04	0.09±0.03	2.14±0.63	0.07±0.03	95.43±0.25	4.47±0.26			
Markers	HDR$^-$ CD33$^+$	HDR$^+$ CD33$^+$	HDR$^+$ CD33$^-$	HLADR$^-$ CD13$^+$	HLADR$^+$ CD13$^+$	HLADR$^+$ CD13$^-$			
%	92.57±0.32	7.28±0.08	0.00±0.00	46.60±0.46	5.74±0.51	6.42±0.53			
Among CD13$^+$ CD116$^+$	CD15$^-$ CD14$^+$ 0.69±0.18	CD15$^+$ CD14$^+$ 0.13±0.07	CD15$^+$ CD14$^-$ 1.99±0.43	CD15$^-$ CD16$^+$ 0.50±0.08	CD15$^+$ CD16$^+$ 0.05±0.00	CD15$^+$ CD16$^-$ 1.93±0.50	CD11b$^-$ CD11c$^+$ 0.02±0.01	CD11$^+$ CD11c$^+$ 0.12±0.04	CD11$^+$ CD11c$^-$ 14.86±0.61
NB4									
Markers	c-KIT$^-$ CD38$^+$	c-KIT$^+$ CD38$^+$	c-KIT$^+$ CD38$^-$	CD34$^-$ CD38$^+$	CD34$^+$ CD38$^+$	CD34$^+$ CD38$^-$	c-KIT$^-$ HLADR$^+$	c-KIT$^+$ HLADR$^+$	c-KIT$^+$ HLADR$^-$
%	42.13±0.71	14.37±3.50	10.23±1.28	28.83±2.57	2.61±0.24	7.43±0.37	16.93±3.61	2.66±0.65	6.71±0.88
Markers	CD15$^-$ CD116$^+$	CD15$^+$ CD116$^+$	CD15$^+$ CD116$^-$	CD11b$^-$ CD11c$^+$	CD11b$^+$ CD11c$^+$	CD11b$^+$ CD11c$^-$	CD11c$^-$ CD14$^+$	CD11c$^+$ CD14$^+$	CD11c$^+$ CD14$^-$
%	13.20±2.86	1.96±0.14	3.15±0.33	5.39±0.71	21.17±2.87	56.63±2.38	9.26±0.33	21.17±1.35	48.03±1.12
MOLM-13									
Markers	HLADR$^-$ CD33$^+$	HLADR$^+$ CD33$^+$	HLADR$^+$ CD33$^-$	c-KIT$^-$ CD34$^+$	c-KIT$^+$ CD34$^+$	c-KIT$^+$ CD34$^-$	c-KIT$^-$ CD38$^+$	c-KIT$^+$ CD38$^+$	c-KIT$^+$ CD38$^-$
%	76.57±1.86	23.43±1.86	0.00±0.00	0.26±0.17	0.09±0.06	7.63±1.29	85.07±0.64	9.21±1.32	0.38±0.11
Markers	HLADR$^-$ CD13$^+$	HLADR$^+$ CD13$^+$	HLADR$^+$ CD13$^-$	CD64$^-$ CD13$^+$	CD64$^+$ CD13$^+$	CD64$^+$ CD13$^-$	CD11b$^-$ CD13$^+$	CD11b$^+$ CD13$^+$	CD11b$^+$ CD13$^-$
%	2.16±0.14	0.85±0.10	21.73±0.42	1.37±0.12	0.01±0.01	0.02±0.00	1.56±0.13	0.04±0.01	0.23±0.01
Markers	CD14$^-$ CD116$^+$	CD14$^+$ CD116$^+$	CD14$^+$ CD116$^-$	CD15$^-$ CD13$^+$	CD15$^+$ CD13$^+$	CD15$^+$ CD13$^-$	CD11c$^-$ CD14$^+$	CD11c$^+$ CD14$^+$	CD11c$^+$ CD14$^-$
%	0.39±0.07	0.10±0.02	0.12±0.03	1.42±0.18	0.18±0.05	7.79±1.63	2.22±0.63	0.01±0.01	0.01±0.01
MV4-11									
Markers	HLADR$^-$ CD33$^+$	HLADR$^+$ CD33$^+$	HLADR$^+$ CD33$^-$	c-KIT$^-$ CD34$^+$	c-KIT$^+$ CD34$^+$	c-KIT$^+$ CD34$^-$	CD38$^-$ CD34$^+$	CD38$^+$ CD34$^+$	CD38$^+$ CD34$^-$
%	14.80±0.78	85.03±0.67	0.00±0.00	0.14±0.06	0.03±0.01	6.02±0.54	0.14±0.06	0.05±0.01	0.63±0.03
Markers	HLADR$^-$ CD13$^+$	HLADR$^+$ CD13$^+$	HLADR$^+$ CD13$^-$	CD14$^-$ CD116$^+$	CD14$^+$ CD116$^+$	CD14$^+$ CD116$^-$	CD15$^-$ CD64$^+$	CD15$^+$ CD64$^+$	CD15$^+$ CD64$^-$
%	2.76±0.18	2.62±0.36	30.30±2.67	0.36±0.05	0.02±0.01	0.13±0.06	0.35±0.10	30.70±3.70	57.93±1.16
Markers	CD14$^-$ CD116$^+$	CD14$^+$ CD116$^+$	CD14$^+$ CD116$^-$	CD64$^-$ CD36$^+$	CD64$^+$ CD36$^+$	CD64$^+$ CD36$^-$	CD13$^-$ CD10$^+$	CD13$^+$ CD10$^+$	CD13$^+$ CD10$^-$
%	0.50±0.16	0.01±0.00	0.17±0.00	0.00±0.00	0.15±0.03	99.43±0.15	0.02±0.01	0.04±0.01	46.13±1.68
K562									
Markers	HDR$^-$ CD13$^+$	HDR$^+$ CD13$^+$	HDR$^+$ CD13$^-$	c-KIT$^-$ CD13$^+$	c-KIT$^+$ CD13$^+$	c-KIT$^+$ CD13$^-$	c-KIT$^-$ CD33$^+$	c-KIT$^+$ CD33$^+$	c-KIT$^+$ CD33$^-$
%	9.77±6.01	0.01±0.00	0.03±0.02	11.93±6.48	2.99±1.44	9.43±1.34	52.97±1.00	8.77±0.45	2.56±0.15
Markers	c-KIT$^-$ CD34$^+$	c-KIT$^+$ CD34$^+$	c-KIT$^+$ CD34$^-$	CD38$^-$ CD33$^+$	CD38$^+$ CD33$^+$	CD38$^+$ CD33$^-$	c-KIT$^-$ CD36$^+$	c-KIT$^+$ CD36$^+$	c-KIT+ CD36$^-$
%	0.42±0.18	0.10±0.06	8.51±0.32	57.70±1.14	0.02±0.01	0.00±0.01	57.90±2.09	17.37±1.42	5.43±0.50
Among c-KIT$^-$ CD36$^+$ cells				Markers	CD71$^+$ GPA$^+$	CD71$^-$ GPA$^+$	CD44$^+$ GPA$^-$	CD44$^+$ GPA$^+$	CD44$^-$ GPA$^+$
				%	1.71±0.17	0.00±0.00	49.03±1.48	1.40±0.15	0.31±0.07
Jurkat									
Markers	CD33$^-$ CD34$^+$	CD33$^+$ CD34$^+$	CD33$^+$ CD34$^-$	c-KIT$^-$ CD34$^+$	c-KIT$^+$ CD34$^+$	c-KIT$^+$ CD34$^-$	CD34$^-$ CD38$^+$	CD34$^+$ CD38$^+$	CD34$^+$ CD38$^-$
%	3.53±0.54	0.03±0.01	0.13±0.02	3.13±0.74	0.84±0.15	0.78±0.29	11.09±1.11	1.20±0.94	3.37±0.44
Among CD38$^+$ CD7$^+$ (1.50±0.40%)									
Markers	CD2$^-$ CD5$^+$	CD2$^+$ CD5$^+$	CD2$^+$ CD5$^-$	CD3$^-$ CD4$^+$	CD3$^+$ CD4$^+$	CD3$^+$ CD4$^-$	CD3$^-$ CD8$^+$	CD3$^+$ CD8$^+$	CD3$^+$ CD8$^-$
%	0.00±0.00	1.48±0.40	0.01±0.00	0.12±0.04	0.67±0.18	0.62±0.16	0.00±0.00	0.01±0.01	1.18±0.31
Among CD38$^-$ CD7$^+$ cells (59.43±2.40%)									
Markers	CD2$^-$ CD5$^+$	CD2$^+$ CD5$^+$	CD2$^+$ CD5$^-$	CD3$^-$ CD4$^+$	CD3$^+$ CD4$^+$	CD3$^+$ CD4$^-$	CD3$^-$ CD8$^+$	CD3$^+$ CD8$^+$	CD3$^+$ CD8$^-$
%	0.36±0.39	56.73±2.55	2.26±0.07	6.84±0.31	21.82±1.12	23.44±1.18	0.01±0.01	0.03±0.01	39.01±2.60

of c-KIT, HLA-DR, CD45, CD116 and CD16. NB4 cells have been utilized to examine the effect of differentiation inducers (8, 18) and examined their effect by morphological observation and surface expression utilizing CD11b antibodies. Although NB4 cells have been also used for apoptosis studies (19, 20), no mention about surface marker expression has been made. Combined with studies on surface markers, these studies will provide us with more information on which cell fractions are most effective in apoptosis. Additional surface markers tested here, such as c-KIT, HLA-DR, CD45, CD116 and CD16, support the robust evidence to define the cells.

MOLM-13 cells that were established from acute monocytic leukemia (M5a) expressed CD33, CD34, CD15, but not CD13 or CD14 (9), and our expression patterns for CD34 and CD14 differed compared with those previously reported (Figures 2 and 3C). This discrepancy also seemed likely to be due to differences in antibody clones of CD34 and CD14 between our study and previous reports. There was no previous clarification of surface phenotype for MV-4-11 cells. p53-Dependent apoptosis and cell cycle was investigated in MOLM-13 cells (21) (22) but without differentiation-based analyses. MOLM-13 and MV-4-11 cells are both CD135- internal tandem duplication (ITD)-positive AMLs, were utilized in CD135-dependent drug-screening studies (23, 24) in which outcomes were evaluated with cell proliferation and phosphorylation of internal signal, such as CD135 itself and Signal transducer and activator of transcription 5 (STAT5), but not with surface phenotype and transcription factor expression. Our results shown in Figure 3C and D suggest that MOLM-13 cells and MV-4-11 are CFU-GM to myeloblasts and myeloblasts, respectively, which implies that these cell lines will also be fit for functional analysis to examine differentiation after the myeloblast state to mature monocytes. Regarding gene expression, as *CEBPα* expression is quite high in MV4-11 cells (Figure 4), reduction of expression would seem to be difficult for this cell line, suggesting it is not suitable for studies of loss-of-function of CEBPα and its downstream genes.

K562 cells are chronic myelogenous leukemia-derived erythroleukemia cells and were not defined on surface marker-based differentiation state when established (11); later studies confirmed the surface phenotype of GPA (25). When examining the differentiation stage of erythroid cells, hemoglobinized cells were stained with benzidine and hemoglobinization was quantified, depending on the staining intensity. Recently Hu *et al*. reported that the distinct stages in erythroid differentiation were clearly separated based on dynamic changes in membrane proteins, such as CD36, CD44, CD71 and GPA using human cord blood-derived erythroid cells (26). Herein, we confirm the expression of these proteins in K562 cells. Concerning gene expression, *GATA1* expression and its target gene effect was checked to investigate its effect on erythroid differentiation in K562 cells (27). *GATA2* and *FOG1* expression was also confirmed (Figure 4) although their expression levels were lower than *GATA1*, suggesting that *GATA2* and *FOG1* expression can be used to trace the differentiation of K562 cells so as to evaluate the differentiation status more accurately.

Jurkat cells were established from acute T-cell leukemia and not defined in terms of surface marker-based differentiation state when they were established (12). Our results revealed that Jurkat cells are differentiated into mature functional T-cells with mature T-cell markers (Figures 2 and 3F), and T-cell-specific genes (*NOTCH1* and *E2A*) were highly expressed (Figure 4), which implies that this cell line is fit for studies that focus on transcriptional regulation, rather than differentiation studies.

The information mentioned herein can be used for establishing standards for choosing appropriate cell lines for functional analyses, and for determination of efficacy after functional analysis and drug screening.

Acknowledgements

We thank Dr. Kasem Kulkeaw, Ms. Tan Keai Sinn and Ms. Chiyoko Nakamichi for discussion, and the Ministry of Education, Culture, Sports, Science and Technology, and the Ministry of Health, Labour and Welfare, and Japan Society for the Promotion of Science (JSPS) bilateral program for grant support. Tomoko Inoue was supported by a research award for young scientists from the Japan Leukaemia Research Fund, a research grant from BioLegend/Tomy Digital Biology and Ogata memorial Science Foundation Research Fund. We thank the English editor for modifying our draft manuscript.

References

1 Okuhashi Y, Itoh M, Nara N and Tohda S: NOTCH knockdown affects the proliferation and mTOR signaling of leukemia cells. Anticancer Res *33*: 4293-4298, 2013.

2 Tomiyasu H, Watanabe M, Sugita K, Goto-Koshino Y, Fujino Y, Ohno K, Sugano S and Tsujimoto H: Regulations of *ABCB1* and *ABCG2* expression through MAPK pathways in acute lymphoblastic leukemia cell lines. Anticancer Res *33*: 5317-5323, 2013.

3 Inoue T, Sugiyama D, Kurita R, Oikawa T, Kulkeaw K, Kawano H, Miura Y, Okada M, Suehiro Y, Takahashi A, Marumoto T, Inoue H, Komatsu N and Tani K: APOA-1 is a novel marker of erythroid cell maturation from hematopoietic stem cells in mice and humans. Stem Cell Rev *7*: 43-52, 2011.

4 Kulkeaw K, Inoue T, Mizuochi C, Horio Y, Ishihama Y and Sugiyama D: Ectopic expression of Hmgn2 antagonizes mouse erythroid differentiation *in vitro*. Cell Biol Int *36*: 195-202, 2012.

5 De Rosa SC, Brenchley JM and Roederer M: Beyond six colors: a new era in flow cytometry. Nat Med *9*: 112-117, 2003.

6 Baumgarth N and Roederer M: A practical approach to multicolor flow cytometry for immunophenotyping. J Immunol Methods *243*: 77-97, 2000.

7 Asou H, Tashiro S, Hamamoto K, Otsuji A, Kita K and Kamada N: Establishment of a human acute myeloid leukemia cell line (Kasumi-1) with 8;21 chromosome translocation. Blood *77*: 2031-2036, 1991.

8 Lanotte M, Martin-Thouvenin V, Najman S, Balerini P, Valensi F and Berger R: NB4, a maturation inducible cell line with t(15;17) marker isolated from a human acute promyelocytic leukemia (M3). Blood *77*: 1080-1086, 1991.

9 Matsuo Y, MacLeod RA, Uphoff CC, Drexler HG, Nishizaki C, Katayama Y, Kimura G, Fujii N, Omoto E, Harada M and Orita K: Two acute monocytic leukemia (AML-M5a) cell lines (MOLM-13 and MOLM-14) with interclonal phenotypic heterogeneity showing MLL-AF9 fusion resulting from an occult chromosome insertion, ins(11;9)(q23;p22p23). Leukemia *11*: 1469-1477, 1997.

10 Lange B, Valtieri M, Santoli D, Caracciolo D, Mavilio F, Gemperlein I, Griffin C, Emanuel B, Finan J, Nowell P and Rovera G: Growth factor requirements of childhood acute leukemia: establishment of GM-CSF-dependent cell lines. Blood *70*: 192-199, 1987.

11 Lozzio CB and Lozzio BB: Human chronic myelogenous leukemia cell-line with positive Philadelphia chromosome. Blood *45*: 321-334, 1975.

12 Schneider U, Schwenk HU and Bornkamm G: Characterization of EBV-genome negative "null" and "T" cell lines derived from children with acute lymphoblastic leukemia and leukemic transformed non-Hodgkin lymphoma. Int J of Cancer *19*: 621-626, 1977.

13 Kwong YL, Chan V, Wong KF and Chan TK: Use of the polymerase chain reaction in the detection of AML1/ETO fusion transcript in t(8;21). Cancer *75*: 821-825, 1995.

14 Markova EN, Kantidze OL and Razin SV: Transcription of the AML1/ETO chimera is guided by the P2 promoter of the AML1 gene in the Kasumi-1 cell line. Gene *510*: 142-146, 2012.

15 Shao HM, Tang YH, Shen Q, Zhu HQ, Ji O, Zhang YC, Ji JM, Jiang PJ, Si YJ and Li ZR: Apoptosis of Kasumi-1 cells induced by puerariae radix flavones and its molecular mechanism. Zhonghua xue ye xue za zhi = Zhonghua xueyexue zazhi *33*: 43-46, 2012 (in Chinese).

16 Klampfer L, Zhang J, Zelenetz AO, Uchida H and Nimer SD: The AML1/ETO fusion protein activates transcription of BCL-2. Proc Natl Acad Sci USA *93*: 14059-14064, 1996.

17 Zapotocky M, Mejstrikova E, Smetana K, Stary J, Trka J and Starkova J: Valproic acid triggers differentiation and apoptosis in AML1/ETO-positive leukemic cells specifically. Cancer Lett *319*: 144-153, 2012.

18 Laurenzana A, Petruccelli LA, Pettersson F, Figueroa ME, Melnick A, Baldwin AS, Paoletti F and Miller WH Jr.: Inhibition of DNA methyltransferase activates tumor necrosis factor alpha-induced monocytic differentiation in acute myeloid leukemia cells. Cancer Res *69*: 55-64, 2009.

19 Han B, Ren Y, Guan L, Wei W, Hua F, Yang Y, Yang T, Cao T, Dong H, Pan H and Xu C: Sodium selenite induces apoptosis in acute promyelocytic leukemia-derived NB4 cells through mitochondria-dependent pathway. Oncol Res *17*: 373-381, 2009.

20 Mozdarani H and Asghari F: Potent reducing effects of vitamin D3 on the frequency of apoptosis induced by arsenic trioxide in NB4 cell line. Arch of Iran Med *13*: 26-33, 2010.

21 Kojima K, Konopleva M, Samudio IJ, Ruvolo V and Andreeff M: Mitogen-activated protein kinase kinase inhibition enhances nuclear proapoptotic function of p53 in acute myelogenous leukemia cells. Cancer Res *67*: 3210-3219, 2007.

22 Kojima K, Konopleva M, Tsao T, Nakakuma H and Andreeff M: Concomitant inhibition of Mdm2-p53 interaction and Aurora kinases activates the p53-dependent postmitotic checkpoints and synergistically induces p53-mediated mitochondrial apoptosis along with reduced endoreduplication in acute myelogenous leukemia. Blood *112*: 2886-2895, 2008.

23 Pauwels D, Klaassen H, Lahortiga I, Kilonda A, Jacobs K, Sweron B, Corbau R, Chaltin P, Marchand A and Cools J: Identification of novel FLT3 kinase inhibitors. Eur J Med Chem *63*: 713-721, 2013.

24 Pietschmann K, Bolck HA, Buchwald M, Spielberg S, Polzer H, Spiekermann K, Bug G, Heinzel T, Bohmer FD and Kramer OH: Breakdown of the FLT3-ITD/STAT5 axis and synergistic apoptosis induction by the histone deacetylase inhibitor panobinostat and FLT3-specific inhibitors. Mol Can Ther *11*: 2373-2383, 2012.

25 Ma J, Hu Y, Guo M, Huang Z, Li W and Wu Y: hERG potassium channel blockage by scorpion toxin BmKKx2 enhances erythroid differentiation of human leukemia cells K562. PLoS One *8*: e84903, 2013.

26 Hu J, Liu J, Xue F, Halverson G, Reid M, Guo A, Chen L, Raza A, Galili N, Jaffray J, Lane J, Chasis JA, Taylor N, Mohandas N and An X: Isolation and functional characterization of human erythroblasts at distinct stages: implications for understanding of normal and disordered erythropoiesis *in vivo*. Blood *121*: 3246-3253, 2013.

27 Fujiwara T, Saitoh H, Inoue A, Kobayashi M, Okitsu Y, Katsuoka Y, Fukuhara N, Onishi Y, Ishizawa K, Ichinohasama R and Harigae H: 3-Deazaneplanocin A (DZNep), an inhibitor of S-adenosylmethionine-dependent methyltransferase, promotes erythroid differentiation. J Biol Chem *289*: 8121-8134, 2014.

Received April 4, 2014
Revised June 9, 2014
Accepted June 10, 2014

ANTICANCER RESEARCH *34*: 4561-4568 (2014)

Dok2 Likely Down-regulates *Klf1* in Mouse Erythroleukemia Cells

YUKA TANAKA[1,2], KASEM KULKEAW[3], TOMOKO INOUE[3], KEAI SINN TAN[3], YOICHI NAKANISHI[2], SENJI SHIRASAWA[1] and DAISUKE SUGIYAMA[3]

[1]*Department of Cell Biology, Faculty of Medicine, Fukuoka University, Fukuoka, Japan;*
[2]*Center for Clinical and Translational Research, Kyushu University Hospital, Fukuoka, Japan;*
[3]*Department of Research and Development of Next Generation Medicine, Kyushu University Faculty of Medical Sciences, Kyushu University, Fukuoka, Japan*

Abstract. *Background/Aim: Docking protein 2 (Dok2) is an adapter protein which is involved in hematopoiesis. However, it still remains unclear how Dok2 functions in regulation of transcription of hematopoietic genes. To address this issue, we knocked-down Dok2 mRNA in mouse erythroleukemia cells which highly express Dok2 intrinsically. Materials and Methods: Mouse erythroleukemia cells were transfected with Dok2 siRNA for 24 h and gene expression of erythroid differentiation-related genes, such as GATA binding protein 1 (Gata1), Krüppel-like factor 1 (Klf1), α-globin and β-globin were assessed by real-time polymerase chain reaction. Results: Among the tested genes, expression of Klf1 exhibited a 1.94-fold increase when compared to the control 24 h after transfection. Immunocytochemistry and chromatin immunoprecipitation assays revealed that Dok2 protein localizes in the nucleus and binds to the promoter region of Klf1 gene. Conclusion: Dok2 is able to control Klf1 expression by transcriptional regulation through directly binding to its promoter region.*

Hematopoiesis is a process which produces mature functional blood cells that maintain homeostasis. During hematopoiesis, hematopoietic stem cells are generated from the mesoderm, and differentiate into progenitor cells, maturing into erythrocytes, granulocytes, macrophages, lymphocytes and platelets (1). This process is strictly regulated by intrinsic and extrinsic molecules and an imbalance in this regulation can give rise to various hematological diseases, such as leukemia (2, 3). Extrinsic molecules include cytokines, chemokines and extracellular matrix proteins which are derived from hematopoietic cell controlling niches (4). Previously, we demonstrated that Delta-like 1 homolog (Dlk1)-expressing hepatoblasts function as niche cells for hematopoietic stem cells by regulating homing and cell differentiation through secretion of extracellular matrix proteins and cytokines (5). In addition to these molecules, hematopoietic cells have also been reported to be regulated intrinsically by transcription factors (1, 6). GATA binding protein 1 (Gata1) and spleen focus-forming virus proviral integration oncogene (Spi1, also known as Pu.1) are well-known transcription factors which regulate the fate of hematopoietic cells. Gata1 is a key transcription factor in erythropoiesis and regulates transcription of Krüpper-like factor 1 (Klf1) and activation of β-globin transcription (7). Ectopic expression of Gata1 in myleomonocytic cells can induce them to differentiate into eosinophil, erythroid, and megakaryocytic cells (8-10). On the other hand, Pu.1 regulates the fate of myeloid cells and is involved in granulocyte, monocyte and lymphocyte development (8, 11). In erythroid cells, Pu.1 acts as an inhibitor of erythroid differentiation by antagonizing Gata1 (1, 12). Thus, proper expression of transcription factors is important to maintain homeostasis of hematopoiesis, and abnormal functioning of these factors are known causes of leukemogenesis (13).

Docking protein 2 (Dok2) (also known as DokR, p56 and Frip), a member of the Dok protein family, is an adapter protein that is a substrate for tyrosine kinase. Dok1, -2 and -3 are selectively expressed in hematopoietic cells among the seven Dok protein members (14-17). Two groups have reported that double knock-out of *Dok1* and *Dok2* in mice induces the abnormal proliferation of myeloid cells due to an increase of proliferation and reduced apoptosis, and is associated with Rat sarcoma virus oncogene (Ras)/mitogen-

Correspondence to: Dr. Daisuke Sugiyama, Department of Research and Development of Next Generation Medicine, Kyushu University Faculty of Medical Sciences, Kyushu University, 3-1-1 Higashi-ku, Fukuoka, 812-8582, Japan. Tel: +81 926426210 ext. 6210, Fax: +81 926426146 e-mail: ds-mons@yb3.so-net.ne.jp

Key Words: Mouse erythroleukemia cells, docking protein 2, Dok2, Klf1, erythropoiesis.

activated protein kinase (Mapk) and Thymoma viral proto-condogene (Akt) activation (18, 19). The role of Dok2 in myeloid leukemia has also been investigated and it was found to act as a suppressor of leukemia (15, 19). Although the function of Dok2 as a member of tyrosine kinase signaling molecules and its roles in regulating myelopoiesis have been extensively investigated, it still remains unclear how Dok2 functions in hematopoietic transcriptional regulation. To address this, we knocked-down *Dok2* gene in mouse erythroleukemia (MEL) cell line, which is a kind of leukemia cell line with the potential to be induced into erythroid cells and is widely used to study erythropoiesis. Herein we present evidence that Dok2 regulates the expression of *Klf1* through directly binding to its promoter region and demonstrated a novel role of Do2 as a mediator of gene transcription in hematopoiesis.

Materials and Methods

Cell lines. Two types of cell lines, MEL cell line (kindly provided by Dr. Brand, Ottawa Health Research Institute, Ottawa, Canada) and Friend erythroleukemia cell line (F5-5, Fl, RIKEN BioResource Center, Ibaraki, Japan) were used in this study. The cells were maintained in RPMI-1640 (Wako Pure Chemical Industries, Osaka, Japan) supplemented with 10% fetal bovine serum (FBS) and 10 U/ml penicillin and 10 mg/ml streptomycin (Sigma-Aldrich, Saint Louis, MO, USA). Cells were passaged every 3-4 days.

siRNA transfection. Knock-down of Dok2 mRNA in MEL cells was performed using siRNAs (Sigma- Aldrich) with transfection reagent, Lipofectamine® 2000 (Life Technologies, Palo Alto, CA, USA). Silencer® Negative Control No.1 siRNA (Ambion, Austin, TX, USA) was used as a control siRNA. Before the transfection, 1.0×10^5 cells were plated in 24-well plate in RPMI-1640 containing 1% FBS. Two microliters of Lipofectamine and 60 pmole of single siRNAs were mixed with 50 μl of Opti-MEM®I Reduced Serum Medium (Life Technologies), respectively, and incubated at room temperature for 20 minutes to form Lipofectamine–siRNA complex. After incubation, Lipofectamine–siRNA complexes were mixed with the cells and incubated at 37oC, 5% CO_2. Cells were collected at 24 hours after transfection and kept in RNAlater (Life Technologies) until gene expression analysis.

May-Grünwald Giemsa staining. Cultured cells were attached onto glass slides (Matsunami glass, Kishiwada, Osaka, Japan) by CytoSpin4 (Thermo Fisher scientific, Waltham, MA, USA) at 450 rpm for 7 min and dried thoroughly. Cells were fixed and stained with May-Grünwald reagent (Muto Pure Chemicals, Tokyo, Japan) at room temperature for 5 min, briefly washed with tap water and incubated with PBS, pH 6.4, for 2 min. Cells were then incubated with 1:18 diluted Giemsa solution (Muto Pure Chemicals) at room temperature for 30 min. After washing with tap water, the slides were dried and were covered with glass coverslips by using MGK-S mounting solution (Matsunami glass). Slides were observed using an Olympus CKX41 microscope (Olympus, Tokyo, Japan).

Immunocytochemistry. Cultured cells were attached onto glass slides (Matsunami glass) by CytoSpin4 (Thermo Fisher scientific) at 450 rpm for 7 min and dried thoroughly. Cells were fixed in 1% paraformaldehyde at 4°C for 30 min. After washing with PBS, cells were incubated with PBS containing 0.05% Triton-X 100 at room temperature for 15 min. After three washes with PBS, the cells were blocked with PBS containing 1% BSA at room temperature for 30 min and incubated overnight at 4°C with goat anti-mouse Dok2 (1:100, Santa Cruz Biotechnology, Dallas, TX, USA) primary antibody. After three washes with PBS, cells were then incubated with donkey anti-goat IgG AlexaFluor488 (1:400, Invitrogen, Carlsbad, CA, USA) and TOTO-3 iodide (1:1500; Invitrogen) at room temperature for 30 min. After a further three washes with PBS, cells were mounted on coverslips with fluorescent mounting medium (Dako Corporation, Glostrup, Denmark) and assessed using a Fluo View 1000 confocal microscope (Olympus).

Flow cytometric analysis. Cells were stained with 1 μg/ml of propidium iodide (PI) (Invitrogen) to distinguish the dead cells and analyzed by BD FACS Aria (BD Bioscience, San Jose, CA, USA).

RNA extraction and real-time polymerase chain reaction (PCR). Total RNA was extracted from cultured cells using RiboPure™ Kit (Ambion), and mRNA was reverse transcribed into cDNA using a High-Capacity RNA-to-cDNA Kit (Life Technologies). Expressions of *Dok2*, *Gata1*, *Klf1*, *α-globin*, *β-globin* and *β-actin* were assessed by using StepOnePlus™ real-time PCR (Life Technologies) with TaqMan® Gene Expression Assays (Life Technologies). mRNA levels were normalized to that of β-actin and relative expression of each gene calculated using a relative standard curve method.

Chromatin immunprecipitation (ChIP) assay. ChIP assay was performed using a Chromatin Immunoprecipitation Assay Kit (Millipore, Bedford, MA, USA) according to the manufacturer's protocol. For ChIP assay, 1×10^6 cells were used. Proteins and genomic DNA were cross-linked by formaldehyde and then sheared by sonication. Immunoprecipitation was carried out overnight with 2 μg of goat anti-mouse Dok2 antibody (Santa Cruz Biotechnology). The amount of *Klf1* and *β-globin* promoter in total input DNA and immunoprecipitated DNA were measured by StepOnePlus™ real-time PCR (Life Technologies) with Fast SYBR® Green Master Mix (Life Technologies). Promoter regions of *Klf1* and *β-globin* gene were identified by the Database of Transcriptional Start Sites Release 8.0 (http://dbtss.hgc.jp/) and were located between 500-1000 bp at 5'-end of transcriptional start sites. Primer sets binding to the promoter regions were designed by Primer Express version 3.0 software (Life Technologies). The sequences of primers were: *Klf1* promoter-specific primers, forward: 5'-TCTGCTCAAGGAG GAACAGAGCTA-3', reverse: 5'-GGCTCCCTTTCAGGCATTA TCAGA-3'; and *β-globin* promoter-specific primers, forward: 5'-GACAAACATTATTCAGAGGGAGTA-3', reverse: 5'-AAGCAA ATGTGAGGAGCAACTGAT-3'.

Statistical analysis. Results are expressed as the mean±SD. Paired samples were compared using Student's *t*-test.

Results

Expression of Dok2 in mouse erythroleukemia cell lines. Gene and protein expression of Dok2 were examined by real-time PCR and immunocytochemistry. Figure 1A shows the relative expression of *Dok2* mRNA in the two types of

erythroleukemia cell lines, F5-5. fl and MEL. *Dok2* was expressed in both cell lines and MEL exhibited 1.37±0.095-fold (p=0.027) higher expression when compared to F5-5. fl. Based on the higher expression of *Dok2* mRNA in MEL cells, we further analyzed the protein expression of Dok2 in MEL cells by immunocytochemistry. We observed that Dok2 (green) is localized in both the cytoplasm and nucleus of MEL cells. There were cells weakly-expressing Dok2 and not all cells expressed Dok2. Greater intensity was detected in cytoplasm compared to in nucleus (Figure 1B).

Loss-of-function of Dok2 in MEL cells. To investigate the function of Dok2 in MEL cells, *Dok2* mRNA was knocked-down by transfection with siRNA for 24 h. Cell morphology after siRNA transfection was observed under microscopy and also assessed by May-Grünwald Giemsa staining. Most of the cells were round and uniform in size, and some cells were swollen and contained vacuoles in their cytoplasm, both in control and *Dok2* siRNA-transfected cells (Figure 2A). May-Grünwald Giemsa staining also showed that vacuoles were present in the cytoplasm (Figure 2B). No obvious morphological differences were observed between control siRNA and Dok2 siRNA-transfected cells. Efficiency of *Dok2* knock-down was assessed by real-time PCR and 61% reduction of *Dok2* mRNA were achieved compared to the control cells (Figure 2C). The effects of *Dok2* knock-down on cell proliferation and cell viability were assessed by flow cytometry. Analysis showed that the live cell numbers were 46854±13719 cells in the control sample and 49828±5734 cells in *Dok2* siRNA-transfected cells (Figure 2D). The cell viability was calculated as percentage based on the number of live PI-negative cells among the total collected cells and was 70.1±5.69% and 66.6±1.73% in control siRNA and Dok2 siRNA transfected cells, respectively (Figure 2E). There were no significant differences of cell number (p=0.76) and viability (p=0.37) between the control siRNA-and *Dok2* siRNA-transfected cells. In summary, *Dok2* knock-down did not affect the proliferation and viability of MEL cells.

Dok2 knock-down induces the expression of erythropoietic transcription factor Klf1. MEL cell line was derived from leukemia cells and its differentiation state is equivalent to that of erythroid progenitors (20). Because of their potential to follow erythroid differentiation, they have been utilized to investigate erythropoiesis. To investigate whether Dok2 is involved in erythroid differentiation, the expression of differentiation-related genes, *Gata1*, *Klf1*, *α-globin* and *β-globin* were assessed by real-time PCR. Slight decrease of *Gata1* mRNA (0.85±0.044-fold, p=0.052) and *β-globin* mRNA (0.91±0.065-fold, p=0.15) expressions were observed after knock-down of *Dok2*. *α-Globin* mRNA expression was significantly down-regulated after Dok2 siRNA transfection and 0.76±0.064-fold (p=0.0050) lower than control siRNA-transfected cells. Among the genes we investigated, only *Klf1* was significantly up-regulated in MEL cells transfected with *Dok2* siRNA when compared to control siRNA (1.94±fold, p=0.0023) (Figure 3A).

Dok2 binds to the promoter region of Klf1. Since the knock-down of *Dok2* caused significant up-regulation of *Klf1* mRNA, we further investigated whether Dok2 directly regulates the expression of *Klf1*. By performing ChIP assay, we examined the binding of Dok2 to the promoter region of *Klf1* and *β-globin*. Figure 4 shows that the expression of Klf1 promoter was enriched 2.44±0.96-fold (p=0.048) in the sample immunoprecipitated with an antibody against mouse Dok2 when compared to the control sample without antibody. On the other hand, expression of *β-globin* promoter was not enriched and was shown to be 0.0016±0.0011-fold ($p=9.4\times10^{-13}$) lower when compared to the control sample without antibody. Thus, we found that Dok2 indeed binds to the promoter region of *Klf1*, but not of *β-globin*.

Discussion

Dok2 has been reported to be localized in the cytoplasm and also the membrane of human embryonic kidney 293T (HEK293T) cell line through its Pleckstrin-homology (PH) and Phosphotyrosine binding (PTB) domains (21). Cytoplasmic Dok2 is phosphorylated under the activated endothelial-specific receptor tyrosine kinase (Tek, also known as Tie2) and further activates Ras GTPase-activating proteins and the adapter protein Non-catalytic region of tyrosine kinase (Nck), which are involved in cell motility (22). Thus, cytoplasmic Dok2 is a molecule of tyrosine kinase signaling. Dok2 comprises a nuclear export sequence, which regulates the translocation of proteins from the nucleus to the cytoplasm, implying the existence of Dok2 in both compartments (23). However, nuclear Dok2 expression and its function have not previously been reported. Herein we showed that Dok2 protein is localized in both the nucleus and cytoplasm of MEL cells by immunocytochemistry (Figure 1B). This implies that nuclear Dok2 regulates gene expression in MEL cells.

Concerning hematopoietic cells, Dok2 is primarily expressed in hematopoietic precursors (15) and is also highly expressed in T-cells of the spleen and thymus, in addition to myeloid cells in bone marrow (24). Double knock-out of *Dok1* and *Dok2* in mice induces the abnormal proliferation of myeloid cells, characterized by an increased percentage of immature granulocytic/monocytic precursors in the spleen and bone marrow, and also leads to hyperplasia of megakaryocytes and myeloid progenitors in bone marrow (18, 19). These changes were accompanied by an increases in Ras/Mapk and Akt activation, which leads to activation of

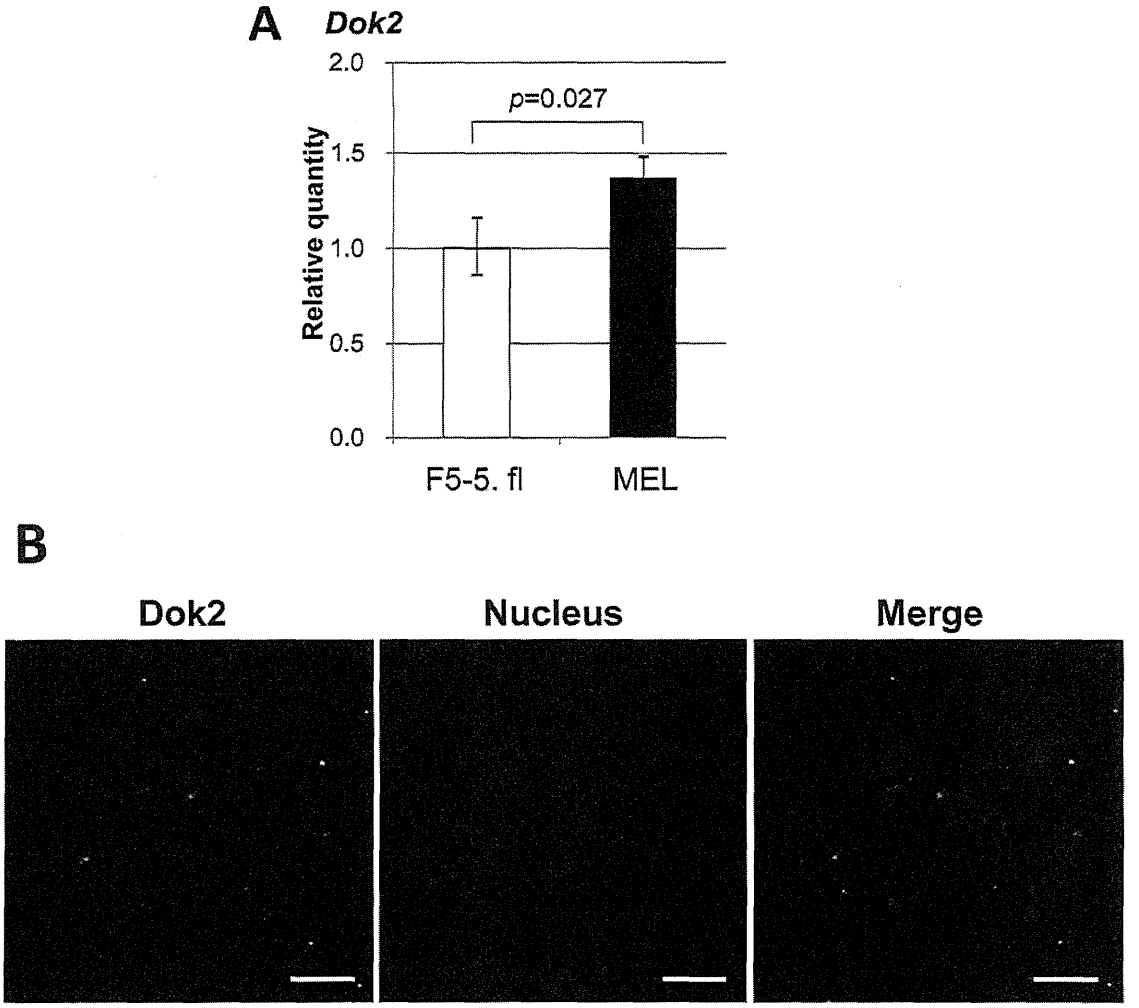

Figure 1. *Expression of Docking protein 2 (Dok2) in MEL cells. A: Relative expression of Dok2 mRNA in F5-5. fl cells and MEL cells examined by real-time polymerase chain reaction. Expression of Dok2 in MEL cells was 1.37±0.095-fold (p=0.027, Student's t-test) higher when compared to F5-5. fl cells. B: Immunocytochemical image of Dok2 protein in MEL cells. Cells were stained with goat antibody to mouse Dok2 (green) and TOTO-3 iodide (blue) for nuclear staining. Dok2 protein was localized in both cytoplasm and nucleus of MEL cells. Scale bar=10 μm.*

anti-apoptotic genes and increases survival of hematopoietic cells (25). Although Dok2 in tyrosine kinase signaling and its role in regulating myelopoiesis have been extensively investigated, the function of Dok2 in transcriptional regulation and its role in erythropoiesis has not been fully investigated. The MEL cell line is a tool widely used in the study of erythropoiesis. Based on gene and protein expression of Dok2 in MEL cells (Figure 1A and 1B), we further investigated the function of Dok2 by using siRNA transfection. Inhibition of *Dok2* expression reportedly accelerates cell proliferation of macrophage-like J774A.1 cells through affecting Rous sarcoma oncogene (Src) family kinase which mediates the induction of a cell proliferation-related gene known as Myelocytomatosis oncogene (*Myc*) (26). However, there was no significant difference in proliferation of MEL cells between control and *Dok2* siRNA-transfected cells (Figure 2D), unlike the previous report. On the other hand, we found a significant up-regulation of *Klf1* mRNA after knock-down of *Dok2* (Figure 3). Klf1 is a transcription factor which is a direct target of Gata1 and plays important roles in erythropoiesis. Three major aspects of Klf1 function have been reported: regulation of erythroid-lineage commitment, switching between γ- and β-globin, and maturation of erythrocytes during terminal

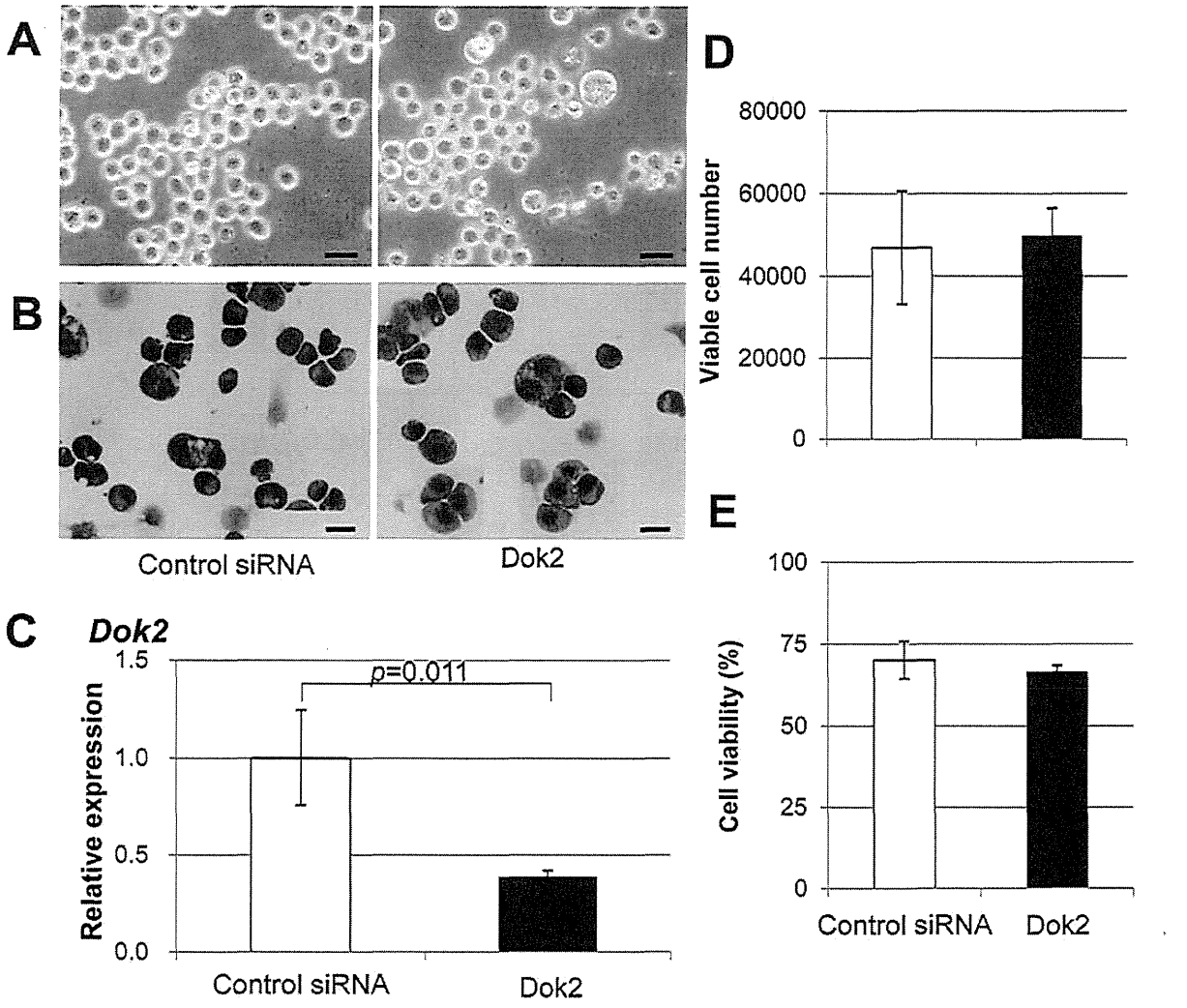

Figure 2. *Loss-of-function of Docking protein 2 (Dok2) in MEL cells. A: Microscopic images of MEL cells cultured with control siRNA (left) and Dok2 siRNA (right) for 24 h. Scale bar=20 μm. B: May-Grünwald Giemsa staining images of MEL cells cultured with control siRNA (left) and Dok2 siRNA (right) for 24 h. Scale bar=20 μm. C: Relative expression of Dok2 mRNA after 24 h of siRNA transfection. Live cell numbers (D) and viability (E) of MEL cells after 24 h of siRNA transfection.*

erythropoiesis (27). The data we presented show that Dok2 could potentially bind to the promoter region of *Klf1*, and that knock-down of *Dok2* altered *Klf1* expression. This suggests that Dok2 transcriptionally down-regulates *Klf1* in MEL cells.

Herein we provide evidence that Dok2 is able to transcriptionally regulate gene expressions in addition to its functions in downstream signaling of tyrosine kinase, and the change of *Klf1* expression after *Dok2* knock-down suggests the possibility that Dok2 is involved in regulation of erythropoiesis and not just myelopoiesis. Although the functions of nuclear Dok2 have not been elucidated, our observation suggests the novel transcription factor-like function of Dok2, and it also suggests that Dok2 is involved in the erythropoietic transcriptional system and in the maintenance of proper erythropoiesis.

Acknowledgements

We thank Ms. Chiyoko Nakamichi and Naoko Kojima for technical support, and the Ministry of Education, Culture, Sports, Science and Technology, the Ministry of Health, Labour and Welfare, and Japan Society for the Promotion of Science (JSPS) bilateral program for grant support. We thank the Mr. Anthony Swain for critical reading of this manuscript.

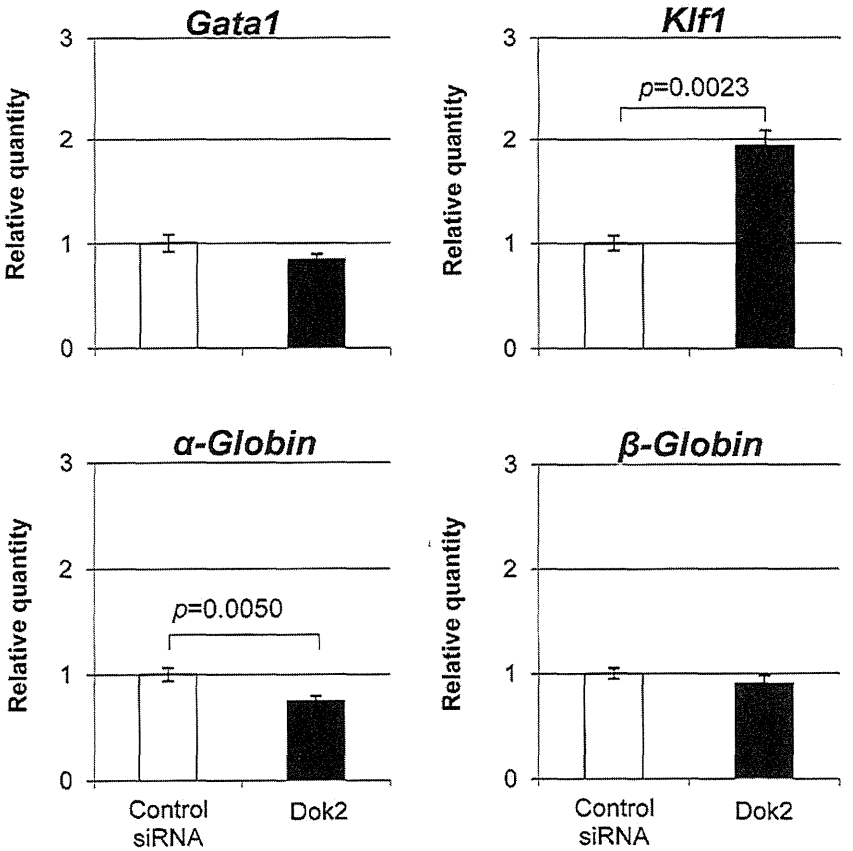

Figure 3. *Gene expression analysis of erythroid differentiation-related genes. Relative expressions of erythroid differentiation-related genes, GATA binding protein 1 (Gata1), Krüppel-like factor 1 (Klf1), α-globin and β-globin after 24 hours of siRNA transfection. mRNA levels were normalized to that of β-actin and relative expression of each gene was calculated using a relative standard curve method. Significant differences were calculated with the Student's t-test.*

References

1 Jagannathan-Bogdan M and Zon LI: Hematopoiesis. Development *140*: 2463-2467, 2013.

2 Iwasaki H: Roles of transcription factors in normal and leukemic hematopoiesis. Rinsho Ketsueki *54*: 1566-1572, 2013.

3 Tsunoda T and Shirasawa S: Roles of ZFAT in haematopoiesis, angiogenesis and cancer development. Anticancer Res *33*: 2833-2837, 2013.

4 Sugiyama D, Inoue-Yokoo T, Fraser ST, Kulkeaw K, Mizuochi C and Horio Y: Embryonic regulation of the mouse hematopoietic niche. Sci World J *11*: 1770-1780, 2011.

5 Sugiyama D, Kulkeaw K and Mizuochi C: TGF-beta-1 up-regulates extracellular matrix production in mouse hepatoblasts. Mech Dev *130*: 195-206, 2013.

6 Steinleitner K, Rampetsreiter P, Koffel R, Ramanathan G, Mannhalter C, Strobl H and Wieser R: EVI1 and MDS1/EVI1 expression during primary human hematopoietic progenitor cell differentiation into various myeloid lineages. Anticancer Res *32*: 4883-4889, 2012.

7 Dore LC and Crispino JD: Transcription factor networks in erythroid cell and megakaryocyte development. Blood *118*: 231-239, 2011.

8 Cantor AB and Orkin SH: Transcriptional regulation of erythropoiesis: an affair involving multiple partners. Oncogene *21*: 3368-3376, 2002.

9 Kulessa H, Frampton J and Graf T: GATA-1 reprograms avian myelomonocytic cell lines into eosinophils, thromboblasts and erythroblasts. Genes Dev *9*: 1250-1262, 1995.

10 Visvader JE, Elefanty AG, Strasser A and Adams JM: GATA-1 but not SCL induces megakaryocytic differentiation in an early myeloid line. EMBO J *11*: 4557-4564, 1992.

11 Scott EW, Simon MC, Anastasi J and Singh H: Requirement of transcription factor PU.1 in the development of multiple hematopoietic lineages. Science *265*: 1573-1577, 1994.

12 Voso MT, Burn TC, Wulf G, Lim B, Leone G and Tenen DG: Inhibition of hematopoiesis by competitive binding of transcription factor PU.1. Proc Natl Acad Sci USA *91*: 7932-7936, 1994.

13 Shima Y and Kitabayashi I: Deregulated transcription factors in leukemia. Int J Hematol *94*: 134-141, 2011.

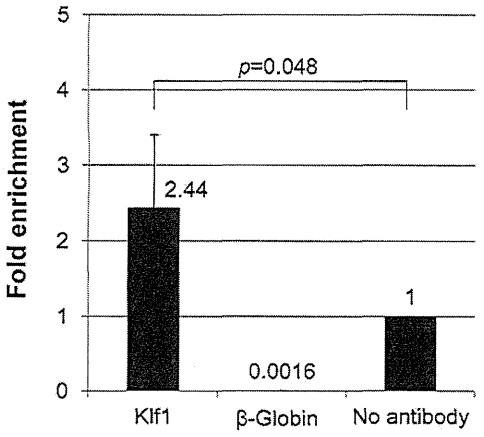

Figure 4. *Chromatin immunoprecipitation (ChIP) assay of Docking protein 2 (Dok2). The amount of Krüppel-like protein 1 (Klf1) and β-globin promoter in total input DNA and immunoprecipitated DNA were measured by real-time polymerase chain reaction. The expression of Klf1 promoter was enriched by 2.44±0.96-fold in the sample immunoprecipitated with antibody to murine Dok2 when compared to the control sample without antibody. Significant differences were calculated with the Student's t-test.*

14 Cong F, Yuan B and Goff SP: Characterization of a novel member of the DOK family that binds and modulates ABL signaling. Mol Cell Biol *19*: 8314-8325, 1999.
15 Di Cristofano A, Carpino N, Dunant N, Friedland G, Kobayashi R, Strife A, Wisniewski D, Clarkson B, Pandolfi PP and Resh MD: Molecular cloning and characterization of p56dok-2 defines a new family of RasGAP-binding proteins. J Biol Chem *273*: 4827-4830, 1998.
16 Jones N and Dumont DJ: The Tek/Tie2 receptor signals through a novel Dok-related docking protein, Dok-R. Oncogene *17*: 1097-1108, 1998.
17 Nelms K, Snow AL, Hu-Li J and Paul WE: FRIP, a hematopoietic cell-specific rasGAP-interacting protein phosphorylated in response to cytokine stimulation. Immunity *9*: 13-24, 1998.
18 Yasuda T, Shirakata M, Iwama A, Ishii A, Ebihara Y, Osawa M, Honda K, Shinohara H, Sudo K, Tsuji K, Nakauchi H, Iwakura Y, Hirai H, Oda H, Yamamoto T and Yamanashi Y: Role of Dok-1 and Dok-2 in myeloid homeostasis and suppression of leukemia. J Exp Med *200*: 1681-1687, 2004.
19 Niki M, Di Cristofano A, Zhao M, Honda H, Hirai H, Van Aelst L, Cordon-Cardo C and Pandolfi PP: Role of Dok-1 and Dok-2 in leukemia suppression. J Exp Med *200*: 1689-1695, 2004.
20 Lachman HM: c-myc protooncogene expression in mouse erythroleukemia cells. Environ Health Perspect *80*: 161-172, 1989.
21 Jones N, Chen SH, Sturk C, Master Z, Tran J, Kerbel RS and Dumont DJ: A unique autophosphorylation site on Tie2/Tek mediates Dok-R phosphotyrosine binding domain binding and function. Mol Cell Biol *23*: 2658-2668, 2003.
22 Kulkarni SV, Gish G, van der Geer P, Henkemeyer M and Pawson T: Role of p120 Ras-GAP in directed cell movement. J Cell Biol 149: 457-470, 2000.
23 Niu Y, Roy F, Saltel F andrieu-Soler C, Dong W, Chantegrel AL, Accardi R, Thepot A, Foiselle N, Tommasino M, Jurdic P and Sylla BS: A nuclear export signal and phosphorylation regulate Dok1 subcellular localization and functions. Mol Cell Biol *26*: 4288-4301, 2006.
24 Yasuda T, Bundo K, Hino A, Honda K, Inoue A, Shirakata M, Osawa M, Tamura T, Nariuchi H, Oda H, Yamamoto T and Yamanashi Y: Dok-1 and Dok-2 are negative regulators of T-cell receptor signaling. Int Immunol *19*: 487-495, 2007.
25 Yen JJ and Yang-Yen HF: Transcription factors mediating interleukin-3 survival signals. Vitam Horm 74: 147-163, 2006.
26 Suzu S, Tanaka-Douzono M, Nomaguchi K, Yamada M, Hayasawa H, Kimura F and Motoyoshi K: p56(dok-2) as a cytokine-inducible inhibitor of cell proliferation and signal transduction. EMBO J *19*: 5114-5122, 2000.
27 Siatecka M and Bieker JJ: The multifunctional role of EKLF/KLF1 during erythropoiesis. Blood *118*: 2044-2054, 2011.

Received April 4, 2014
Revised June 9, 2014
Accepted June 10, 2014

blood

2014 123: 1586-1595
doi:10.1182/blood-2013-09-526087 originally published online January 17, 2014

Mutations in Krüppel-like factor 1 cause transfusion-dependent hemolytic anemia and persistence of embryonic globin gene expression

Vip Viprakasit, Supachai Ekwattanakit, Suchada Riolueang, Nipon Chalaow, Chris Fisher, Karen Lower, Hitoshi Kanno, Kalaya Tachavanich, Sasithorn Bejrachandra, Jariya Saipin, Monthana Juntharaniyom, Kleebsabai Sanpakit, Voravarn S. Tanphaichitr, Duantida Songdej, Christian Babbs, Richard J. Gibbons, Sjaak Philipsen and Douglas R. Higgs

Blood (print ISSN 0006-4971, online ISSN 1528-0020), is published weekly by the American Society of Hematology, 2021 L St, NW, Suite 900, Washington DC 20036.

Regular Article

RED CELLS, IRON, AND ERYTHROPOIESIS

Mutations in Krüppel-like factor 1 cause transfusion-dependent hemolytic anemia and persistence of embryonic globin gene expression

Vip Viprakasit,[1] Supachai Ekwattanakit,[2,3] Suchada Riolueang,[3] Nipon Chalaow,[3] Chris Fisher,[4] Karen Lower,[5] Hitoshi Kanno,[6] Kalaya Tachavanich,[1] Sasithorn Bejrachandra,[7] Jariya Saipin,[7] Monthana Juntharaniyom,[8] Kleebsabai Sanpakit,[1] Voravarn S. Tanphaichitr,[1] Duantida Songdej,[4] Christian Babbs,[4] Richard J. Gibbons,[4] Sjaak Philipsen,[9] and Douglas R. Higgs[4]

[1]Department of Pediatrics & Thalassemia Centre, [2]Department of Medicine, and [3]MSc-PhD in Immunology Program, Department of Immunology, Faculty of Medicine Siriraj Hospital, Mahidol University, Bangkok, Thailand; [4]Medical Research Council Molecular Haematology Unit, Weatherall Institute of Molecular Medicine, Headington, Oxford, United Kingdom; [5]Department of Hematology and Genetic Pathology, School of Medicine, Flinders University, Adelaide, Australia; [6]Department of Blood Transfusion Medicine and Cell Processing, Women's Medical College, Tokyo, Japan; [7]Department of Transfusion Medicine, Faculty of Medicine Siriraj Hospital, Mahidol University, Bangkok, Thailand; [8]Department of Pediatrics, Khon Kaen Regional Hospital, Ministry of Public Health, Khon Kaen, Thailand; and [9]Erasmus University Medical Center-Cell Biology, Rotterdam, The Netherlands

Key Points

- KLF1 mutations cause severe congenital hemolytic anemia associated with a deficiency of red cell pyruvate kinase.
- A severe KLF1 deficiency causes hereditary persistence of embryonic globin synthesis.

In this study, we report on 8 compound heterozygotes for mutations in the key erythroid transcription factor Krüppel-like factor 1 in patients who presented with severe, transfusion-dependent hemolytic anemia. In most cases, the red cells were hypochromic and microcytic, consistent with abnormalities in hemoglobin synthesis. In addition, in many cases, the red cells resembled those seen in patients with membrane defects or enzymopathies, known as chronic nonspherocytic hemolytic anemia (CNSHA). Analysis of RNA and protein in primary erythroid cells from these individuals provided evidence of abnormal globin synthesis, with persistent expression of fetal hemoglobin and, most remarkably, expression of large quantities of embryonic globins in postnatal life. The red cell membranes were abnormal, most notably expressing reduced amounts of CD44 and, consequently, manifesting the rare In(Lu) blood group. Finally, all tested patients showed abnormally low levels of the red cell enzyme pyruvate kinase, a known cause of CNSHA. These patients define a new type of severe, transfusion-dependent CNSHA caused by mutations in a *trans*-acting factor (Krüppel-like factor 1) and reveal an important pathway regulating embryonic globin gene expression in adult humans. (*Blood*. 2014;123(10):1586-1595)

Introduction

Anemia is a major public health problem affecting more than 1.5 billion people worldwide.[1] The major causes of anemia include malnutrition, particularly iron and folate deficiency, and infections including malaria and HIV. However, a substantial proportion of patients with anemia have inherited red cell defects, with mutations found in genes encoding red cell enzymes, membrane proteins, heme, and globins. For example, every year more than 300 000 affected individuals are born with severe anemia resulting from inherited hemoglobinopathies.[2] However, after screening for mutations in genes currently known to underlie red cell defects, there are many cases of inherited anemia in which either the patient's clinical and hematological phenotype is not consistent with their described genotype or the cause of the anemia remains completely unknown.

Here we report on 8 unrelated patients who displayed severe, transfusion-dependent neonatal anemia with red cell abnormalities ranging from a thalassemia-like morphology (with hypochromic microcytic red cells) to that usually associated with chronic nonspherocytic hemolytic anemias (CNSHA) with anisopoikilocytosis, fragmented cells, and a high reticulocyte count. In addition, all of the probands had a remarkable persistence of fetal and embryonic globin synthesis. We found that the parents of these children also had significantly raised levels of fetal hemoglobin (HbF) consistent with previous observations in heterozygotes for *KLF1* mutations. In the light of emerging evidence that mutations in *KLF1*, the gene encoding erythroid Krüppel-like factor (EKLF), give rise to persistent postnatal γ-globin expression in humans[3-7] and embryonic globin expression in mice,[8,9] we sequenced this gene in the probands. We discovered that all of them are compound heterozygotes for mutations in *KLF1*. Only 3 compound heterozygotes for KLF1 mutations have been previously reported, and their phenotypes have been only partially described.[6,7] The transcription factor KLF1 is a master regulator of terminal erythroid differentiation, controlling expression of many key pathways and structures including cell division, the cell membrane and cytoskeleton, iron metabolism, and heme and globin synthesis.[5] Here, we show that individuals with mutations affecting both alleles of *KLF1* exhibit severe anemia, in which 1 or more of these pathways is significantly disrupted, giving rise to a wide spectrum of hematological phenotypes. The syndromes described here add to an increasing

Submitted September 6, 2013; accepted January 10, 2014. Prepublished online as *Blood* First Edition paper, January 17, 2014; DOI 10.1182/blood-2013-09-526087.

The online version of this article contains a data supplement.

The publication costs of this article were defrayed in part by page charge payment. Therefore, and solely to indicate this fact, this article is hereby marked "advertisement" in accordance with 18 USC section 1734.

list of *trans*-acting mutations that cause unusual forms of inherited anemia.[10-13] Most important, all such patients show persistent expression of substantial amounts of embryonic hemoglobins in adult life. Despite extensive characterization of the patterns of globin expression in millions of patients during the last 30 years, persistent expression of embryonic globins in adults has very rarely been reported.

Materials and methods

Blood samples

Blood was collected from all patients and parents after they signed a written informed consent. Patients receiving regular blood transfusion had a period of at least 10 weeks without blood transfusions before sample collection. This study was approved by the local ethical committees at Siriraj Hospital, Bangkok, Thailand, and at the John Radcliffe Hospital, Oxford, United Kingdom. This study also was conducted in accordance with the Declaration of Helsinki.

Hematology and blood serum factors

Routine hematology was determined using an automated red blood cell counter (Sysmex F280). Hemoglobin was analyzed by liquid chromatography, using an automated hemoglobin analyzer (HB Gold; Cumbria) and by isoelectric focusing (IEF) (Resolve; PerkinElmer). Abnormal hemoglobins were excised from the IEF gel, digested with trypsin and analyzed using a matrix-assisted laser desorption/ionization time-of flight (MALDI-TOF/TOF) mass spectrometer (Ultralex Bruker Daltonics). Routine biochemical parameters were measured using standard techniques.

Red cell phenotype analysis

Samples were tested for the Lutheran blood group using a gel card (DiaMed) with other additional blood group antigens including P1, Le^a, Le^b, k, Kp^a, Kp^b, Jk^a, Jk^b, M, N, S, s, Fy^a, and Fy^b, as per manufacturer's instructions. The presence of Band-3 (AE-1 anion exchanger) on the red blood cell surface was estimated by the eosin-5-maleimide binding assay.[14]

Red blood cell enzyme activities for glucose-6-phosphate dehydrogenase, 6-phosphogluconate dehydrogenase, pyruvate kinase (PK), hexokinase, glucose phosphate isomerase, phosphofructokinase, aldolase, and phosphoglycerate kinase were assessed as described previously.[15] Because reticulocytes contain higher amounts of these enzymes than mature red blood cells, a calculation for the net activities of each enzyme by correction for the reticulocyte number was performed to compare the results with our standard reference range.[15]

Flow cytometry to assess the proportions of HbF,[16] Hb Bart's (γ4), Hb ζ (ζ), and CD44 was performed on mature erythrocytes ($CD71^-$) and circulating erythroblasts ($CD71^+$) of patients and parents. In addition, leukocytes were stained with CD44 and CD45 after lysis of erythrocytes with fluorescence-activated cell sorter lysis solution (BD Biosciences). The antibodies used were anti-HbF directly conjugated to fluorescein isothiocyanate (FITC; Caltag), anti-Hb Bart's (γ4), anti-Hb ζ (ζ), CD44 PE, CD45 Per CP, and CD71 PE-Cy5 (BD Biosciences), as well as isotype controls (mouse immunoglobulin G FITC and FITC-conjugated F[ab']2 anti-mouse globulin; Dako).

Molecular characterization of the globin, KLF1 and PKLR genes

We performed multiplex Gap-PCR[17] and amplification-refractory mutation system–PCR assays for detection of common α-thalassemia mutations.[18] Sequence analysis was performed on 1.3 kb of the α2- and α1-, 1.81 kb of the ζ-, 2.2 kb of the ε-, and 2.85 kb of the β-globin genes and hypersensitive site 2 of the β-globin locus control region (630 bp), all promoters of the ^{G}γ- (807 bp), ^{A}γ- (802 bp), and β-globin (1044 bp) genes, using standard techniques. All primer sequences are summarized in supplemental Table 6, available on the *Blood* Web site. A multiplex ligation-dependent probe amplification assay was performed to exclude large rearrangements of the β-globin cluster.[19] The *KLF1* and *PKLR* genes were amplified and sequenced as previously described.[20,21]

Primary erythroid cell culture and expression analysis

Erythroid cells were obtained using a 2-phase culture system[22] and harvested once they were double-positive for CD71 and glycophorin A. RNA was extracted, reverse transcribed to generate double-stranded cDNA (Superscript Double Stranded cDNA Synthesis Kit; Invitrogen), and labeled with dCTP-Cy3 as the test sample; 500 ng sonicated genomic DNA was labeled with dCTP-Cy5 as the input (Bioprime DNA Labeling System; Invitrogen). Both test and input samples were applied to a custom-designed Agilent expression array, as described.[23] cDNA was analyzed by real-time PCR assays obtained from Applied Biosystem's Assay-on-Demand resource. Expression was calculated relative to a reference gene (PABPC1) and normal Thai subjects (n = 6) as controls, using the Offal method.[24]

Results

Clinical presentation

All 8 patients reported here presented with neonatal jaundice requiring phototherapy and, in 5 cases, blood transfusion. Most patients were anemic at presentation with marked hepatosplenomegaly. By 1 year of age, all patients had received at least a single blood transfusion, and subsequently, all of them required regular transfusion during the first years of life. Three patients required splenectomy caused by hypersplenism. A summary of the clinical presentations and disease severity of the 8 probands is presented in supplemental Table 1 and associated footnotes.

Hematological evaluation

Full blood counts were determined for all 8 index patients and their parents and available siblings (Table 1). Despite transfusion, the levels of Hb in the probands (range, 3.0–8.3 g/dL) were low in some cases (Table 1). With a single exception (P1), all of the patients had hypochromic red cells (mean cell hemoglobin [MCH], 20.1–24.8 pg), suggesting abnormalities in the heme and/or globin synthesis pathways. All of the patients showed raised numbers of reticulocytes (5%–16%) and many nucleated red blood cells in the peripheral blood, suggesting a significant hemolytic component to the anemia. The morphology of the peripheral blood was variable. In some patients (P1, Figure 1A; P2–P4, supplemental Figure 1), the peripheral blood resembled that seen in patients with thalassemia, with marked hypochromasia, microcytosis, anisopoikilocytosis, target cells, and fragmented red cells. In those patients who coinherited globin gene defects, the severity of the changes was much greater than normally associated with these genotypes. In contrast, the peripheral blood of others (P5, Figure 1B; P6–P8, supplemental Figure 1) resembled that seen in nonspherocytic hemolytic anemia, with numerous fragmented red blood cells, schistocytes, and acanthocytes.

The parents and siblings of the 8 affected individuals were not anemic (Hb, 11.4–15.6 g/dL), although many of them had abnormal red cell indices because they are carriers for common forms of thalassemia in Thailand (see following). In the 6 individuals (highlighted in gray in Table 1) who were not carriers of hemoglobinopathies (αα/αα:β/β), the red cell indices were either unremarkable or marginally hypochromic and microcytic (average MCH, 27.1 + 1.71 pg [range, 25.1–29.3 pg]; average mean corpuscular volume, 81.9 + 5.57 fL [range, 75.4–89.5 fL]).

Table 1. Hematological parameters, globin and *KLF1* genotypes in Thai families with unusual forms of hereditary hemolytic anemia

												Hemoglobin typing (%)					Globin genotypes		*KLF1* genotypes	
Cases	Sex	Age (y, mo)	Hb (g/dL)	Hct (%)	RBC (×106/μL)	MCV (fL)	MCH (pg)	MCHC (g/dL)	RDW (%)	Retic. (%)	NRBC (/100WC)	Hb A	Hb F	HbA2/E	Hb Portland-1	Hb Bart's	αα/αα	β/β	Allele 1	Allele 2
Family A																				
P1	F	4*	3.0	8.5	1.06	80.1	28.1	35.1	16.2	6.0	248	70.1	16.2	3.1	ND	ND	αα/αα	β/β	R331W	G335R
		6.2†	6.9	25.1	2.36	106.2	29.1	27.4	19.0	16.0	1510	38.5	51.8	2.0	4	2				
Father	M	37	13.4	40.8	4.56	89.5	29.3	32.7	13.3	2.0	0	82.3	1.5	2.7	0	0	αα/αα	β/β	W	G335R
Mother	F	34	12.2	37.4	4.69	79.7	26.0	32.6	13.5	1.0	0	82.5	3.0	2.7	0	0	αα/αα	β/β	R331W	W
Family B																				
P2	M	0.6‡	5.9	18.2	2.76	65.9	21.4	32.4	NA	5.0	26	72.3¶	16.6	3	8.1§	0	−SEA/αα	βE/β	G176RfsX179	R301H
		12‖	7.5	25.7	3.72	69.1	20.1	29.1	17.8	9.0	181	16.9	39.1	4.3	16.2	14.7				
Father	M	36	15.6	46.3	5.88	78.8	26.5	33.6	13.6	0.5	0	65.1	3.6	2.0	0	0	αα/αα	β/β	W	R301H
Mother	F	23**	11.4	37	6.41	57.1	17.8	30.9	ND	ND	0	74.9	3.4	21.7	0	0	−SEA/αα	βE/β	G176RfsX179	W
Brother	M	10	12.5	37.8	5.67	66.7	22.1	33.1	17.4	1.0	0	54.0	6.9	26.9	0	0	αα/αα	βE/β	G176RfsX179	W
Family C																				
P3	M	0.2	5.2	19.0	NA	74.0	21.0	28.0	26.0	11.0	124	ND	ND	ND	ND	ND	αα/αα	βE/	−154C/T	A298P
		1.10††	7.7	24.5	3.35	73.1	23.0	31.4	26.2	14.0	57	21.0	52.8	22.7	+	+		βE		
Father	M	31	14.0	42.1	5.45	77.3	25.7	33.3	14.5	1.0	0	68.4	0.5‡‡	31.1	0	0	αα/αα	βE/β	W	A298P
Mother	F	32	11.6	35.1	5.0	70.4	23.2	33.0	14.4	0.5	0	72.0	4.3‡‡	23.5	0	0	αCSα/αα	βE/β	−154C/T	W
Family D																				
P4	M	1.2¶¶	6.5	21.1	2.98	70.6	21.7	30.7	37.1	10.0	100	78.7	5.5	8.3	7.3	0.2	−SEA/-	βE/β	Q58X	A298P
		5.4§§	6.6	21.5	3.09	69.5	21.4	30.9	24.3	5.0	49	70	2.6	9.5	17.6	0.3	α3.7			

Data of hemoglobin (Hb) analyses showed here derived from liquid chromatography in which HbA_2 and Hb E were eluted at the same window and could not separate based on this methodology. Hb Portland-1 ($\zeta_2\gamma_2$) and Hb Bart's (γ_4) have not been quantified in every patient (supplemental Table 1 and associated clinical text). Individuals without globin gene mutations are highlighted in gray.

F, female; M, male; MCH, mean cell hemoglobin; MCHC, mean cell hemoglobin concentration; MCV, mean corpuscular volume; NA, not available; ND, not determined; RDW, red cell distribution width; P, patient; Retic., reticulocyte count; W, wild type for *KLF1* gene sequences.

*This study was done when this patient was first referred to our hospital and 2 months after the last blood transfusion.

†This study was performed 1 year after splenectomy and when the patient was free from blood transfusion. Of note, P1 is the only patient with a normal MCH (28 pg), suggesting that the coinheritance of thalassemia makes a significant contribution to the defects in Hb synthesis seen in our patients.

‡This study was performed when the patient (P2) was first seen at our hospital in 1997 at age 6 months.

¶The patient received less than 1 month of blood transfusion before this study and has been on regular transfusion since.

§At first diagnosis, this abnormal hemoglobin was identified as Hb Bart's.

‖This study was performed 6 months after splenectomy.

**The mother of P2 died of lung cancer on February 18, 2004.

††The patient was withheld from regular blood transfusion for 4 months before this study.

‡‡HbF measurements were variable even within the same individual; at 2 years before this study, the Hb F levels in the mother and the father were 1.5% and 1.2%, respectively by low-pressure liquid chromatography (LPLC; same laboratory). Moreover, the Hb F levels measured were different based on the techniques used; in the mother, Hb F levels were 4.3%, 3.4%, and 3.3%, and those of the father were 0.5%, 0.9%, and 0%, based on LPLC, high-performance liquid chromatography, and capillary electrophoresis, respectively. Using capillary electrophoresis, we were able to discriminate between Hb E and HbA_2; interestingly, the levels of HbA_2 in the mother were 3.8% (with 20.8% Hb E) compared with 5.4% (with 26.1% Hb E) in the father. In addition, 0.2% of Hb Constant Spring eluted at retention time 305 s was identified in the mother.

¶¶This study was done 2 months after blood transfusion, when this patient was first referred.

§§This study was performed 3 months after the regular blood transfusion was held.

‖‖This study was performed when this patient was withheld from blood transfusion for 10 weeks.

***This study was performed 6 months after splenectomy.

†††This study was performed when this patient first presented at our hospital.

‡‡‡This study was performed after the patient was free from blood transfusion for 2 years.

¶¶¶This study was performed when this patient first presented at age 2 months.

§§§This evaluation was done when the patient was first referred to our center, and the last blood transfusion was performed around 12 weeks before this study.

Table 1. (continued)

Cases	Sex	Age (y, mo)	Hb (g/dL)	Hct (%)	RBC (×106/μL)	MCV (fL)	MCH (pg)	MCHC (g/dL)	RDW (%)	Retic. (%)	NRBC (/100WC)	Hemoglobin typing (%)					Globin genotypes		*KLF1* genotypes	
												Hb A	Hb F	HbA2/E	Hb Portland-1	Hb Bart's	αα/αα	β/β	Allele 1	Allele 2
Father	F	28	14.0	42.5	6.98	60.9	20.1	32.9	17.2	1.5	0	75	2.6	22.4	0	0	−SEA/αα	βE/β	Q58X	W
Mother	M	27	12.1	34.6	4.81	72.0	25.2	35	15.5	1.0	0	70.2	1.8	28	0	0	αα/-α3.7	βE/β	W	A298P
Family E																				
P5	M	18‖‖	3.4	11.1	1.37	81.0	24.8	30.6	25.4	10.1	113	62.7	29.0	4.6	10.2	6.0	αα/αα	β/β	G176RfsX179	A298P
		23***	8.2	27.4	3.38	81.1	24.2	29.9	22.4	6.0	86									
Father	M	58	14.4	43.7	5.4	81.0	26.6	33.0	14.6	1.0	0	82.8	0.9	3.8	0	0	−α3.7/αα	β/β	W	A298P
Mother	F	55	14.1	42.7	4.85	88.0	29.1	33.1	13.9	1.0	0	82.8	3.1	2.8	0	0	αα/αα	β/β	G176RfsX179	W
Family F																				
P6	F	0.9†††	6.6	21.0	2.94	71.0	22.3	31.3	23.8	10.0	8	33.3	49.8	2.4	13.7	0.8	αCSα/αα	β/β	G176RfsX179	A298P
		5.7‡‡‡	8.3	27.1	3.82	70.9	21.6	30.5	23.4	10.0	13	45.0	50.0	3.0	+	+				
Father	M	34	13.2	40.1	5.5	73.0	24.0	32.9	14.4	6.0	0	83.0	0.2	2.7	0	0	αCSα/αα	β/β	W	A298P
Mother	F	30	13.9	41.5	5.2	80.0	26.6	33.5	14.4	2.0	0	94.8	2.4	2.8	0	0	αα/αα	β/β	G176RfsX179	W
Brother 1	M	11	11.8	36.7	5.2	70.4	22.7	32.3	15.1	1.0	0	96.4	0.5	3.1	0	0	αCSα/αα	β/β	G176RfsX179	W
Brother 2	M	8	11.7	35.1	4.7	75.4	25.1	33.3	15.1	1.0	0	94.9	2.4	2.7	0	0	αα/αα	β/β	W	A298P
Family G																				
P7	M	0.2¶¶¶	5.7	17.8	2.66	67.0	21.2	31.9	21.9	11.0	146	33.9	54.6	2.2	14.3	0.3	−α3.7/αα	β/β	G176RfsX179	A298P
Mother	F	22	11.9	36.4	4.59	79	26.0	32.8	14.4	1.0	0	85.8	0	2.6	0	0	−α3.7/αα	β/β	G176RfsX179	W
Family H																				
P8	M	4§§§	5.4	16.3	2.18	75.0	24.7	33.1	20.4	10	26	69.0	19.0	10.0	+	+	αα/αα	βE/β	G176RfsX179	A298P
Father	M	32	13.5	45.2	5.1	66.0	19.9	29.9	16.1	2.0	0	95.9	0.9	3.2	0	0	−SEA/αα	β/β	W	A298P
Mother	F	30	12.7	40.7	5.53	74.0	23.0	31.5	15.1	2.0	0	66.6	4.7	28.7	0	0	αα/αα	βE/β	G176RfsX179	W

Data of hemoglobin (Hb) analyses showed here derived from liquid chromatography in which HbA_2 and Hb E were eluted at the same window and could not separate based on this methodology. Hb Portland-1 ($\zeta_2\gamma_2$) and Hb Bart's (γ_4) have not been quantified in every patient (supplemental Table 1 and associated clinical text). Individuals without globin gene mutations are highlighted in gray.

F, female; M, male; MCH, mean cell hemoglobin; MCHC, mean cell hemoglobin concentration; MCV, mean corpuscular volume; NA, not available; ND, not determined; RDW, red cell distribution width; P, patient; Retic., reticulocyte count; W, wild type for *KLF1* gene sequences.

*This study was done when this patient was first referred to our hospital and 2 months after the last blood transfusion.

†This study was performed 1 year after splenectomy and when the patient was free from blood transfusion. Of note, P1 is the only patient with a normal MCH (28 pg), suggesting that the coinheritance of thalassemia makes a significant contribution to the defects in Hb synthesis seen in our patients.

‡This study was performed when the patient (P2) was first seen at our hospital in 1997 at age 6 months.

¶The patient received less than 1 month of blood transfusion before this study and has been on regular transfusion since.

§At first diagnosis, this abnormal hemoglobin was identified as Hb Bart's.

‖This study was performed 6 months after splenectomy.

**The mother of P2 died of lung cancer on February 18, 2004.

††The patient was withheld from regular blood transfusion for 4 months before this study.

‡‡HbF measurements were variable even within the same individual; at 2 years before this study, the Hb F levels in the mother and the father were 1.5% and 1.2%, respectively by low-pressure liquid chromatography (LPLC; same laboratory). Moreover, the Hb F levels measured were different based on the techniques used; in the mother, Hb F levels were 4.3%, 3.4%, and 3.3%, and those of the father were 0.5%, 0.9%, and 0%, based on LPLC, high-performance liquid chromatography, and capillary electrophoresis, respectively. Using capillary electrophoresis, we were able to discriminate between Hb E and HbA_2; interestingly, the levels of HbA_2 in the mother were 3.8% (with 20.8% Hb E) compared with 5.4% (with 26.1% Hb E) in the father. In addition, 0.2% of Hb Constant Spring eluted at retention time 305 s was identified in the mother.

¶¶This study was done 2 months after blood transfusion, when this patient was first referred.

§§This study was performed 3 months after the regular blood transfusion was held.

‖‖This study was performed when this patient was withheld from blood transfusion for 10 weeks.

***This study was performed 6 months after splenectomy.

†††This study was performed when this patient first presented at our hospital.

‡‡‡This study was performed after the patient was free from blood transfusion for 2 years.

¶¶¶This study was performed when this patient first presented at age 2 months.

§§§This evaluation was done when the patient was first referred to our center, and the last blood transfusion was performed around 12 weeks before this study.

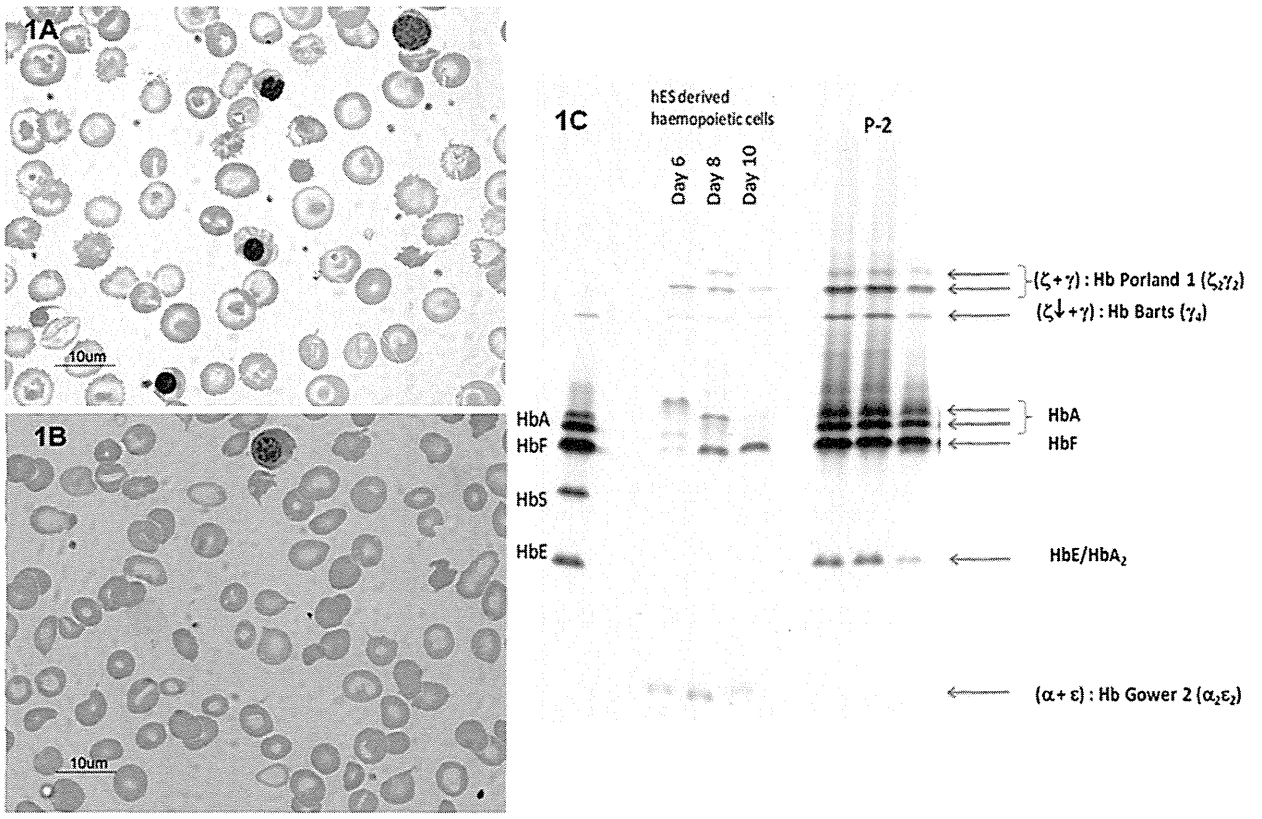

Figure 1. Wright stained peripheral blood pictures from 2 index patients who had normal α- and β-globin genes. In P1 (A), a marked hypochromic microcytosis with anisopoikilocytosis, acanthocytes, and numerous nucleated red blood cells was observed; in P5 (B), a blood picture with numerous fragmented red blood cells with schistocytes and numerous nucleated red blood cells was seen, similar to nonspherocytic hemolytic anemia. Both smears were performed after splenectomy in both patients and were free from blood transfusion. Peripheral blood features in other patients are available in supplemental Figure 1. (C) IEF study of embryonic globins identified in a patient with *KLF1* mutations. Comparing hemoglobin profiles from the patient, P2, with control human embryonic stem cell (hES)-derived hematopoietic cells reveals 3 distinct abnormal hemoglobin protein bands separated at a more cathodic position than HbA and HbF. These hemoglobin species were similar to those of patients (P2, in triplication), as they were separated to the same isoelectric points. These hemoglobin bands were subsequently identified by mass spectrometry to be Hb Portland-1 ($\zeta_2\gamma_2$) and Hb Bart's (γ_4). Of note, a different level of embryonic protein expression during erythroid differentiation from embryonic to fetal erythropoiesis in hES cells from day 6 to day 10 was observed (*Right*). Moreover, a fast-moving hemoglobin specie of Hb Gower 2 ($\alpha_2\varepsilon_2$) was identified in this erythroid cell model but not from the patient. The standard hemoglobin controls are shown on the far left lane.

Analysis of hemoglobin

Hemoglobin analysis revealed abnormally high levels of HbF (average, 38%; range, 2.6%–54.6%) in all patients, consistent with increased F cells by flow cytometry (heterocellular pattern; supplemental Table 2). Two unanticipated hemoglobins present at levels of up to 18% were observed in all patients. Using IEF and MALDI-TOF mass spectrometry (Figure 1B, supplemental Figure 2, and Table 1), these hemoglobins were identified as the γ chain tetramer Hb Bart's (γ_4) and the embryonic hemoglobin Portland I ($\zeta_2\gamma_2$). The presence of both Hbs was further confirmed by flow cytometry (supplementary Table 2). Hb Gower 2 ($\alpha_2\varepsilon_2$) was not detected at significant amounts, and other minor bands (which may correspond to Hb Gower 1, $\zeta_2\varepsilon_2$) were not examined further. Increased levels of embryonic ζ and ε-globin mRNA expression were observed by analysis of cDNA extracted from the peripheral blood of patient P2, using a tiled microarray (Figure 2A-B), as well as in other 5 patients using real-time PCR assays (Figure 2C). Interestingly, significantly increased proportions of HbF were observed in 14 of 15 parents from the index families (range, 0.2%–4.7%; average (SD), 2.34% + 1.34%; Table 1), but none of the parents expressed Hb Bart's or embryonic globins, as assessed by chromatography and IEF (Table 1). Detection of F-cells, anti-ζ, and anti-Bart's using flow cytometry was variable in these parents and siblings (supplemental Table 2).

Analysis of structural proteins commonly mutated in red cell disorders

Only 2 of the probands (P1 and P5) were found to have entirely structurally normal α- and β-globin genes. The other patients were single or double heterozygotes for common mutations of the globin genes. However, their clinical severity far exceeded that seen in their relatives or other carriers of thalassemia. All patients, and many of the parents, had unexplained increased levels of HbF, which could have been linked to a deletion or deletions involving the β-globin locus.[25] However, we excluded the possibility of mutations lying within the *cis*-regulatory regions involved in γ-globin gene expression using multiplex-ligation probe assays and sequencing analysis (supplementary Figure 3 and supplementary Table 3).

Although the hematologic profiles in these patients were complex, 4 patients (P5–P8) were initially diagnosed with red cell enzymopathies.[26] These patients were tested for 8 common red cell enzyme activities and were found to have significant PK deficiency (<50% of normal after reticulocyte count correction). On the basis of these findings, we evaluated the PK status of the remaining 4 patients (P1–P4), in whom we did not initially suspect any enzymopathy. All had significantly reduced levels of PK activity (supplementary Table 2). However, DNA sequencing of the coding region including the intron/exon boundaries of the *PKLR gene*,

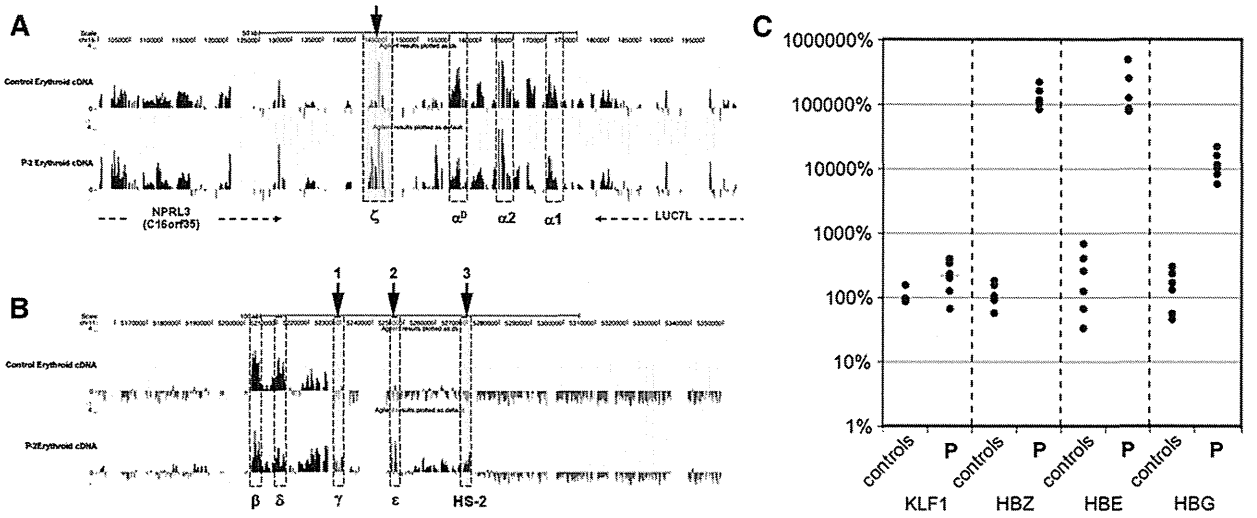

Figure 2. Expression profiles of the primary erythroid culture cells from patients with *KLF1* mutations. An expression profiling of the α(A) and β(B) globin gene clusters using Agilent tiled oligonucleotide array was performed in P2 erythroid cells compared with normal control. (A) A distinctive higher peak of ζ globin gene expression (dark arrow with gray box highlighted) was observed in the patient (bottom) compared with a normal cDNA from a control sample (top). (B) Two unique upregulated expression peaks of γ-globin (arrow 1) and ε-globin (arrow 2) genes in the patient were shown. Another upregulating expression of noncoding mRNA (arrow 3) was observed at the β-globin locus control region (β-globin locus control region, hypersensitive site 2 [HS-2]), all with dark arrows and gray boxes highlighted. However, the meaning of this observation on the downstream globin gene regulation and hereditary persistence of embryonic globin in the patient remains unclear. There was no significant change on expression of other nearby genes on both clusters. (C) Expression profile of erythroid specific genes from primary erythroid cell samples from 5 patients (P) with *KLF1* mutations and normal controls (n = 6), using qPCR and Taqman probe hybridization, showing a marked increase of fetal and embryonic globin mRNA expression (HBZ, ζ-globin gene; HBE, ε-globin gene; HBG, γ-globin gene) in all patients with relatively normal expression of *KLF1*. This suggests that although most patients carry a single nonsense mutation, which could result in haplo-insufficiency, a missense mutation on the other allele might produce stable transcripts that could be detected at similar levels to normal. Alternatively, our mRNA analysis used might not be sensitive enough to demonstrate a minor perturbation in the level of *KLF1* expression. With the exception of BCL11A (B-cell CLL/lymphoma 11A [zinc finger protein] from 1 patient [P2]), there was no change in expression compared with normal controls of other erythroid-specific genes, including CD71, SOX6, ERAF, GYPA, glycophorin A, and EPOR (erythropoietin receptor; supplementary Figure 5).

encoding the red cell PK enzyme, failed to identify any causative mutations.

We next evaluated expression of proteins present on the red cell membrane and in the cytoskeleton. Extensive minor blood group analysis in 5 index families is summarized in supplementary Table 4. All patients had the rare In(Lu) blood group phenotype (a^-/b^-). In addition, all parents and siblings, except one (the father of P2), also expressed this rare blood group phenotype. The In(Lu) phenotype results from the suppression of 2 cell surface glycoproteins, BCAM and CD44, which carry the Lutheran and Indian blood group antigens, respectively.[21,27] It has previously been shown that carriers of KLF1 mutations have the In(Lu) phenotype and that expression of CD44 was significantly reduced in the erythrocytes of the majority of patients tested (supplementary Table 2), but not in their leukocytes, whereas expression of the integral protein Band 3 evaluated by eosin-5-maleimide binding assay appeared unchanged compared with control samples (supplementary Table 2).

Identification of mutations in the KLF1 genes

Although the levels of embryonic (ζ and ε) and fetal (γ) globin RNA and protein are significantly elevated in all probands, analysis of their globin gene haplotypes showed that they have inherited quite different combinations of α- and β-globin haplotypes (supplemental Figure 6 and supplemental Figure 7). Given that elevations in embryonic globins resulting from *cis*-acting mutations have never been described previously, it seemed very unlikely that these patients had inherited a variety of *cis*-mutations causing persistent embryonic gene expression. To analyze this in further detail, the embryonic ζ and ε genes and the fetal γ genes were sequenced in all patients, but no changes (other than common single nucleotide polymorphisms) were found.

These observations led us to hypothesize that the genetic lesion in these patients may lie in a *trans*-acting factor involved in erythroid differentiation and maturation. Therefore, we DNA sequenced the coding region and intron/exon boundaries of 2 key erythroid transcription factors, *GATA1* and *KLF1*, in a single proband (P2). No likely pathogenic changes were identified in the *GATA1* gene; however, we found 2 changes in *KLF1*, a c.902G>A transition leading to a p.R301H substitution and a 7 bp insertion (c.525_526InsCGGCGCC), resulting in a frameshift (p.G176RfsX179) and disruption of the protein before the zinc finger DNA binding domain (Figure 3 and Table 1). Each of these sequence changes has a separate parental origin. The c.902G>A change has been previously reported to underlie elevated levels of HbF, as has a similar 7bp insertion (leading to p.G176AfsX179).[6] To gain further genetic evidence that compound heterozygosity for deleterious *KLF1* alleles may underlie the abnormalities reported here, we DNA sequenced this gene in the 7 remaining patients. Surprisingly, we found all 7 to be compound heterozygotes for likely pathogenic *KLF1* changes (Figure 3, Table 1, and supplemental Figure 4). Four previously unreported coding changes were identified: a c.172C>T transition causing a premature truncation at position 58 in the protein (p.Q58X) before the zinc finger domains, a c.892G>C transversion predicted to alter alanine in the first zinc finger (p.A298P), a c.991C>T transition leading to a p.R331W amino acid change in the second zinc finger, and a c.1003G>A change leading to a p.G335R substitution that alters a glycine residue just outside the second zinc finger domain. All 3 novel missense changes identified alter residues that are highly conserved throughout evolution (Figure 3). We also identified a C>T change 154 bp 5′ of the initiating ATG codon

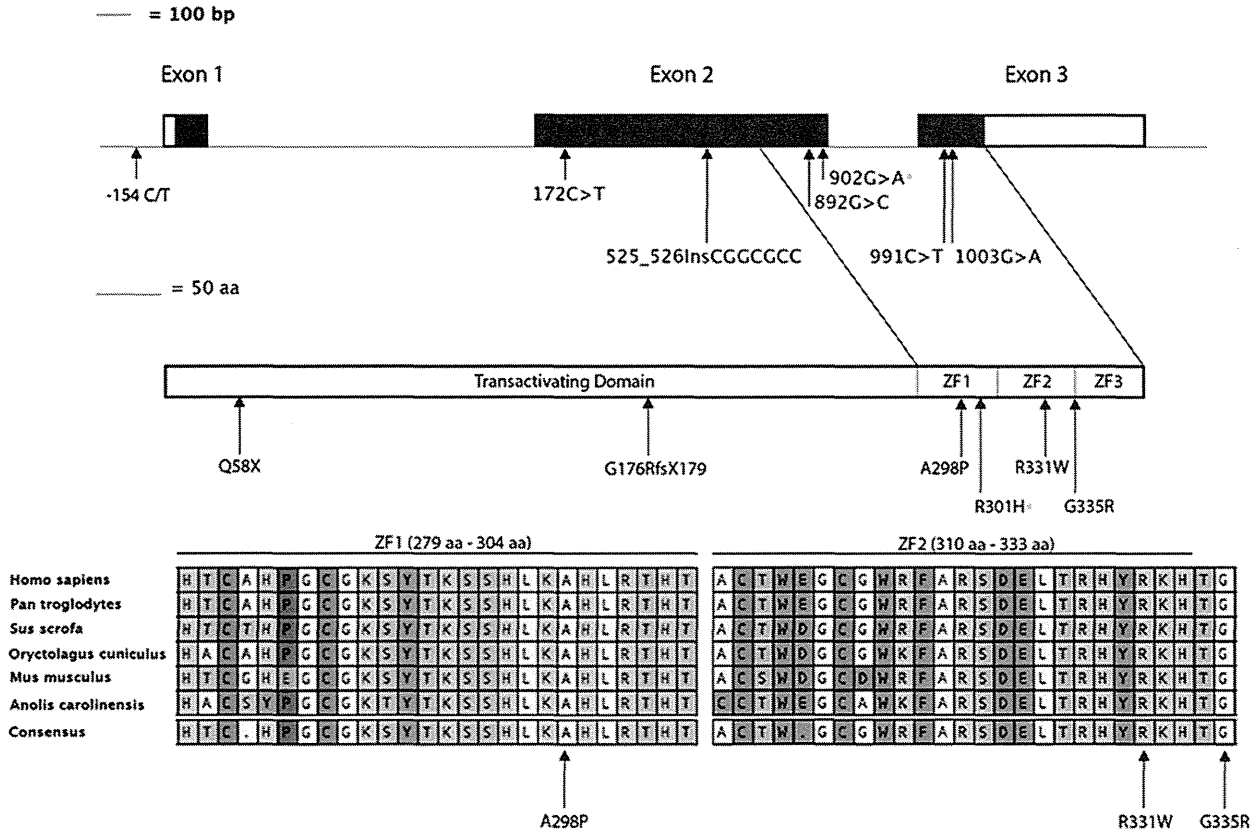

Figure 3. *KLF1* gene structure, identification of variants in cases reported in this study, and their location within conserved domains. (top) Schematic representation of *KLF1*; exons are shown to scale, with the coding sequence in black and untranslated regions in white, and the locations of nucleotide changes identified here. Below this is a diagram representing the KLF1 protein with the previously annotated transactivating domain and the 3 zinc finger domains encoded by exons 2 and 3. The locations of all mutations identified in this study are shown; all changes are novel except for the previously reported R301H substitution (asterisk). (bottom) A position and conservation of amino acid residues found to be changed in the probands reported here (arrows). The entire first and second zinc finger sequences are shown with interspecies conservation, and the extent of each zinc finger domain is shown above each region. The cysteine and histidine residues involved in the coordination of zinc are denoted by the # symbol, and the residues that contact specific bases in DNA are marked with a + symbol.

(−154 C>T) in a single patient (P3). This region has previously been reported to include the promoter region of *KLF1*[20], and the change is predicted to alter the binding of transcription factors tumor suppressor p53, paired box 5, and epidermal growth factor receptor–specific transcription factor (http://alggen.lsi.upc.es/cgi-bin/promo_v3/promo/promoinit.cgi?dirDB=TF_8.3). This change was absent in 100 population-matched control individuals and is also absent from single nucleotide polymorphism database (http://www.ncbi.nlm.nih.gov/SNP/), suggesting it is rare. These genetic data, taken together with the patient's phenotypic abnormalities, strongly suggest this change disrupts expression of the associated *KLF1* transcript, as seen in a single patient (P1) tested (Figure 2C).

All parents and some siblings studied were heterozygotes for the *KLF1* changes present in probands. The 4 coding changes are extremely rare and are listed neither in dbSNP136 (http://www.ncbi.nlm.nih.gov/projects/SNP/) nor in ~13 000 alleles from African- and European-Americans listed in the Exome Variant Server (http://evs.gs.washington.edu/EVS/). In addition, both of these *KLF1* changes were absent from 296 normal chromosomes from population-matched control individuals. The p.Q58X change is predicted to prematurely truncate the KLF1 protein, and the 3 novel missense changes alter invariant residues in the first and second zinc finger regions (Figure 3) and may disrupt DNA binding. Altered binding of KLF1 in vivo may underlie the observed PKLR deficiency in these patients as the WT KLF1 protein binds the promoter region of the *PKLR* gene in human erythroid progenitor cells (Figure 4).

KLF1 target genes

The predominant hematologic phenotypes in these compound heterozygotes appear to arise from the effects of KLF1 on the globin genes (causing abnormal globin synthesis) and the genes encoding CD44 (causing the In(Lu) phenotype) and PK (causing CNSHA). Of interest, on the basis of reanalysis of previously published data (supplemental Table 5), these erythroid genes are among the most sensitive to changes in the levels of KLF1. Others have previously analyzed the binding of KLF1 to *cis*-elements surrounding the globin genes and the CD44 gene.[28] Here we have analyzed binding of KLF1 to the PKLR gene (based on data from Su et al[29] showing that it specifically binds the erythroid promoter of this gene; Figure 4). This is clearly consistent with mutations in the *KLF1* gene causing severe downregulation of the levels of PK resulting in CNSHA. It was of interest that all patients with the CNSHA phenotype and PK deficiency (P5–P8) have exactly the same combination of mutations (p.G176RfsX179;p.A298P; Table 1). Finally, it was of interest that in a single case (P2) that could be analyzed, the level of *BCL11A* mRNA was reduced (supplementary Figure 5) when both alleles of the *KLF1* gene are mutated. This is consistent with previous observations showing that

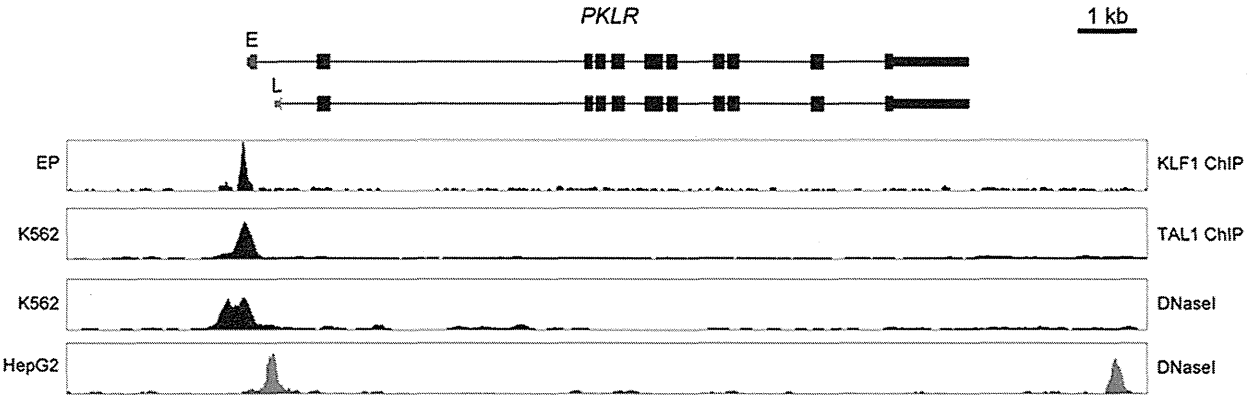

Figure 4. Binding of KLF1 to the *PKLR* gene. (top) a representation of the *PKLR* gene structure including the start of the erythroid (E)-specific transcript (dark gray) and the liver (L)-specific first exon in light gray. (bottom) KLF1 binding and DNase I hypersensitive sites in cultured human erythroid progenitors (EP), K562; erythroleukemia cell line, HepG2; and liver cells line, as indicated.

this gene is a direct target of KLF1 and is normally involved in the silencing of γ-globin synthesis.[3,4] Following on from observations of the effect of BCL11A deficiency on embryonic globin expression in the mouse[30] and the anecdotal observation that embryonic expression is increased in a patient with congenital dyserythropoietic anemia type IV associated with a dominantly acting KLF1 mutation (E325K),[30] it seems plausible that when KLF1 is downregulated to a critical level, it also has a similar effect on human embryonic globin expression.

Discussion

We have defined a new cause of severe anemia in pediatric patients who are compound heterozygotes for mutations in the key erythroid transcription factor KLF1. The cardinal features of this condition are transfusion-dependent anemia associated with abnormalities in red cell enzymes (eg, PK deficiency), red cell membranes [In(Lu) phenotype], and an abnormal pattern of globin synthesis with very high levels of HbF and readily detectable levels of embryonic globins in adult life.

It appears that *KLF1* mutations may cause a wide spectrum of phenotypes, which may have been expected from its pleiotropic role in erythropoiesis. The principles emerging from current studies suggest that both the levels of KLF1 and the type of mutated protein produced may exert different effects on KLF1 target genes and, consequently, cause a wide range of red cell phenotypes. The nonsense mutation G176RfsX179 was identified in 5 of the families reported here and is similar to the G176AfsX179 reported in 2 individuals from Korea and Vietnam,[6] but no patients have inherited 2 nonsense mutations. It seems likely that this would lead to embryonic or early fetal lethality resulting from severe in utero anemia, as occurs in null mice.[32] This warrants further studies of KLF1 in families with unexplained, recurrent hydrops fetalis or fetal loss, which cannot be accounted for by other conditions.

It has previously been shown that a mutation (E325K) in the second zinc finger domain of KLF1 causes a significant alteration in DNA binding, especially at the β-globin genes, resulting in a severe red cell and clinical phenotype (congenital dyserythropoietic anemia), even when only a single allele is mutated.[30] It is thought that this mutation acts as a dominant negative, gain-of-function mutation. In contrast, the patients described here have newly defined phenotypes (severe thalassemia or CNSHA) associated with KLF1 mutations. It is of interest that all patients with CNSHA had exactly the same genotype (Table 1, Family E-H), suggesting that the phenotype is largely driven by the *KLF1* genotype, rather than by epistatic effects. Of particular interest is that the A298P mutation lies in a key residue of the first zinc finger of KLF1, which determines the binding specificity of the protein.[33]

Given the widespread effects of KLF1 in erythropoiesis summarized here, it seems unlikely that this study and that of Satta et al[7] have captured the full spectrum of these inherited anemias. Despite the apparent relationship between genotype and phenotype, it is interesting that all 3 previously described compound heterozygotes for *KLF1* mutations (p.S270X/p.K332Q)[7] and (G176Afs179X; L51R)[6] had similarly raised levels of HbF (22%, 31%, and 9.5%), but rather milder anemia (Hb, 12.1, 11.5, and 13.7 g/dL), than the patients described here. However, 2 of these patients were adults (older than 30 years)[7] when analyzed and also harbored a mutation (p.K322Q) that, in vitro, had only a mildly decreased DNA binding affinity to the promoters tested (~2 fold), and therefore may represent a relatively mild hypomorphic allele.[34] The age of the third patient is not reported. We have noticed (anecdotally) that anemia in our patients has improved with age, requiring progressively less transfusion. It will be important to follow-up all patients with *KLF1* mutations to determine whether severity may be truly age-dependent.

During the last few years, the role played by KLF1 in globin gene regulation has become clearer.[5,35] Almost since its discovery, it has been known that KLF1 is an activator of β-globin expression, but more recently, it also has been shown to play a role in silencing γ-globin expression, probably by regulating BCL11A and its interaction with Sox6 at the γ-globin gene.[3,36,37] Mutations in *KLF1* therefore reduce β-globin expression (causing β thalassemia)[32] and de-repress γ-globin expression, thereby increasing the synthesis of HbF.[4] However, the relationship between these effects and the mutations that downregulate *KLF1* are not straightforward: Not all heterozygotes for *KLF1* mutations produce increased levels of HbF,[38] and in those who do, the level can be very different, even in the presence of the same mutation, demonstrating variable penetrance.[7,37,38] These observations have led to speculation about the role of KLF in regulating the levels of HbF.[36] The data presented here clarify the situation because all report that compound heterozygotes produce substantial amounts of HbF, putting beyond doubt that KLF1 plays a major role in normally repressing γ-globin synthesis in humans in vivo. Reactivation of γ-globin synthesis by downregulating KLF1 might provide a way to ameliorate the effect of severe β-hemoglobinopathies.[3,39]