

α -1,6-Fucosyltransferase (FUT8) Inhibits Hemoglobin Production during Differentiation of Murine and K562 Human Erythroleukemia Cells^{*[S]}

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Background: The overall view of erythropoiesis remains unclear.

Results: Overexpression of α -1,6-fucosyltransferase inhibits hemoglobin production in murine and human erythroleukemia cells; down-regulation of α -1,6-fucosyltransferase promotes hemoglobin production and erythroid differentiation of human erythroleukemia cells.

Conclusion: Core fucosylation plays an important role in hemoglobin production and erythroid differentiation.

Significance: This might be the first finding that glycosylation negatively regulates erythroid differentiation.

Erythropoiesis results from a complex combination of the expression of several transcription factor genes and cytokine signaling. However, the overall view of erythroid differentiation remains unclear. First, we screened for erythroid differentiation-related genes by comparing the expression profiles of high differentiation-inducible and low differentiation-inducible murine erythroleukemia cells. We identified that overexpression of α -1,6-fucosyltransferase (*Fut8*) inhibits hemoglobin production. FUT8 catalyzes the transfer of a fucose residue to N-linked oligosaccharides on glycoproteins via an α -1,6 linkage, leading to core fucosylation in mammals. Expression of *Fut8* was down-regulated during chemically induced differentiation of murine erythroleukemia cells. Additionally, expression of *Fut8* was positively regulated by c-Myc and c-Myb, which are known as suppressors of erythroid differentiation. Second, we found that FUT8 is the only fucosyltransferase family member that inhibits hemoglobin production. Functional analysis of FUT8 revealed that the donor substrate-binding domain and a flexible loop play essential roles in inhibition of hemoglobin production. This result clearly demonstrates that core fucosylation inhibits hemoglobin production. Third, FUT8 also inhibited hemoglobin production of human erythroleukemia K562 cells. Finally, a short hairpin RNA study showed that *FUT8* down-regulation induced hemoglobin production and increase of transferrin receptor/glycophorin A-positive cells in human erythroleukemia K562 cells. Our findings define FUT8 as a novel factor for hemoglobin production and demonstrate that core fucosylation plays an important role in erythroid differentiation.

Erythropoiesis is intricately regulated by several lineage-specific factors (1). Pro-erythroblasts/colony-forming unit-erythroid cells, which arise from megakaryocytic/erythroid progenitors (MEPs),² are stimulated by erythropoietin (Epo), differentiate into erythroblasts, and finally mature to become erythrocytes/red blood cells (2–4). Binding of Epo to the Epo receptor (EpoR) leads to phosphorylation and activation of receptor-associated Janus-kinase 2 (JAK2) and initiation of the EpoR signaling cascade (5, 6). Epo also stimulates phosphorylation and activation of GATA-binding protein 1 (GATA-1) (7). The transcription factor GATA-1 is highly expressed in MEPs and is well known as a specific regulator of erythroid differentiation. GATA-1 induces the expression of erythroid genes such as glycophorin A, *EpoR*, and hemoglobin (8). In embryos of GATA-1 null mice, embryonic erythroid cells are arrested at an early pro-erythroblast-like stage of their development (9) and die thereafter by apoptosis. The transcription factor PU.1 is a hematopoietic-specific member of the ETS family that is required for the development of lymphoid and myeloid lineages (10). However, PU.1 interacts directly with GATA-1 and blocks terminal erythroid differentiation of murine erythroleukemia (MEL) cells by repression of GATA-1-mediated transcriptional activation (11). The transcription factor erythroid Kruppel-like factor (EKLF/KLF1) plays a crucial function during erythropoiesis (12). EKLF/KLF1 and KLF2 bind the c-Myc (*Myc*) promoter and regulate expression of the *Myc* gene (13). Down-regulation of *Myc* expression at the late stage of erythropoiesis is essential for terminal erythroid maturation (14). GATA-1 represses *Myc* and c-Myb (*Myb*) expression during erythroid differentiation (15, 16). As described above, erythropoiesis is predominantly

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² The abbreviations used are: MEP, megakaryocytic/erythroid progenitor; FUT8, α -1,6-fucosyltransferase; MEL, murine erythroleukemia; Epo, erythropoietin; EpoR, erythropoietin receptor; HMBA, hexamethylene bisacetamide; TSA, trichostatin A; HD, high differentiation-inducible; LD, low differentiation-inducible; RRM, R365A/R366A double mutant; FLM, D368A/K369A/V370A/G371A/T372A quintuple mutant; TGF- β , transforming growth factor β 1; SH, Src homology.

regulated by Epo stimulation and by transcriptional control with the development-specific transcription factor GATA-1.

Friend leukemia integration 1 (FLI-1) is one of the well known regulators of erythropoiesis. FLI-1, a member of the ETS family of transcription factors that was originally identified in MEL cells, has a role in erythroleukemia induction (17). FLI-1 is also expressed in normal hematopoietic cells and suppresses erythroid differentiation (18). However, FLI-1 activates megakaryocytic differentiation (19). Both FLI-1 and EKLF/KLF1 bind to GATA-1 and are functionally antagonistic to the activation of megakaryocytic and erythrocytic gene promoters (20). Moreover, they control megakaryocytic and erythrocytic differentiation of MEPs. A recent study showed that microRNA-145 (miR-145) represses *Fli-1* (21). That study also revealed the effects of miR-145 on megakaryocytic and erythroid differentiation. This finding indicates the existence of a novel differentiation regulator in addition to existing transcription factors, cytokines, and cytokine receptors. Thus, the overall view of erythroid differentiation is not yet clear.

MEL and human erythroleukemia K562 (K562) cells are widely used for the study of erythroid differentiation. MEL cells, which were isolated from Friend virus-infected mice, provide a model system for the study of erythroblast differentiation and leukemogenesis (22). The addition of chemicals, such as dimethyl sulfoxide (DMSO), hexamethylene bisacetamide (HMBA), and trichostatin A (TSA), is known to induce differentiation of MEL cells to erythroblasts that highly express hemoglobin (23–25). These chemicals act as initiators of the synthesis of β -globin and other erythroid-specific proteins (24). Expression of *Myc* and *Myb* genes are down-regulated during MEL cell differentiation, and overexpression of these genes blocks differentiation (26, 27). Furthermore, DMSO-resistant MEL cell clones were isolated and used for the study of erythroid differentiation (28, 29). K562 cells were isolated from the blood of patients with chronic myelogenous leukemia. Sodium butyrate and hemin also induce erythroid differentiation of K562 cells (30–32). In a phenotypic analysis of K562 cells, the rate of formation of transferrin receptor (CD71)/glycophorin A-positive cells was increased by hemin-mediated induction of differentiation (33). During erythroid differentiation, CD71 is highly expressed in the pre-proerythroblast and the erythroblasts that follow, whereas glycophorin A expression is delayed relative to CD71 and correlates with the transition from the pro-erythroblast to the basophilic normoblast (34). Although MEL and K562 cells are erythroleukemia cells, these cells are useful models for the study of erythroid differentiation because they can be induced to differentiate like normal erythroid cells.

We here used DNA microarrays to screen for erythroid differentiation-related genes to identify novel regulators of hemoglobin production and erythroid differentiation. We compared the expression profile of high differentiation-inducible (HD) and low differentiation-inducible (LD) MEL cells during differentiation induced by three different chemicals. We selected the consistently down-regulated genes in HD MEL cells as candidate differentiation suppressors and then overexpressed these genes in HD MEL cells to analyze for inhibition of hemoglobin

production. We found that overexpression of α -1,6-fucosyltransferase (*Fut8*) inhibited hemoglobin production in the MEL cell. We then performed functional analysis of FUT8 to understand the mechanism of suppression of hemoglobin production and erythroid differentiation, as observed in MEL and K562 cells.

EXPERIMENTAL PROCEDURES

Cell Culture—MEL cells (T-3-Cl-2-0.fl) (35) and K562 cells were cultured in RPMI 1640 medium (Nissui Pharmaceutical) with 10% fetal calf serum (Sigma-Aldrich) at 37 °C with 5% CO₂. Both cell lines were provided by RIKEN BioResource Center (Ibaraki, Japan). HD and LD MEL cells were obtained by recloning the original MEL cells. Erythroid differentiation was determined by benzidine staining. PLAT-E cells (36) and PLAT-A cells were cultured in Dulbecco's modified Eagle's media (Nissui Pharmaceutical) with 10% fetal calf serum (Sigma-Aldrich) at 37 °C with 5% CO₂. PLAT-E and PLAT-A cells were kindly provided by Dr. Kitamura (University of Tokyo, Japan).

Microarray Experiments—The cDNA microarray was prepared in our laboratory using NIA mouse 15k and 7.4k cDNA clone sets (37–39). These cDNA clone sets were provided by Riken BioResource Center. Microarray preparation, RNA labeling, and hybridization procedures are described in the [supplemental material](#).

For comparison of HD and LD MEL cell expression profiles, HD and LD MEL cells were cultured for 6, 12, 24, and 36 h with 1.0% DMSO, 3.0 mM HMBA, or 15 nM TSA, respectively. For expression analysis of HD MEL cells during differentiation, HD MEL cells were cultured for 1, 6, 12, 24, and 36 h with 1.5% DMSO, 5.0 mM HMBA, or 30 nM TSA, respectively. Total RNA from MEL cells was extracted using the RNeasy mini kit (Qiagen).

Hybridization images were scanned using a ScanArray 5000 (PerkinElmer Life Sciences). The fluorescence intensity of the cDNA microarray was quantified using QuantArray 3.0 software (PerkinElmer Life Sciences). The cDNA microarray data were analyzed using GeneSpring 6.0 (Silicon Genetics). Intensity differences between Cy3 and Cy5 were normalized by the LOWESS method. The expression ratio for each time experiment is expressed as the average of quadruplicate experiments, including two dye swaps. The complete microarray data were archived at the National Center for Biotechnology Information Gene Expression Omnibus (accession numbers GSE40754 and GSE43849) (www.ncbi.nlm.nih.gov).

Generation of Targeted Genes Overexpressing Cells—Total RNA of MEL and K562 cells was extracted using the RNeasy mini kit. cDNA was synthesized from the total RNA using SuperScript III reverse transcriptase (Invitrogen). The targeted genes were amplified by PCR using KOD Plus (Toyobo); the primers are described in the [supplemental material](#). The PCR products were purified by isopropyl alcohol precipitation and restricted using restriction enzymes as described in the [supplemental material](#). The DNA fragments were purified using the Wizard SV Gel and PCR Clean-Up System (Promega). The purified DNA fragments of the targeted genes were cloned into the vector pMXs-CMVp-IRES-EGFP that contains an

enhancer of the cytomegalovirus promoter in the 5'-long terminal repeat sequence. This vector is a modification of the vector pMXs-IRES-EGFP (40) that was kindly provided by Dr. Kitamura. The cloned vectors were introduced into PLAT-E or PLAT-A cells using Lipofectamine 2000 (Invitrogen), and culture supernatants containing virus were collected 48 h after transfection. MEL and K562 cells were grown in the culture supernatant supplemented with 0.08–4 $\mu\text{g}/\text{ml}$ Polybrene to aid infection. The infected cells were cloned using the limiting dilution method, and targeted gene overexpression was verified by measuring of the expression of enhanced green fluorescent protein using FACSCalibur (BD Biosciences).

Site-directed Mutagenesis—Site-directed mutagenesis of mouse *Fut8* and human *FUT8* was performed with Splicing by Overlap Extension PCR. The primer sequences are listed in the [supplemental material](#). Additional procedures are the same as described above.

Western Blotting—FUT8 was detected in Western blotting using goat anti-FucT-VIII antibody (S-17; catalog no. sc-34629, Santa Cruz Biotechnology) and HRP-conjugated rabbit anti-goat antibody (catalog no. 61-1620, Zymed Laboratories Inc.). GAPDH was detected with mouse anti-GAPDH antibody (clone 6C5; catalog no. AM4300, Ambion) and HRP-conjugated goat anti-mouse antibody (catalog no. 62-6520, Zymed Laboratories Inc.). The experimental conditions were different between overexpression and knockdown studies. The experimental details are described in the [supplemental material](#).

Reverse Transcription (RT)-PCR—Total RNA from MEL and K562 cells was extracted using the RNeasy mini kit. cDNA was synthesized from the total RNA using SuperScript III reverse transcriptase. PCR was performed using GoTaq Flexi DNA polymerase (Promega). The primers for RT-PCR analysis are described in the [supplemental material](#).

Transient Transfection of FUT8 Short Hairpin RNA (shRNA)—shRNAs for *FUT8* were designed by siDirect. shRNA sequences are described in the [supplemental material](#). Forward and reverse oligonucleotides were denatured for 5 min at 95 °C and annealed for 1 h at 37 °C. The annealed, double-stranded oligonucleotide was ligated into the piGENE hU6 puro vector (iGENE Therapeutics) that had been restricted with BspMI. The plasmids containing the shRNA constructs were then restricted with EcoRI and BamHI, and the DNA containing both the shRNA and hU6 promoter sequence was introduced into the EcoRI and BamHI-digested piGENE U6 Rep vector (iGENE Therapeutics) that included *EBNA1* genes and OriP sequence. The piGENE U6 Rep T7STOP vector (iGENE Therapeutics) was used as a negative control vector. These vectors were transfected into K562 cells by electroporation using Bio-Rad Gene Pulser II (Bio-Rad). The transfected cells were cultured with 2 $\mu\text{g}/\text{ml}$ puromycin (Sigma-Aldrich) for 2 weeks.

Flow Cytometric Analysis—Flow cytometric analysis was performed using FACSCalibur. The cells were stained with anti-human CD235a (glycophorin A) FITC (catalog no. 11-9987; eBioscience) and anti-human CD71 (transferrin receptor) PE (catalog no. 12-0719; eBioscience).

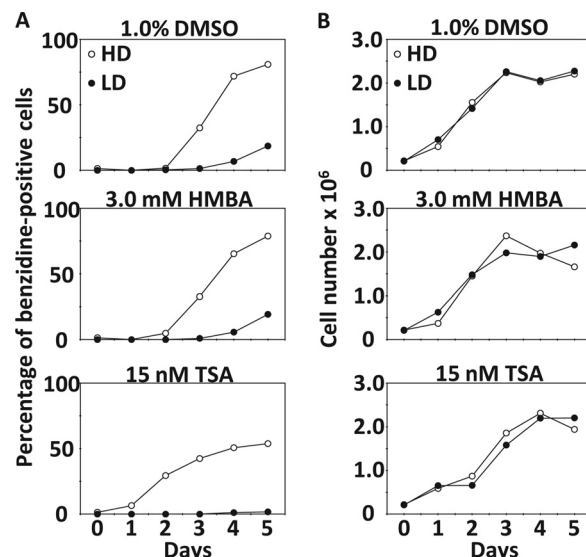


FIGURE 1. Hemoglobin-positive ratios and growth of HD and LD MEL cells after treatment with 1.0% DMSO, 3.0 mM HMBA, or 15 nM TSA. A, hemoglobin-positive ratio was measured with benzidine stain each day for 5 days after chemical treatment. B, daily cell counts/ml in culture.

RESULTS

Comparison of HD and LD MEL Cell Expression Profiles using cDNA Microarray—We screened for differentiation-related genes in MEL cells using cDNA microarray analysis. Prior to microarray analysis, we selected clones of HD and LD MEL cells by recloning from the original cells. HD MEL cells were more highly differentiated than LD MEL cells by treatment with 1.0% DMSO, 3.0 mM HMBA, or 15 nM TSA (Fig. 1A). The concentrations of each chemical were reduced for comparable microarray analysis, because general concentrations, 1.5% DMSO, 5.0 mM HMBA, or 30 nM TSA, were affecting the growth of LD MEL cells (data not shown). The rate of cell differentiation was determined by benzidine staining of hemoglobin. There were no differences in cell growth between HD and LD cells in chemical treatment (Fig. 1B). Next, we compared the expression profiles of HD and LD MEL cells during differentiation induced by 1.0% DMSO, 3.0 mM HMBA, or 15 nM TSA using a cDNA microarray that was prepared from NIA mouse 15k and 7.4k cDNA clone sets. We compared gene expression profiles at 6, 12, 24, and 36 h after chemical treatment. In this microarray study, we defined up-regulated and down-regulated genes of expression ratio as >1.5 and <0.66 , respectively. Table 1 lists the genes from HD MEL cells that are consistently and significantly ($p < 0.05$) up-regulated or down-regulated at more than one time point by treatment with each of the three chemicals. These genes were considered as candidates of differentiation-related genes. We considered that not all of differentiation-related genes were continuously up-regulated or down-regulated during differentiation. In MEL cells differentiation, *c-Myc* mRNA decreases at 2–12 h after DMSO treatment, but it increases to pretreatment levels at 18 h (41).

Fut8 Overexpression Inhibits Hemoglobin Production in MEL Cells—We investigated an association between the down-regulated genes and hemoglobin production in an overexpression study. *Idi1*, *Plek*, *Fut8*, *Rnpc2*, *Cst3*, *Gstt3*, and *Spn* were selected as candidate differentiation suppressor genes. Because these

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TABLE 1

Difference in gene expression between HD and LD MEL cells after drug treatment

Microarray results were filtered at an expression ratio of >1.5 or <0.66 , $p < 0.05$, at more than one time point, and genes consistently up-regulated or down-regulated among the three chemical treatments were selected. The expression ratios are the average of quadruplicate experiments.

Clone name	Gene symbol	Expression ratio (HD MEL cells/LD MEL cells)											
		DMSO				HMBA				TSA			
		6 h	12 h	24 h	36 h	6 h	12 h	24 h	36 h	6 h	12 h	24 h	36 h
H3038C06	<i>Zfp951</i>	0.48	0.38	0.39	0.24	0.42	0.53	0.42	0.25	0.62	0.64	0.48	0.45
H3078E08	unknown	0.49	0.39	0.43	0.26	0.47	0.53	0.44	0.26	0.60	0.64	0.50	0.43
H3049A07	<i>G430049J08Rik</i>	0.53	0.48	0.47	0.33	0.46	0.50	0.41	0.26	0.64	0.63	0.38	0.35
H4070E06	Unknown	0.50	0.44	0.36	0.22	0.48	0.54	0.43	0.21	0.58	0.64	0.52	0.59
H3062A11	<i>Clip2</i>	0.59	0.46	0.51	0.33	0.45	0.51	0.47	0.29	0.76	0.69	0.46	0.41
H4016D11	Unknown	0.59	0.55	0.46	0.34	0.55	0.61	0.49	0.27	0.60	0.59	0.46	0.49
H3061H10	<i>Tmem123</i>	0.66	0.62	0.73	0.48	0.47	0.61	0.45	0.27	0.67	0.61	0.44	0.36
H3104H09	<i>Spc24</i>	0.60	0.50	0.51	0.48	0.49	0.56	0.55	0.35	0.64	0.70	0.54	0.50
H3073G11	<i>Rtp3</i>	0.62	0.58	0.63	0.39	0.49	0.66	0.48	0.28	0.77	0.63	0.48	0.51
H3078A01	<i>G430049J08Rik</i>	0.62	0.56	0.68	0.38	0.56	0.58	0.53	0.28	0.66	0.65	0.53	0.56
H3046C10	<i>Idi1</i>	0.68	0.56	0.95	0.61	0.50	0.57	0.80	0.61	0.50	0.49	0.53	0.56
H4046D12	<i>Plek</i>	0.59	0.58	0.84	0.63	0.52	0.46	0.76	0.44	0.53	0.57	0.68	0.86
H3037D02	<i>G430049J08Rik</i>	0.74	0.90	0.62	0.62	0.75	0.60	0.55	0.45	0.66	0.66	0.49	0.51
H3078G11	<i>Drd3</i>	0.76	0.85	0.94	0.58	0.63	0.66	0.59	0.32	0.75	0.68	0.49	0.54
H4019B10	<i>Fut8</i>	0.78	0.65	0.81	0.37	0.63	0.58	0.62	0.31	0.60	0.66	0.87	0.95
H3078G10	<i>Rtp3</i>	0.78	0.79	0.81	0.59	0.68	0.75	0.72	0.31	0.69	0.72	0.57	0.56
H4073E05	<i>Rnpc2</i>	0.72	0.77	0.89	0.66	0.46	0.71	0.85	0.57	0.72	0.93	0.55	0.48
H3033F11	<i>Cst3</i>	0.65	0.83	0.80	0.60	0.63	0.65	0.66	0.65	0.65	0.73	0.75	0.78
H4040B02	<i>Gstt3</i>	0.76	0.90	1.18	0.47	0.80	0.70	0.67	0.48	0.98	0.66	0.59	0.59
H3138C09	<i>Rnf128</i>	0.91	0.69	0.78	0.62	0.84	0.66	1.02	0.75	0.96	1.03	0.74	0.62
H3032F06	<i>Hbb-y/Hbb-b1</i>	0.61	0.69	0.79	0.92	0.57	0.68	1.14	1.54	0.62	0.60	0.69	0.96
H4003H10	<i>Spn</i>	0.80	0.82	1.14	0.62	0.88	0.90	1.00	0.66	0.65	0.64	0.84	0.98
H3113C01	<i>Hbb-y/Hbb-b1</i>	0.69	0.59	0.87	1.07	0.68	0.75	1.39	1.59	0.60	0.68	0.86	0.96
H3119H11	<i>Wdfy3</i>	0.83	0.84	2.03	0.83	1.03	0.94	0.77	0.64	0.59	0.74	0.69	0.81
H4020E10	<i>Il4ra</i>	0.95	0.89	0.90	0.60	1.06	1.00	1.00	0.54	0.88	1.17	1.33	1.66
H3028F05	<i>Odc1</i>	1.06	1.13	1.51	1.17	0.92	1.30	1.09	1.59	0.99	1.01	1.24	1.76
H4062C02	<i>Mcm4</i>	0.92	1.09	1.57	1.13	1.04	1.08	1.44	1.50	1.04	1.15	1.50	1.67
H3024H12	<i>Hspa8</i>	0.93	1.06	1.59	1.19	0.87	1.08	1.67	1.37	0.95	1.09	1.48	2.02
H4006F09	<i>Hat1</i>	1.12	0.94	1.68	1.27	1.08	1.12	1.69	1.38	1.01	1.55	1.30	1.61
H3006C07	Unknown	1.23	1.24	1.50	1.16	1.24	1.65	1.37	1.57	0.96	1.06	1.39	1.80
H4003E09	<i>Car2</i>	1.05	1.15	1.63	1.34	1.01	1.08	1.82	1.65	0.87	0.90	1.79	2.19
H3130A05	<i>Psm2</i>	1.26	1.20	1.67	1.44	1.19	1.17	1.85	1.50	1.27	1.11	1.48	2.10
H3018A08	<i>Cct8</i>	1.40	1.30	1.75	1.40	1.24	1.52	1.89	1.49	1.37	1.42	1.34	1.69
H4034F12	<i>Fbxo45</i>	1.33	1.28	2.14	1.21	1.22	1.43	1.65	1.30	1.30	1.63	1.39	2.09
H3093D05	<i>Trfr</i>	1.26	1.42	1.77	1.51	1.30	1.28	1.72	2.06	1.23	1.17	1.62	1.68
H3123F09	<i>Guf1</i>	0.98	1.12	0.96	1.60	1.25	1.11	1.23	2.41	0.93	1.00	1.59	4.07
H3122H09	<i>Hba-a1</i>	0.97	1.19	1.10	1.69	1.21	1.07	1.37	2.85	1.13	1.01	1.44	3.50
H3126A10	<i>Hba-a1</i>	1.03	1.13	1.06	1.59	1.11	1.10	1.36	2.93	1.21	1.09	1.51	3.55
H3026B04	<i>Ppat</i>	1.84	1.49	1.81	1.65	1.37	1.47	1.78	1.76	1.21	1.65	1.22	1.63
H3126G09	<i>Hba-a1</i>	1.00	1.08	1.05	2.13	1.18	0.95	1.35	3.39	1.05	1.06	1.34	3.65
H3125H07	<i>Hba-a1</i>	0.97	1.08	1.11	1.99	1.23	1.15	1.55	3.45	0.90	1.10	1.62	3.78
H3121B01	<i>Hba-a1</i>	1.04	1.23	1.31	2.05	1.43	1.11	1.63	3.81	1.06	1.08	1.68	3.97
H3124F12	unknown	0.99	0.99	1.25	1.64	1.26	1.07	1.42	4.07	1.29	1.17	1.79	4.58
H3122H11	<i>Hba-a1</i>	1.02	1.20	1.29	2.03	1.25	1.30	1.40	4.25	1.17	1.03	1.85	4.27
H3045A12	<i>Hba-a1</i>	1.03	1.16	1.59	2.09	1.43	1.16	1.81	4.45	1.19	1.31	2.30	5.50
H3123A01	<i>Hba-a1</i>	1.06	1.40	1.90	2.73	1.25	1.32	2.27	5.18	1.35	1.01	1.64	4.09
H3083F09	<i>Phf1,Rsbn1</i>	1.10	1.09	1.58	1.87	1.47	1.16	2.16	4.91	1.20	1.47	2.47	5.70
H3123E05	<i>Hba-a1</i>	1.34	1.30	1.90	2.72	1.51	1.49	2.33	5.70	1.42	1.22	2.02	4.85

genes were strongly down-regulated in HD MEL cells during chemically induced differentiation, annotations on these cDNA clones were available at that time. Each gene was introduced into HD MEL cells using the retroviral vector pMXs-CMVp-IRES-EGFP, thereby constructing MEL cell lines in which the candidate gene was overexpressed. After cell cloning, we measured the hemoglobin-positive ratio of the overexpressing cells 4 days after 1.5% DMSO treatment. We observed that the rate of appearance of benzidine-positive cells in clones overexpressing *Fut8* was significantly ($p < 0.01$) less than that in control cell clones that contained the empty vector (Fig. 2A). There were no differences in cell growth between clones overexpressing *Fut8* and control cell clones (Fig. 2B). These data indicate that the low hemoglobin-positive rate of MEL cells overexpressing *Fut8* was caused by *Fut8*-mediated differential suppression of hemoglobin production and not by differential suppression of growth. Overexpression of the other genes did not show statis-

tically significant differential suppression of hemoglobin production in MEL cells (data not shown).

Fut8 Expression Is Down-regulated during MEL Cell Differentiation—We analyzed the expression profile of *Fut8* and other erythroid differentiation-related genes during chemically induced differentiation of HD MEL cells by another microarray study. We compared gene expression before and after chemical treatment. Fig. 3 shows expression profiles of representative cDNA clones of erythroid differentiation-related genes and *Fut8*. *Fut8* expression was rapidly down-regulated after 1.5% DMSO, 5.0 mM HMBA, or 30 nM TSA treatments, the same as *Myc* and *Myb* that were known as suppressors of erythroid differentiation. However, hemoglobin genes (*Hba-a1* and *Hbb-y/Hbb-b1*) were up-regulated during MEL cell differentiation. Especially, up-regulation of hemoglobin genes with TSA treatment was faster than DMSO or HMBA treatment. In the same conditions, *Fut8* decreased at 6–12 h,

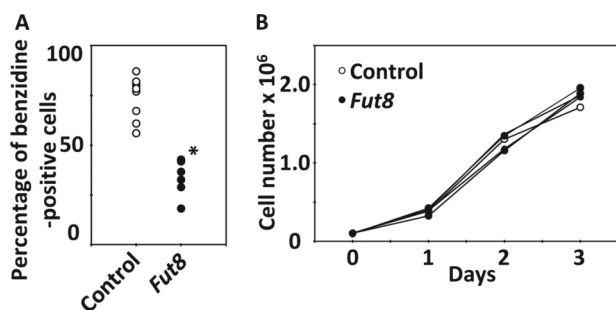


FIGURE 2. Hemoglobin-positive ratios and growth of MEL cells overexpressing *Fut8*. A, hemoglobin-positive ratios of MEL cells containing the empty vector (control), and *Fut8* overexpression 4 days after 1.5% DMSO treatment. Individual circles mean different cell clone; there were nine control cell clones, and six *Fut8*-overexpressing clones were analyzed. The asterisk indicates statistically significant difference ($p < 0.01$). B, daily cell counts/ml in culture without DMSO. One control clone and five *Fut8*-overexpressing clones were analyzed.

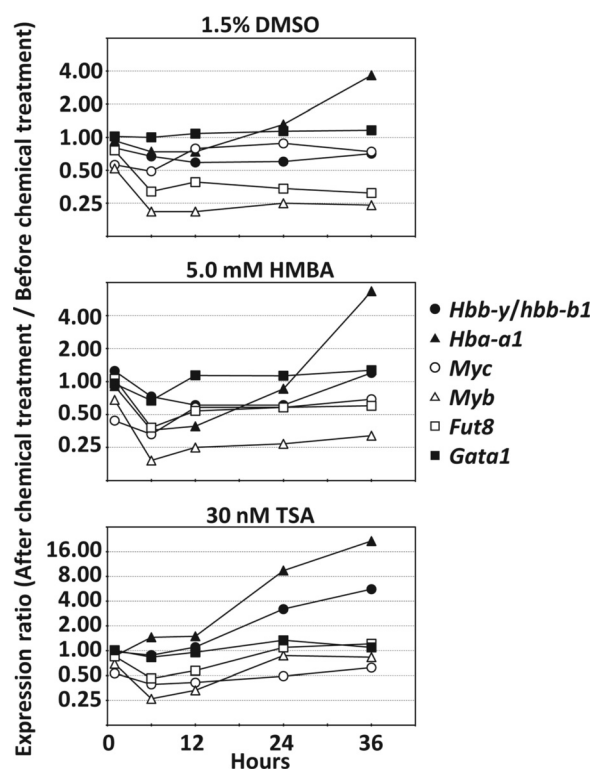


FIGURE 3. Expression profile of *Fut8* and other erythroid differentiation-related genes during HD MEL cells differentiation with 1.5% DMSO, 5.0 mM HMBA, or 30 nM TSA treatment. The expression ratios are the average of quadruplicate experiments.

but it increased to before TSA treatment levels at 24 h. These data indicate that temporary down-regulation of *Fut8* at an early time point is important for hemoglobin expression, and continuous down-regulation of *Fut8* is not necessary for hemoglobin expression. Significant expression change of *Gata-1* was not detected. The expression profile indicates that *Fut8* expression is well correlated to *Myb* expression among the three chemical treatments.

Overexpression of *Myc* and *Myb* Increase *Fut8* Expression—We investigated whether *Myc* and *Myb* were effect on *Fut8* expression. We constructed *Myc*- and *Myb*-overexpressing MEL cell clones and measured *Fut8* expression by RT-PCR and Western blotting. As a result, *Fut8* was more highly expressed

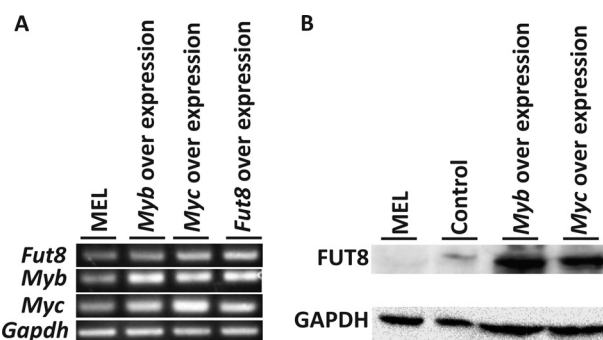


FIGURE 4. Effect of *Myb* and *Myc* for *Fut8* expression. A, RT-PCR analysis of *Fut8*, *Myb*, and *Myc* overexpression in MEL cells. B, Western blot analysis of FUT8 in *Myb*- and *Myc*-overexpressing MEL cells.

in *Myc*- and *Myb*-overexpressing MEL cells than in normal MEL cells and empty vector introduced cells (Fig. 4, A and B). This result indicates that *Fut8* expression was positively regulated by *Myc* and *Myb*.

***Fut8* Is the Only Suppressor of Hemoglobin Production among the *Fut* Gene Family—**We also performed overexpression studies of other *Fut* family genes to determine that suppression of hemoglobin production was caused not by general function of fucosyltransferase but by the unique function of FUT8. We cloned the *Fut1*, *Fut2*, *Fut4*, *Fut7*, *Fut9*, *Fut11*, *Pofut1*, and *Pofut2* genes into the same vector, and these genes were overexpressed in HD MEL cells as described for *Fut8* above. These overexpression clones were treated with 1.5% DMSO for 4 days, and the rates of appearance of benzidine-positive cells were determined. However, these clones did not show statistically significant differential suppression of hemoglobin production compared with control cell clones that contained the empty vector (Fig. 5). These results indicate that *Fut8* is the only fucosyltransferase that is capable of inhibition of hemoglobin production in MEL cell.

FUT8-mediated Core Fucosylation Is Essential for Hemoglobin Production—We next investigated whether the FUT8-mediated differential suppression of hemoglobin production could be cancelled by mutation of FUT8 functional domains. The structure of FUT8 has an SH3 domain, an SH3-binding domain, a donor substrate-binding domain, and a flexible loop. Arg-365 and Arg-366 are essential for substrate binding (42), and the flexible loop, which includes Asp-368 and Lys-369, is also essential for enzyme activity (43). On the basis of these findings, we constructed FUT8 mutants of the donor substrate-binding domain (the R365A/R366A double mutant (RRM)) and the flexible loop (the D368A/K369A/V370A/G371A/T372A quintuple mutant (FLM)) and introduced them into MEL cells (Fig. 6A). Both FUT8 mutant proteins were overexpressed in MEL cells (Fig. 6B). We observed that introduction of the RRM or FLM mutations into FUT8 cancelled the FUT8-mediated differential inhibition of hemoglobin production in MEL cells (Fig. 6C). These results strongly indicate that FUT8-mediated core fucosylation is associated with hemoglobin production in MEL cells.

FUT8 Overexpression Inhibits Hemoglobin Production in K562 Cells—We next investigated whether the results with MEL cells were consistent between species. We investigated whether *FUT8* functions as an inhibitor of hemoglobin production in humans using K562 cells. We constructed overexpres-

FUT8 Inhibits Hb Production in MEL and K562 Differentiation

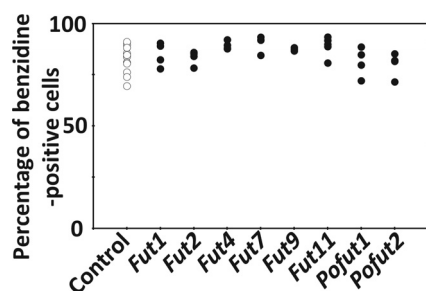


FIGURE 5. Hemoglobin-positive ratios of MEL cells overexpressing *Fut* family genes. Hemoglobin-positive ratio of MEL cells containing the empty vector (control) or overexpressing *Fut* family genes 4 days after 1.5% DMSO treatment. Individual circles denote different cell clones. The following were analyzed: 12 control cell clones; four clones overexpressing *Fut1*; four clones overexpressing *Fut2*; four clones overexpressing *Fut4*; four clones overexpressing *Fut7*; three clones overexpressing *Fut9*; five clones overexpressing *Fut11*; four clones overexpressing *Pofut1*, and four clones overexpressing *Pofut2*.

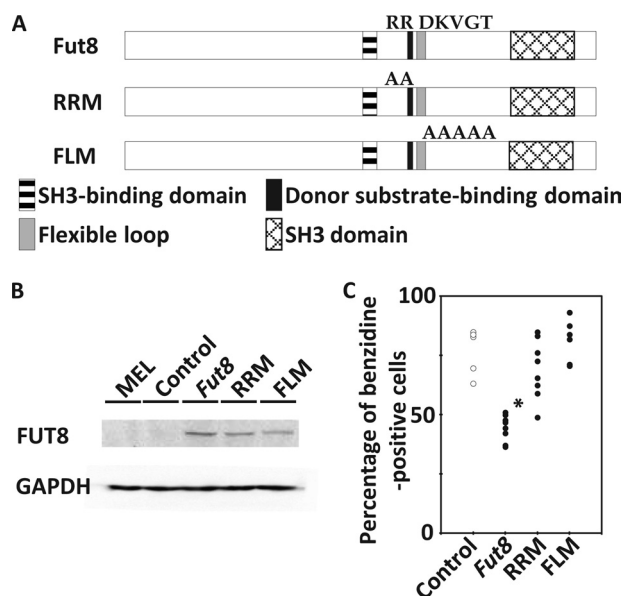


FIGURE 6. Hemoglobin-positive ratios of MEL cells with overexpression of *Fut8* mutant. A, constructs of *FUT8* mutant. RRM means R365A/R366A mutant of *FUT8*. FLM means flexible loop (D368A/K369A/V370A/G371A/T372A) mutant of *FUT8*. B, Western blot analysis of overexpression of *FUT8* and *FUT8* mutant. C, hemoglobin-positive ratio of MEL cells with the empty vector (control), overexpression of *Fut8* and *Fut8* mutants 4 days after 1.5% DMSO treatment. Individual circles indicate different cell clone. Four control cell clones, nine clones overexpressing *Fut8*, eight clones overexpressing RRM, and six clones overexpressing FLM were analyzed. The asterisk indicates statistically significant difference ($p < 0.01$).

sion clones containing wild-type *FUT8* or its donor substrate-binding domain mutant RRM (Fig. 7A). Differentiation was induced in these clones by treatment with 100 μ M hemin, and the rate of appearance of benzidine-positive cells was determined 4 days after treatment. We observed that *FUT8* overexpression also inhibited hemoglobin production during hemin-inducible differentiation of K562 cells (Fig. 7B). The RRM mutant did not inhibit hemoglobin production. This result indicates that *FUT8* is also involved in the inhibition of hemoglobin production of human cells.

shRNA-mediated *FUT8* Knockdown Promotes Hemoglobin Production and Differentiation of K562 Cells—We examined the effect of *FUT8* knockdown using shRNA constructs in K562 cells. Three different *FUT8* shRNA sequences were designed

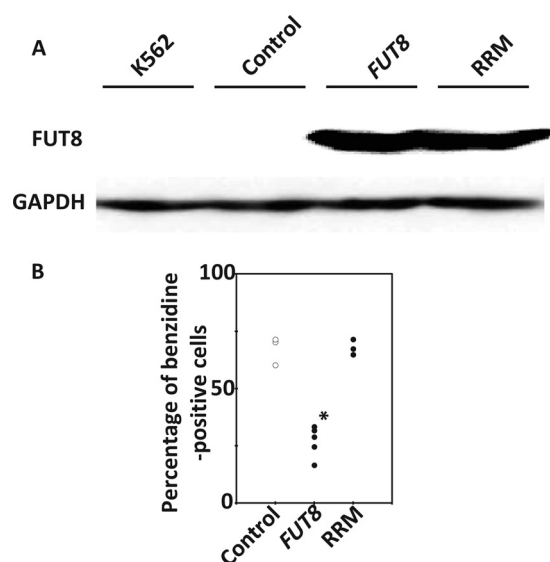


FIGURE 7. Hemoglobin-positive ratios of K562 cells overexpressing *FUT8* and *FUT8* mutants. A, Western blot analysis of *FUT8* and *FUT8* mutant overexpression. B, hemoglobin-positive ratios of K562 cells containing the empty vector (control), overexpressing *FUT8*, and overexpressing *FUT8* mutant 4 days after 100 μ M hemin treatment. Individual circles denote different cell clones. Three control cell clones, five clones overexpressing *FUT8*, and three clones overexpressing RRM were analyzed. The asterisk indicates statistically significant difference ($p < 0.01$).

and transiently introduced into K562 cells. The cells containing each shRNA construct were selected with puromycin for 2 weeks after transfection, and then the decreases in *FUT8* mRNA and protein expression were confirmed by RT-PCR and Western blotting, respectively (Fig. 8A and B). Using benzidine staining, we observed that the hemoglobin-positive rates of the cells into which shRNA constructs had been introduced were higher than cells into which a negative control vector had been introduced (Fig. 8C). The introduction of shRNA constructs did not affect cell growth, except for SH3, which may be related to the high differentiation ratio of cells into which the SH3 construct had been introduced (Fig. 8D). Furthermore, we performed flow cytometric analysis of K562 cells using glycophorin A and CD71 as markers of erythroblast differentiation. Flow cytometric analysis showed that the rate of appearance of glycophorin A- and CD71-positive cells was increased in cells into which shRNA constructs had been introduced (Fig. 8E). The increase in the rate of coexpression of these markers indicates that *FUT8* regulates not only hemoglobin production but also the differentiation of pre-erythroblasts to basophilic normoblasts and polychromatic erythroblasts.

DISCUSSION

DNA microarray is a powerful tool to understand biological phenomena regulated by multiple genes, such as cell differentiation, because it can comprehensively analyze gene expression at one time point. However, it also detects the noise of gene expression, such as responses to the stresses of chemical addition and cell proliferation that occur during chemical induction of the differentiation of cell lines. In this study, we compensated for the shortcomings of our microarray experiments by comparing the expression profiles of HD and LD MEL cells. The only difference between these cells was their ability to differen-

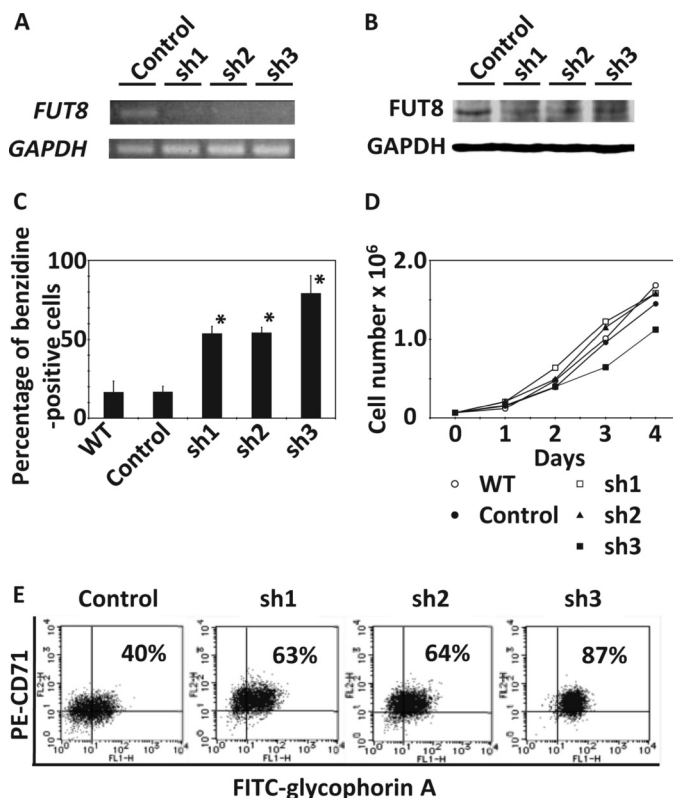


FIGURE 8. shRNA-mediated *FUT8* knockdown in K562 cells. Negative control vector (control) or *FUT8* shRNA constructs (*sh1*, *sh2*, and *sh3*) were transfected into K562 cells by electroporation. The cells containing each construct were selected with puromycin for 2 weeks after transfection, and then these cells and nontransfected K562 cells (WT) were analyzed. *A*, RT-PCR analysis of *FUT8* expression. *B*, Western blot analysis of *FUT8* expression. *C*, hemoglobin-positive ratios of K562 cells. The mean values and S.D. bars for triplicate experiments are shown. The asterisk indicates statistically significant difference ($p < 0.01$). *D*, daily cell counts/ml in culture. *E*, flow cytometric analysis of CD71 and glycophorin A.

tiate in response to chemicals that induce cell differentiation. We also effectively selected for differentiation-related genes by focusing on the genes consistently regulated among the three chemical inducers.

Next, we evaluated the direct relationship between the targeted genes and differentiation by gain-of-function, which was performed using an overexpression study. We observed that *Fut8* overexpression inhibited hemoglobin production during MEL cell differentiation; the same effect was observed in human K562 cells.

Fut8 is a member of the fucosyltransferase gene family. Fucosyltransferases are involved in various biological and pathological processes in eukaryotic organisms, including tissue development, angiogenesis, fertilization, cell adhesion, inflammation, and tumor metastasis (44). There are two main types of fucosyltransferases in the mouse. One of the main types of fucosyltransferases catalyzes fucosylation of *N*-linked glycans at asparagine residues. FUT1 and FUT2 transfer fucose to galactose in an α -1,2 linkage. FUT3 and FUT5 transfer fucose to *N*-acetylglucosamine in an α -1,3 or α -1,4 linkage. FUT4, FUT6, FUT7, FUT9, FUT10, and FUT11 transfer fucose to *N*-acetylglucosamine in an α -1,3 linkage. Finally, FUT8 transfers fucose to *N*-acetylglucosamine in an α -1,6 linkage. Another main type of fucosyltransferase in mice catalyzes fucosylation of *O*-linked

glycans at serine or threonine residues. There are two *O*-fucosyltransferases, POFUT1 and POFUT2. In this study, *FUT8* was the only fucosyltransferase that showed inhibition of hemoglobin production. This indicates that core fucosylation plays a key role in hemoglobin production and cell differentiation because *FUT8* is the only fucosyltransferase that adds fucose to the core asparagine-linked glycans.

To evaluate whether *FUT8*-mediated core fucosylation directly affects hemoglobin production, we performed mutagenesis studies of the donor substrate-binding domain and the flexible loop that are essential for core fucosylation activity. We observed that *FUT8*-mediated inhibition of hemoglobin production was cancelled by these *FUT8* mutations in MEL cells. Moreover, the same results were observed in K562 cells. This is the first report that the regulation of core fucosylation is essential for hemoglobin production and erythroid differentiation. In the development of other tissues, it has previously been reported that *FUT8*-mediated core fucosylation of signal-transducing receptors positively regulates the function of the receptors. However, there have been no reports of *FUT8*-mediated negative regulation of differentiation. *Fut8* knock-out mice lack core fucosylation in both their epidermal growth factor receptors and platelet-derived growth factor receptors, and they experience early death during postnatal development ($\sim 70\%$ *Fut8* null mice) or growth retardation (45). The lack of core fucosylation of transforming growth factor β 1 (TGF- β) receptors led to abnormal lung development and an emphysema-like phenotype in *Fut8* null mice (46). In B cell development, the loss of core fucosylation in both α 4 β 1 integrin and vascular cell adhesion molecule 1 led to a decrease in binding between pre-B cells and stromal cells, which impaired the generation of pre-B cells in *Fut8* null mice (47). There were no reports about *Fut8* null mice of defective of erythroid phenotypes, because it seem that down-regulation of *Fut8* promotes erythroid differentiation. These reports predict that signal-transducing receptors other than EpoR may play a role in erythroid differentiation and that core fucosylation of these receptors may play a key part in this role. We think the TGF- β receptor is one of the candidate targets of *FUT8*. This receptor was identified as the target of core fucosylation (46), and TGF- β induces hemoglobin production in K562 and other human erythroleukemia cells (48, 49).

FUT8 knockdown studies using shRNA in K562 cells indicated that the decrease in *FUT8* expression was important for hemoglobin production and differentiation. In the microarray experiments, we also observed that *Fut8* expression was decreased during HD MEL cell differentiation. Our results indicate that *Fut8* is regulated downstream of Myc and Myb. Both transcription factors were already known as regulators of erythroid differentiation (13–16, 26, 27). The upstream sequence of human *FUT8* has binding sites for MYB and GATA-1 (50). In a study exploring the regulation of erythroid gene expression by GATA-1, *Fut8* expression was rapidly down-regulated after GATA-1 induction (51). This result also indicates that *Fut8* expression is down-regulated downstream of GATA-1.

In contrast, the enzyme activity of *FUT8* was increased during megakaryocytic differentiation (52). This fact is the

opposite to our findings that a decrease in FUT8 progressed differentiation of pre-erythroblasts to basophilic normoblasts and polychromatic erythroblasts. FUT8 may be involved in the decision whether MEPs differentiate into erythrocytes or megakaryocytes, a role similar to FLI-1, which suppresses erythroid differentiation but activates megakaryocytic differentiation.

In conclusion, expression profile analysis of the differential differentiation ability of MEL cells using DNA microarrays identified FUT8 as a suppressor of differentiation and demonstrated that FUT8-mediated core fucosylation regulates hemoglobin production and erythroid differentiation. In a previous erythropoiesis study, several transcription factors and cytokines, including GATA-1 and erythropoietin, were shown to regulate hemoglobin production and erythroid differentiation. In addition, our findings clearly demonstrate that modification of proteins by glycosylation is important for hemoglobin production and erythroid differentiation. We are currently exploring the target proteins of FUT8-mediated core fucosylation. Further study of FUT8-mediated core fucosylation during erythroid differentiation will help to clarify the additional details of the mechanism of differentiation.

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