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Review of *ABO* Expression and Variations based on Transcriptional Regulation of the ABO Blood Group Gene

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Abstract
Background and Summary: We review the transcriptional regulation of *ABO* expression and discuss variants in the promoter and erythroid cell-specific regulatory region in individuals with weak ABO phenotypes such as B_m, A_m, B_3, and A_3. We also review the molecular mechanisms responsible for variations in *ABO* expression in development and disease including the cell type-specific expression of *ABO* during erythroid cell differentiation, and reduction of A- or B-antigens in cancer cells or on red blood cells in patients with leukemia. Although the relationship between ABO blood group antigens and diseases has been characterized, the physiological significance of the ABO blood group system remains unclear. ***Key Messages:*** This review discusses accumulated knowledge of the *ABO* gene regulation and potential reasons for conservation of ABO during evolution.

Introduction

The ABO blood group system, termed system number 001 by the International Society of Blood Transfusion, was discovered by Karl Landsteiner [1, 2], which is the most important blood group system in transfusion medicine and transplantation. Many pioneering studies have revealed weak ABO subgroup phenotypes such as A_3, A_x, A_m, A_{el}, B_3, B_x, B_m, and B_{el} subgroups [3], which are predicted to result from extensive polymorphism of the *ABO* gene. The system is composed of two carbohydrate antigens, A and B, and their antibodies. Biochemical and molecular genetic studies have clarified the molecular basis of the ABO blood group system [3].

After delineation of the antigen structures, subsequent investigations were carried out to identify the gene encoding the glycosyltransferases by protein purification and production of enzyme-specific antibodies. Prof. Sen-ichiro Hakomori and his colleagues including Dr. Fumi-ichiro Yamamoto at the Biomembrane Institute, University of Washington, partially purified A-transferase from human lung and identified a partial amino acid sequence of the protein [4], followed by elucidation of the human *ABO* gene structure [5–7]. These discoveries resulted from their pursuit of a molecular basis for loss of ABH-antigens in cancer cells including those in the urinary tract and lung, and for biosynthesis of A-antigens in gastric cancer cells of an individual with type O blood group [8–11].

Following identification of the gene structure, subsequent genetic analyses reported multiple nonsynonymous substitution variants in coding exons and splicing sites of the *ABO* gene in individuals with weak ABO phenotypes, and numerous allelic variants involving synonymous substitutions in individuals with ordinary ABO phenotypes [12–22], leading to the classification of over 200 *ABO* alleles

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[23]. However, variants in the coding region and splicing site of the *ABO* gene in same samples with A_3 and B_m phenotypes have not been observed [24, 25] whereas two nonsynonymous substitutions were reported in exon 7 of individuals with an A_m analog to B_m [26, 27]. The B_m phenotype is characterized by a divergence in the amount of B-antigen between RBCs and saliva [28]. Compared to individuals with the ordinary secretory-type B phenotype, those with the secretory-type B_m phenotype exhibit a trace amount of B-antigen on RBCs but similar amounts of B-antigen in saliva [3]. In addition, serum B-transferase activity in individuals with the B_m phenotype is approximately half that of individuals of the type B group. As the B_m and A_1B_m types accounted for half of the observed weak phenotypes in Japanese individuals [29], the molecular basis for these phenotypes requires further exploration in Japan.

Many phenomena other than those described above have been well-characterized because of the long history of research on ABO blood groups [3]. For example, A- and B-antigens are known to be expressed in a cell type-specific manner whereby they are expressed on cells of erythrocyte and epithelial lineage, but not in fibroblasts (shown in Fig. 1). Moreover, these antigens are expressed during cell differentiation. Early in vitro cell differentiation experiments demonstrated that expression of A-antigens on cells of erythroid lineage increased with cell differentiation, and A-antigen expression was observed in blast-forming and colony-forming unit-erythroid cells [30–34]. In addition, weak A-antigen expression on RBCs of patients with acute myeloid leukemia is well documented [3]. Bianco et al. [35] reported that loss of ABH-antigens in RBCs of patients with myeloid malignancies including acute myeloid leukemia and myelodysplastic syndrome (MDS) and myeloproliferative disorders including chronic myeloid leukemia was a frequent phenomenon. However, the molecular basis for these phenomena remained elusive since they could not be delineated based on the nucleotide sequences of the *ABO* coding region. Therefore, it was necessary to elucidate the transcriptional regulation of *ABO*.

Gene expression is the process by which the information encoded in a gene leads to a function. The process by which the information encoded in the *ABO* gene results in functional expression of A- and B-antigens involves many stages, such as access of transcription machinery to regulatory regions in the *ABO* gene, transcription from *ABO*, RNA splicing, production of glycosyltransferases and their retention in the Golgi apparatus, and transfer of GalNAc or Gal to the H-antigen by A- or B-transferase to produce A- or B-antigens, respectively (shown in Fig. 1) [3]. Transcription, which is the synthesis of RNA from template DNA, requires a DNA region called a promoter, which defines the transcription start site and direction of transcription [3]. There are also DNA regions that activate or repress transcription, called enhancer or suppressor regions, respectively. Furthermore, DNA-binding transcription factors that attach or assemble on promoter and enhancer regions, act as an "on/off switch" to control when and where RNA molecules and proteins are produced and as a "volume control" to determine the quantity of these products. Even when the promoter and enhancer regions are separated by distance in the DNA sequence, they are spatially close to each other due to the loop structure of DNA (shown in Fig. 1). This review focuses on the transcriptional regulation of *ABO* and discusses variations in *ABO* expression.

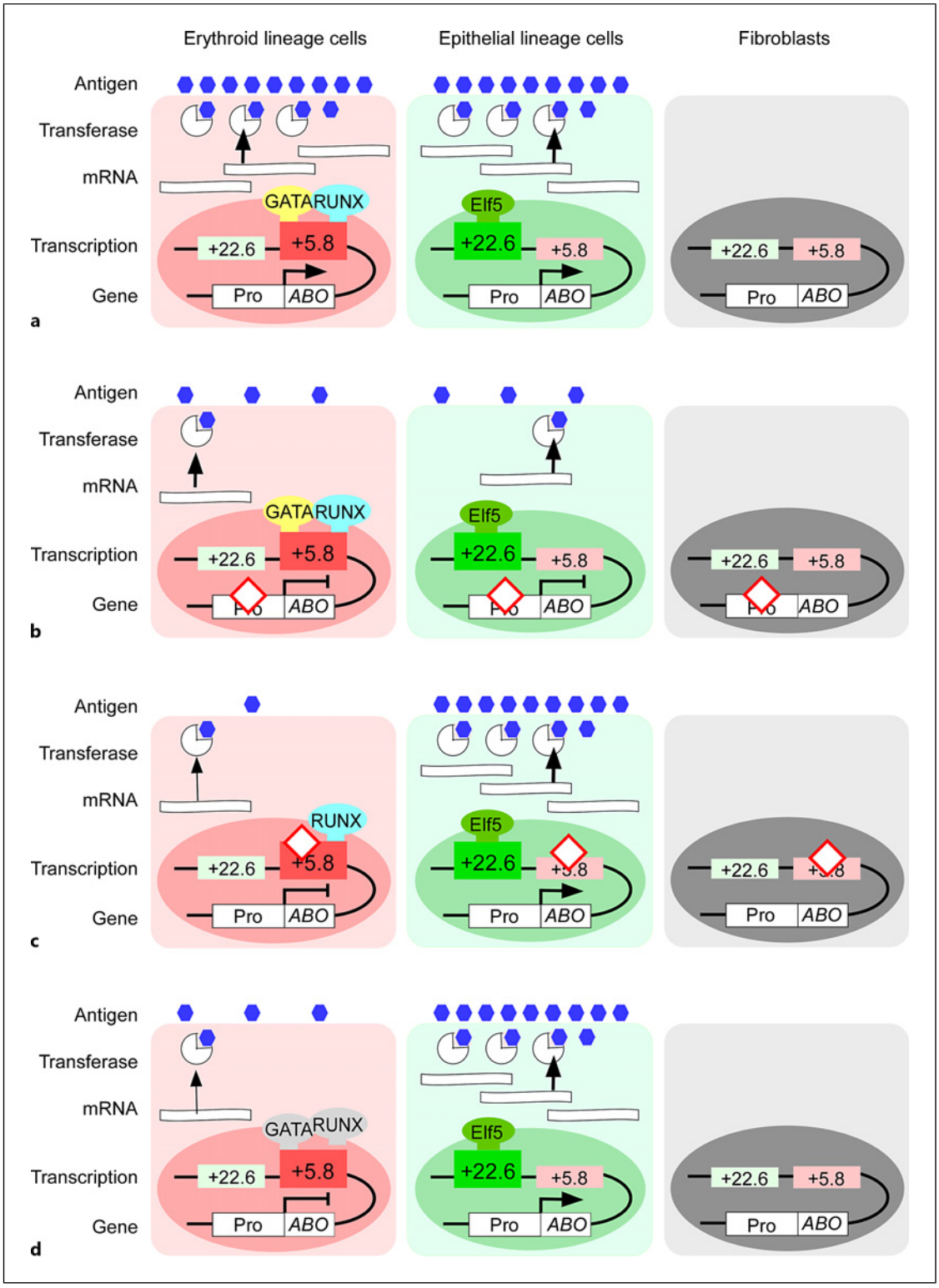
Structure of *ABO*

The human *ABO* gene is located on the long arm of chromosome 9 at 9q34. Yamamoto et al. [5] demonstrated that *ABO* is composed of seven exons spanning approximately 19.5-kb of genomic DNA (shown in Fig. 2a) and that two critical single-base substitutions in the last coding exon between *A*- and *B*-alleles result in amino acid substitutions responsible for the different donor substrate specificity between A- and B-transferases. Furthermore, they showed that a single-base deletion in exon 6 of the *O*-allele causes a frameshift, resulting in failure to produce a protein with the catalytic activity necessary for production of A- or B-antigens [36]. As many variants have been reported in the coding region of individuals with ordinary and weak ABO phenotypes, we refer readers to other literature and databases for a comprehensive understanding of these variants [3, 23].

Transcriptional Regulatory Regions of the Human *ABO* Gene

ABO *Promoter*

A region of approximately 1.3-kb around exon 1 of the *ABO* gene has the characteristics of a CpG island (shown in Fig. 2a). 5′-RACE demonstrated that transcription of *ABO* started immediately upstream of the translation start site on exon 1 (shown in Fig. 3a) [7, 39, 40]. Transcription was also observed from exon 1a located on the 5′ edge of the CpG island of the *ABO* gene [39], although the transcript level from exon 1a was much lower than that from exon 1. In addition, transcription in the opposite direction of the *ABO* transcription, termed *ABOAS*, was initiated from the 3′ edge of the CpG island, although its significance was unclear [41]. Using in vitro experiments, including luciferase assays and electrophoretic mobility assays



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(EMSAs), promoter activity was demonstrated in the region from −150 to −2 relative to the translation start site in cell type-independent manner. In addition, the transcription factor Sp1 was shown to bind to the promoter region functioning as a positive transelement (shown in Fig. 3a) [42, 43]. In contrast, genetic analyses of weak ABO phenotypes revealed variants such as single nucleotide substitutions and deletions in the promoter region of the *ABO* gene in individuals with A_3 and B_3 phenotypes, suggesting that

the promoter region may be involved in transcriptional regulation of *ABO* in a cell type-independent manner (shown in Fig. 3a) [44–49].

Investigations in human cultured cancer cells indicated that DNA methylation of the *ABO* promoter may be associated with repression of transcription. Cells lacking expression of the A-antigen displayed hypermethylation of the *ABO* promoter, while promoter region hypomethylation was observed in the cells expressing the A- or B- antigen [39, 50, 51]. Subsequent investigations using clinical specimens from cancerous tissues in the bladder and oral cavities demonstrated that the lack of ABH-antigen expression in cancer cells could be attributed to gene deletion, promoter methylation, and unknown causes [52, 53]. Similarly, Bianco-Miotto et al. [54] reported that DNA methylation of the *ABO* promoter underlies the loss of *ABO* allelic expression in a significant proportion of patients with leukemia. Thus, *ABO* transcription appears to be regulated through epigenetic mechanisms.

Erythroid Cell-Specific Positive Regulatory Region

Using genome annotation data such as DNase I-sensitive sites, chromatin modifications, and transcription factors identified by the ENCODE project, as well as in vitro experiments including luciferase assays, EMSAs, and ChIP assays, a positive regulatory region, termed the +5.8-kb site (502 bp), was identified approximately 5.8-kb downstream of the translation start site of the *ABO* gene (shown in Fig. 2a, 4a). This site, which functions as a positive regulatory region in an erythroid cell-specific manner, was shown to bind the transcription factor RUNX1 and erythroid cell-specific transcription factors GATA-1/2 [37, 55, 56]. In addition, the site contained two GATA-binding sites, both of which were required for its transcriptional activity [37].

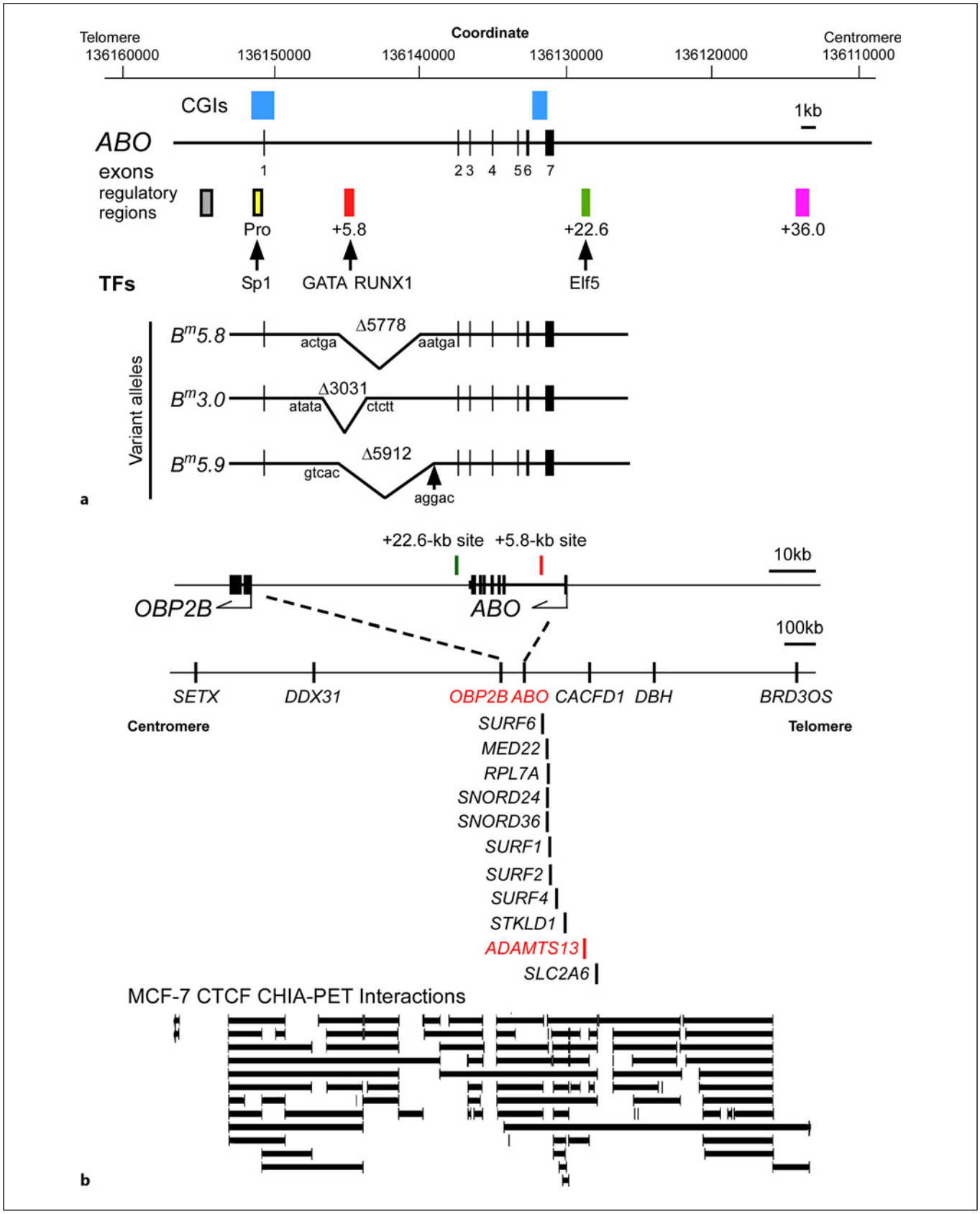
Genetic analysis revealed a 3.0-kb deletion (*B^{m}3.0*) and a 5.8-kb deletion (*B^{m}5.8*) including the +5.8-kb site in Japanese individuals with the B_m phenotype (shown in Fig. 2a) [30, 37]. In contrast, the 5.8-kb deletion was not observed in 1,005 individuals with the standard ABO blood types [37]. Moreover, a single nucleotide substitution in the downstream GATA-binding site within the +5.8-kb site was reported in an individual with the B_m phenotype (shown in Fig. 3b) [55]. Subsequently, single nucleotide substitutions were observed in the GATA motif of individuals with weak ABO phenotypes such as A_m [48, 59–61]. Further, a deletion or single nucleotide substitutions in and around the RUNX1 motif were reported in individuals with weak phenotypes including A_m, A_3, and B_3 [38, 45, 48, 56, 62–64]. Next-generation sequencing in individuals with weak ABO phenotypes revealed a 5.9-kb deletion encompassing the +5.8-kb site (*B^{m}5.9*), single nucleotide substitutions in the upstream and downstream GATA-binding sites, and a single nucleotide substitution near the putative C/EBP binding site [65–67]. Collectively, these findings suggest that *ABO* transcription may be regulated by the +5.8-kb site in an erythroid cell-specific manner.

Investigation into the sequence of the +5.8-kb site in 113 Japanese individuals showed a relationship between specific *ABO* alleles and six haplotypes of the site, which were classified on the basis of six polymorphic nucleotides (shown in Fig. 4a), with the exception of 3.5% of the alleles where genetic recombination was found between *O*- and *B*-alleles, *A*- and *O*-alleles, *A*- and *B*-alleles [57]. Similarly, allelic genetic recombination was recently observed using long-read sequencing [68].

In vitro differentiation of CD34-positive cells obtained from peripheral blood mononuclear cells to erythroid cells demonstrated that expression of *ABO*, *GATA-2*, and *RUNX1* in undifferentiated hematopoietic cells decreased with differentiation or maturation (shown in Fig. 5) [69]. Conversely, *FUT1* and *GATA*-1 expression increased during differentiation. These findings suggest that decline of *ABO* expression might be attributed to reduced expression of the transcription factors GATA-2 and RUNX1 during erythroid cell differentiation.

Investigations of leukemia-associated genes were recently carried out for 13 patients with MDS, including 2 patients with mixed field agglutination of RBCs in response to anti-A antibodies [70, 71]. Compared to patients who did not demonstrate reduction of the A- or B-antigen, a single 2-bp deletion in *RUNX1* was found in 1 patient with A-antigen reduction while the other patient exhibited single nonsynonymous substitutions in *RUNX1* and *GATA-2*. Taking the report by Bianco-Miotto et al. [54] into consideration, leukemogenesis is likely accompanied by *ABO* promoter methylation as well as somatic mutations in *RUNX1* and *GATA-2*,

Fig. 1. Diagrams of *ABO* expression. **a** *ABO* expression in individuals with ordinary blood types. *ABO* is expressed in a cell type-specific manner: *ABO* is expressed in cells of erythroid and epithelial lineage, but not in fibroblasts. *ABO* is located on the long arm of chromosome 9, and its transcription is dependent upon the constitutive promoter and cell type-specific regulatory regions such as the +5.8-kb site and +22.6-kb site, followed by production of transferase which synthesizes A- or B-antigens. The +5.8-kb site binds transcription factors RUNX1 and GATA-1/2, whereas the +22.6-kb site interacts with transcription factor Elf5. Those are not expressed in fibroblasts. Hexagon represents A- or B-antigen; Disk indicates transferase; Ribbon denotes transcript or mRNA; curved arrow indicates transcription, while curved arrow with stop represents reduced transcription; Diamonds denote variants in the DNA. **b** *ABO* expression in individuals of weak ABO phenotype with variant in ABO promoter. **c** *ABO* expression in individuals of weak phenotype with variant in the +5.8-kb site. **d** *ABO* expression in individuals with MDS with reduced A-antigen expression on RBCs. RUNX1 or GATA-2 in gray indicates mutated protein.



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resulting in decreased transcription of the *ABO* gene and reduced production of A- or B-antigen (shown in Fig. 1d). Thus, it is likely that weak A-antigen expression on RBCs is due to mutations involved in MDS pathogenesis since recurrent mutations of *RUNX1* and *GATA-2* were previously reported in patients with MDS. Furthermore, when combined with observations from in vitro experiments, the evidence indicates that it is plausible that transcriptional regulation of *ABO* is dependent on GATA-2 and RUNX1 during erythroid cell differentiation.

Epithelial Cell-Specific Positive Regulatory Region

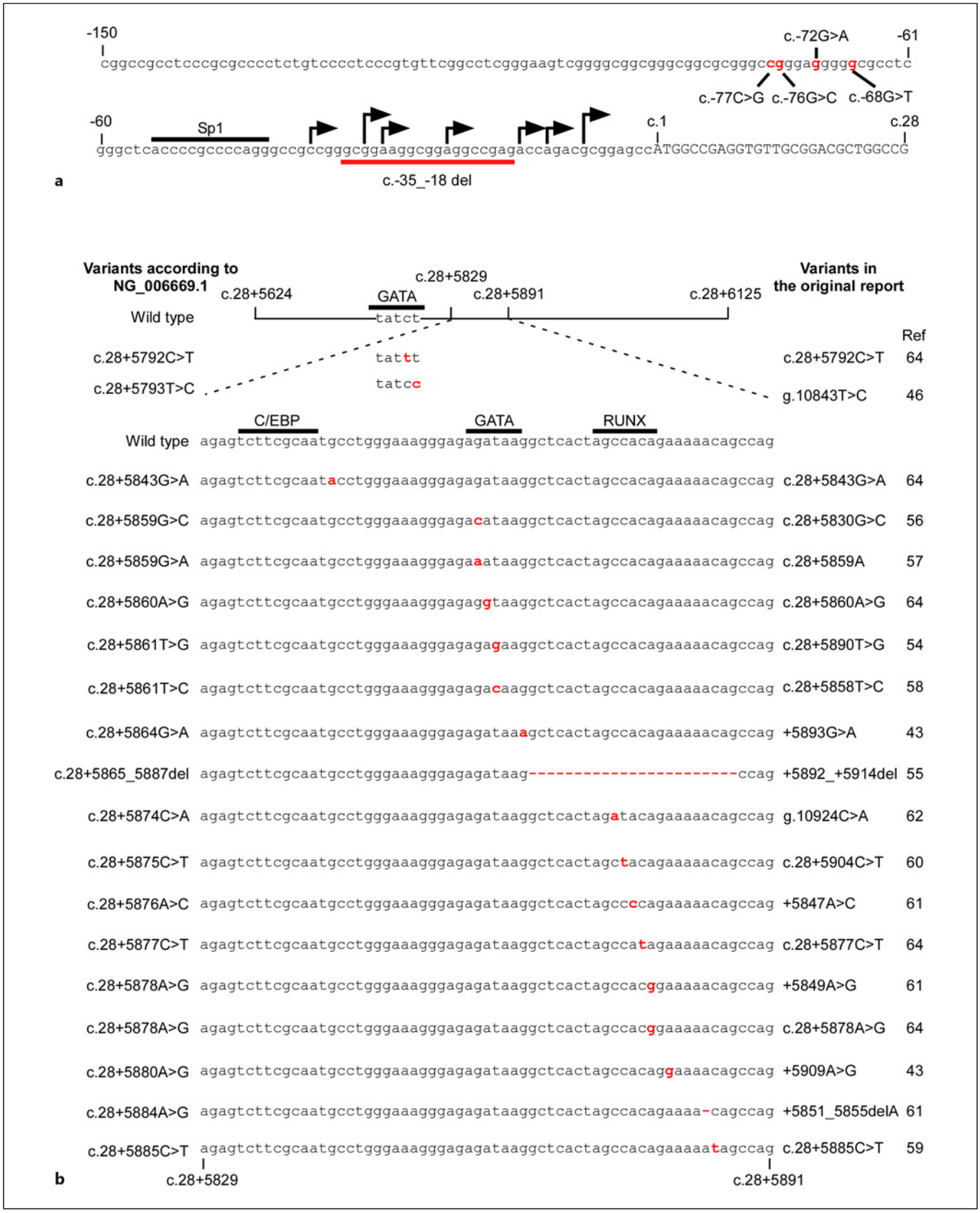
Using the genome annotation data and the in vitro experiments described above, a positive regulatory region, termed the +22.6-kb site, was identified approximately 2.6-kb downstream of *ABO* (shown in Fig. 2a, 4b). The site was shown to bind the epithelial cell-specific transcription factor Elf5 and functioned as a positive regulatory region in an epithelial cell-specific manner [58]. When biallelic deletion of the site was generated in the gastric cancer cell line KATOIII using CRISPR/Cas9, *ABO* expression was reduced to one-third that of the wild-type control cells. Similarly, shRNA knockdown of *ELF5* led to the loss of one-third of the *ABO* transcripts and half of the Elf5 protein levels in knockdown cells relative to control cells. These findings suggest that *ABO* transcription could be regulated by the downstream +22.6-kb site in an epithelial cell-specific manner.

Other Transcriptional Regulatory Regions

Initial luciferase reporter assays in KATOIII cells identified a positive regulatory region 3.8-kb upstream from the translation start site (shown in Fig. 2a) [42, 72]. The region comprises 4 tandem copies of a 43-bp repeat unit which was demonstrated to bind the positive transcription factor, CCAAT-binding factor/NF-Y, through the CCAAT motif. However, similar regulatory activity was not observed in the erythroleukemia cell lines HEL and K562 [37, 43], suggesting that this site is unlikely to be involved in the transcriptional regulation of *ABO* in erythroid cells. Genetic population studies revealed that both *B*- and *O*-alleles were linked to 4 tandem copies of the 43-bp repeat unit and that the A^1 allele was not linked to this tandem repetitive element [73, 74]. Seltsam et al. [75] observed unexpected variations in the CCAAT-binding factor/NF-Y enhancer region that includes the repeat units, in four individuals with weak B phenotypes suggesting that the sequence variations in the regulatory region might cause weak blood group B phenotypes. In contrast, Thuresson et al. [76] reported a hybrid allele between O^2 and *B* that lacked three repeat units, although the *B* transcript level was similar to that in fresh peripheral blood samples from normal controls. Interestingly, transcripts from A^1 and A^2 alleles could not be detected in peripheral blood but detected higher levels than transcripts from *B*- and *O*-alleles in CD34+ cells from healthy marrow donors [42]. Therefore, whether *ABO* transcription is influenced by the enhancer region in erythroid cells remains controversial.

A negative regulatory region from −307 to −151 relative to the translation start site was identified just upstream of the proximal *ABO* promoter by the initial luciferase reporter assays in KATOIII cells, suggesting that *ABO* transcription might be regulated by this negative element (shown in Fig. 2a) [77]. Although EMSAs revealed a nuclear factor from KATOIII cells bound to the region, the identity of the factor is unknown. Thus, significance of the negative regulatory region remains elusive. In addition to the described regulatory regions, genome annotation data and in vitro experiments including luciferase assays indicate a positive regulatory region 36-kb downstream from the translation start site (shown in Fig. 2a) [37, 58], but it have not yet been characterized.

Fig. 2. Schematics of transcriptional regulation of *ABO* expression. **a** Map of the 50-kb region of genomic DNA surrounding human *ABO* including structures of wild-type *ABO* and variants with a large deletion in intron 1. Vertical lines and squares indicate exons. In addition, blue squares indicate locations of CpG islands over the structure of *ABO*. Below the structure, gray square indicates the CCAAT-binding factor/NF-Y enhancer region, yellow square represents the proximal promoter, red square denotes the +5.8-kb site, green square indicates the +22.6-kb sites, and purple represents the DNase I hypersensitive site region +36.0. Pro, promoter: +5.8, the +5.8-kb site: +22.6, the +22.6-kb site: +36.0, region +36.0. Also shown are transcription factors binding to transcriptional regulatory regions. Below the genomic structure of the ordinary *ABO*, variant alleles with a large deletion including the +5.8-kb site are shown. V-shaped line represents deletion of the sequence. According to HGVS nomenclature using the nucleotide sequences of accession number NG_006669.1 and NM_020469.1 as a reference, $B^m3.0$ is represented as c.28+4077_7107del, $B^m5.8$ as c.28+5110_10889del, and $B^m5.9$ as c.28+5443_11354del, while the variant descriptions were +4105_+7136del [30], +5137_+10914del [37], c.28+5443_29-1655del [38], respectively, in the original paper. Five nucleotides flanking the deletions are shown. **b** Schematic illustration of the relationship among *ABO* and the genes around *ABO*. The top diagram represents the genomic regions including *OBP2B*, *ABO*, the +5.8-kb site, and the +22.6-kb site. The *ABO* exons are indicated by lines or a solid box, and the *OBP2B* exons are denoted by boxes. The middle diagram indicates genes around *ABO*. The bottom diagram represents the locations of CTCF-mediated chromatin interactions determined by chromatin interaction analysis with paired-end tag sequencing (ChIA-PET) data extracted from MCF-7 cells which were constructed using publicly available data from ENCODE Chromatin Interactions tracks (http://genome.ucsc.edu). Vertical lines at the ends of horizontal lines correspond to CTCF binding sites, so that TAD might be formed in the regions shown by horizontal lines.



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Epigenetic Regulation of Human *ABO*

As previously mentioned, DNA methylation of the *ABO* promoter may be involved in suppression of *ABO* transcription in malignant cells [50, 51]. Histone deacetylase inhibitors (HDACIs) such as sodium butyrate, panobinostat, vorinostat, and sodium valproate were observed to suppress *ABO* expression in K562 and KATOIII cells, leading to decreased expression of B-antigens on the surface of KATOIII cells [78, 79]. These findings suggest that *ABO* transcription might be regulated epigenetically.

ABO suppression by HDACIs might reduce the risk of acute humoral rejection directed against donor-oriented A/B-antigens on endothelial cells of liver arteries or bile ducts in ABO-incompatible (ABOi) liver transplantation [80]. As the most severe form of rejection can lead to graft loss, any adverse effects resulting from ABOi liver transplantation could potentially be ameliorated through decreased expression of A- or B-antigens on endothelial cells by use of HDACIs. Similarly, *ABO* suppression by HDACIs could potentially reduce the risk of developing venous thromboembolism and coronary heart disease, as genome-wide association studies have reported associations with the *ABO* locus and diseases such as venous thrombosis, coronary artery disease, gastric cancer, gastric and duodenal ulcers, norovirus infection, malaria infection severity, and severe COVID-19 [81–83]. The incidence of venous thrombosis is known to be lower in individuals with type O blood group compared to other blood types, whereas addition of A- or B-antigens to von Willebrand factor (vWF) has been suggested to increase its blood level. Therefore, it is possible that the risk of developing venous thromboembolism and coronary heart disease might be reduced by decreasing the A- or B-antigens in vWFs using HDACIs. Further research is needed to elucidate transcriptional regulation of the *ABO* gene and provide potential clinical applications.

Topologically Associating Domain around Human *ABO*

One major type of chromatin organization is a self-interacting domain termed the topologically associating domain (TAD) or contact domain [84–86]. In mammalian cells, TAD boundaries are usually demarcated by the chromatin architectural protein CCCTC-binding factor (CTCF) and cohesin. The TAD boundaries preferentially remain stable across cell types, while a small subset of boundaries show cell-type specificity. In addition, the two interacting DNA sites bound by the CTCF protein and occupied by the cohesin complex form chromosome loop structures: some TADs involve a single loop, while others include multiple loops. These loops frequently contain more than one gene, and a feature which could facilitate the co-regulation and co-expression of genes located within the same loop. Furthermore, enhancer sharing, in which a single enhancer affects the transcription of multiple genes, has been reported. On the basis that a topological domain boundary is characterized by binding of transcription factors CTCF and cohesin, the boundaries were identified at approximately 38-kb on the centromere side and 38-kb on the telomere side of exon 1 of *ABO* from the genome annotation data (shown in Fig. 2b). This internal 76-kb region was inferred to be a TAD formed around *ABO* [87]. The topological domain included *ABO* and the gene encoding Odorant Binding Protein 2B (OBP2B) located 66-kb on the centromere side of *ABO*. Consistent with this assumption, the expression of *ABO* and *OBP2B* was downregulated in KATOIII cells with biallelic deletion of the +22.6-kb site, suggesting that both genes were transcriptionally regulated by the +22.6-kb site in coordinated manner [87].

Genome annotation data suggested that the +22.6-kb site interacts with the transcription start site of the gene encoding ADAMTS-13, which is located 178.5-kb on the telomere side from the site (shown in Fig. 2b) [87]. ADAMTS-13 cleaves vWF, which is synthesized in the vascular endothelium and secreted into the bloodstream

Fig. 3. Alignment of variants within the regulatory regions in weak phenotypes. **a** Nucleotide sequence of the 5′-flanking region in *ABO*. Shown is the sequence from position −150 to c.+28 relative to the translation start ATG site of *ABO*. The upper-case letters denote the coding sequences of exon 1, and the lower case letters the noncoding genomic sequence. High arrows above the sequence indicate the transcription initiation sites that were determined by 5′-RACE using human pancreas cDNA by Yamamoto et al. [7], and low arrows denote the transcription initiation sites that were determined by in vitro erythroid culture of AC133-CD34+ cells [39]. The proximal *ABO* promoter is located between −150 and −2 relative to the ATG translation start site [42, 43]. The recognition motif for transcription factor Sp1 is indicated by an overbar. Variants in *ABO* promoter were found in individuals with A_3 and B_3: Nucleotide substitutions at −77, −76, −72, and −68 are indicated in red, and the deletion between −35 and −18 is denoted by an underbar. **b** Alignment of variants within the +5.8-kb site found in weak phenotypes. The top diagram represents the +5.8-kb site including a variant of the upstream GATA motif. The bottom diagram indicates the sequences between c.28+5829 and c.28+5891 in intron 1 of *ABO*. The motifs for transcription factors are indicated by overbars, whereas the putative C/EBP recognition motif has not been investigated. The nucleotide substitutions that were found in the weak phenotypes are shown in red, and the nucleotide deletion of 23-bp is indicated by horizontal lines in red. Those variants are described on the left side of the nucleotide sequence according to HGVS nomenclature using the nucleotide sequences of accession numbers NG_006669.1 and NM_020469.1 as a reference. The variant descriptions in the original reports and the corresponding references are shown on the right side of the sequence.

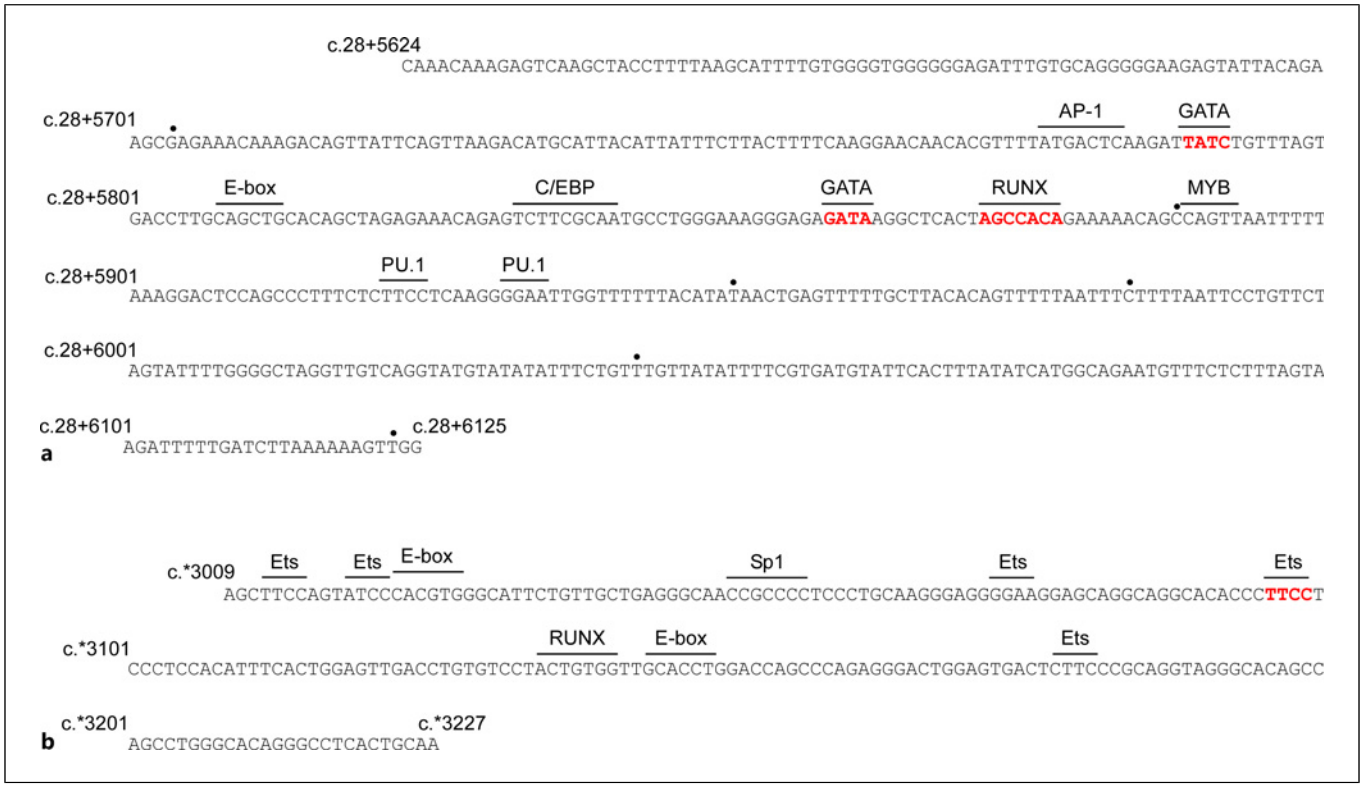


Fig. 4. Nucleotide sequences of the +5.8-kb and +22.6-kb sites. Nucleotide sequences of the +5.8-kb site and the +22.6-kb site are shown in panels **a** and **b**, respectively. The +5.8-kb site is located between c.28+5624 and c.28+6125, and the +22.6-kb site between c.*3009 and c.*3227. Positions of these nucleotides are described according to HGVS nomenclature using the nucleotide sequence accession numbers NG_006669.1 and NM_020469.1 as a reference. The sequence was derived from a haplotype *ABOInt1*01*, whereas dots over nucleotides indicate those with single nucleotide polymorphisms [57]. The motifs for several relevant transcription factors and E-box are indicated by overbars. The nucleotides in red were observed to be involved in binding to transcription factors such as GATA-1/2, RUNX1, and Elf5 [36–58].

as a macromolecule. vWF acts as a molecular glue by adhering and aggregating platelets to damaged subendothelial tissue. ADAMTS-13 cleaves vWF to an appropriate length, preventing its binding to platelets in the bloodstream. As described above, vWF levels in the circulation are influenced by the addition of A- or B-antigens [82]. Since the transcription start sites of *ABO* and *ADAMTS13* were reported to interact with the +22.6-kb site, it is plausible that transcription of both of these genes that are associated with regulation of vWF are regulated by the +22.6-kb site.

Relationship between *ABO* and *FUT1* Expression

Results from in vitro differentiation of CD34-positive cells into erythroid cells indicated that co-expression of *ABO* and *FUT1* could be crucial for synthesis of A- or B-antigens (shown in Fig. 5) [69]. Variants have been reported in the *ABO* promoter and in the +5.8-kb site in individuals with A_3 or B_3 blood types, in whom mixed field agglutination of RBCs is observed when exposed to anti-A or -B antibodies [3]. Based on this premise, Takahashi et al. [45] suggested that variants in those regulatory regions could result in decreased levels of transcript and of glycosyltransferase and could shorten the duration during which A- or B-transferase, as well as FUT1 concentrations, are adequate to promote synthesis of A- or B-antigens in sufficient quantities. Adequate levels of A- and B-antigens are required for hemagglutination by the corresponding antibodies, such that variation in RBC antigen expression could lead to mixed field agglutination of RBCs.

The tail of monkeys and uricase that converts uric acid to 5-hydroxyisourate are thought to have been abolished or inactivated during evolution of monkeys such as prosimians, New World monkeys and Old World monkeys to apes including chimpanzees, gorillas, and gibbons. Furthermore, the RBCs of monkeys appear to lack A- or B-antigens, whereas these antigens are expressed at lower levels in apes [88–90]. One putative mechanism leading to increased expression of the H-antigen on human RBCs is the insertion of a short interspersed nuclear element (SINE), one of several

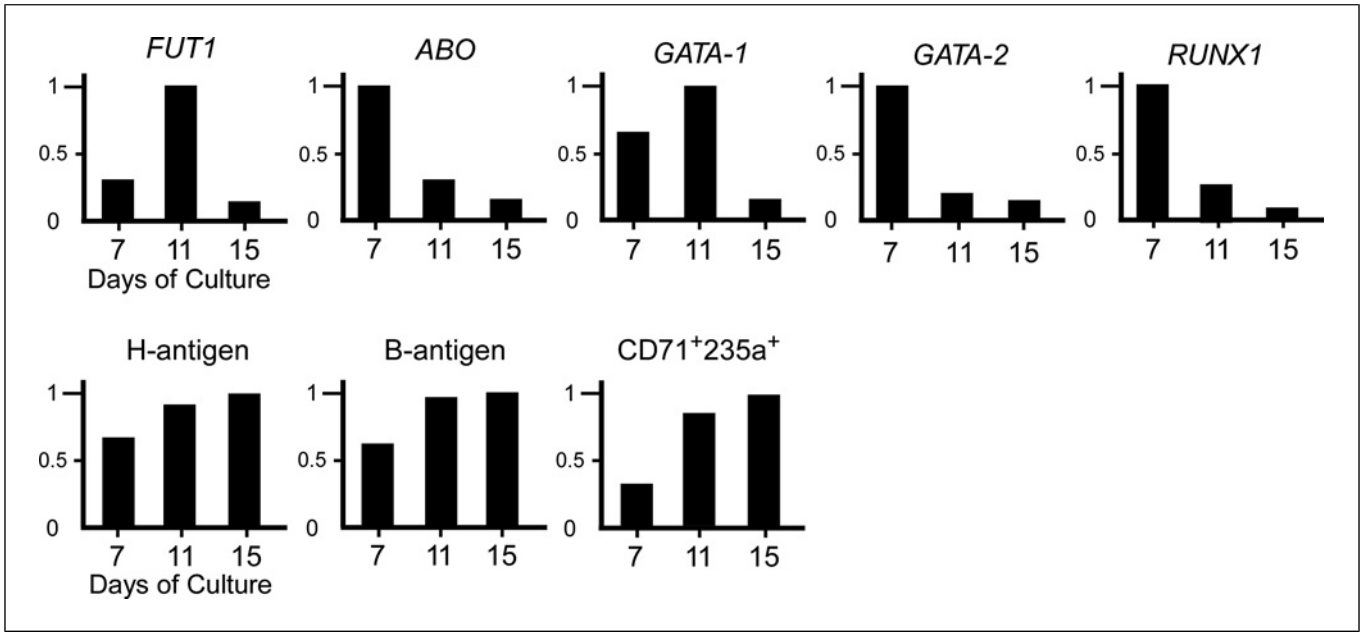


Fig. 5. Schematic illustration of the expression of cell surface antigens and genes during the course of in vitro erythroid cell differentiation. The diagrams were constructed using the expression of genes such as *FUT1*, *ABO*, *GATA-1*, *GATA-2*, and *RUNX1* as well as the cell surface expression of H-antigen and B-antigen 7, 11, and 15 days after in vitro erythroid cell differentiation from $CD34^+$ cells as previously reported [69]. The time course of the relative expression of individual genes or antigens is indicated. The maximum gene expression or cell population showing antigen expression during erythroid differentiation is expressed as 1.0 on the vertical axis, whereas the relative expression ratios at other time points are calculated for individual genes and antigens. Although the original report [69] did not show change in the ratios of cells expressing CD71 antigen and CD235 antigen as control, those are shown in the diagram.

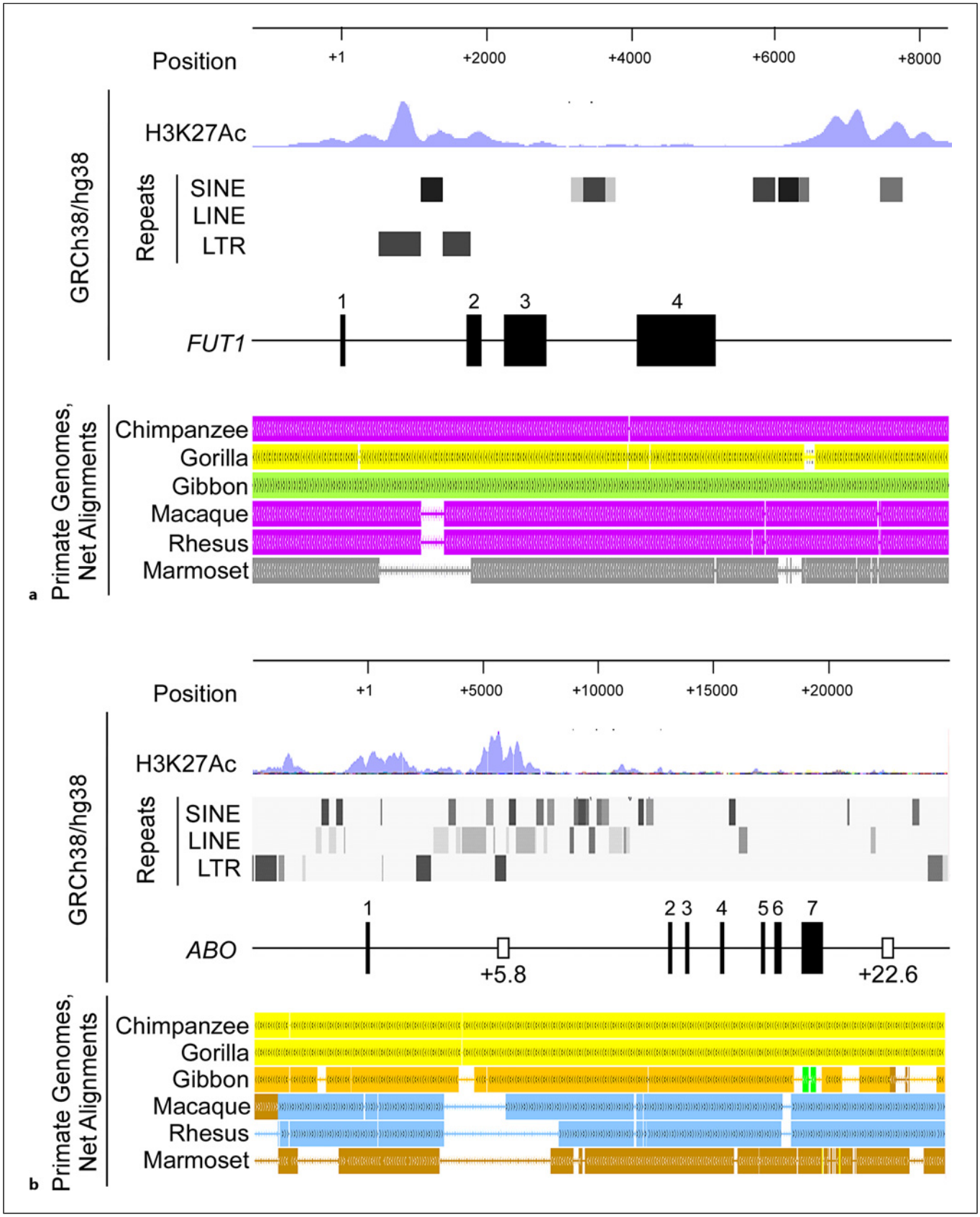
transposable elements, in the first intron of *FUT1* since the SINE was conserved in Hominoidea including humans and apes, but was absent from monkeys or non-primate mammals (shown in Fig. 6a) [91]. Moreover, comparison of the +5.8-kb site in humans with the corresponding sites in apes and monkeys indicated that the sites in Hominoidea contained a long terminal repeat (LTR), another transposable element, which was replaced with SINE in monkeys (shown in Fig. 6b) [92]. Thus, the SINE may have been replaced by LTR during the evolution of monkeys to apes, resulting in formation of the +5.8-kb site, transcription from *ABO* in cells of erythroid lineage, production of the glycotransferases, and emergence of A- or B-antigens on RBCs.

Human *FUT1* is located on the long arm of chromosome 19 at 19q13. *FUT1* is composed of four exons spanning 7.4 kb, although transcriptional regulation has not been delineated. However, ENCODE Candidates *Cis*-regulatory Elements and RepeatMasker indicated distal enhancer-like signatures in the LTRs flanking the SINE of the first intron in human *FUT1* (shown in Fig. 6a) [93]. The presence of many GATA motifs in the LTRs combined with observations from the in vitro experiments of differentiation of CD34-positive cells into erythroid cells, indicate that the regulatory potential of the LTRs might be dependent upon GATA-1. Since the +5.8-kb site includes the LTR which is involved in the regulation of *ABO* expression in cells of erythroid lineage, Sano et al. [92] suggested that LTRs could contribute to the *cis*-regulatory network thereby facilitating cooperative expression of the genes involved in the production of carbohydrate chains with A- or B-antigens at the non-reducing end. Further research on the relationship between the LTR at the +5.8-kb site of *ABO* and the LTRs flanking the SINE in intron 1 of *FUT1* is needed to clarify the mechanism underlying the expression network of genes involved in ABH-antigen production on human RBCs.

Molecular Basis Underlying Regulation of *ABO* Expression

Molecular Basis for Cell-Specific Expression of ABO

ABO transcription is regulated by a constitutive proximal promoter and a cell-specific regulatory region such as the +5.8-kb site or the +22.6-kb site (shown in Fig. 1a). Luciferase reporter assays and genetic studies showed that the erythroid cell-specific regulatory activity of the +5.8-kb site was dependent upon binding of the erythroid cell-specific transcription factor GATA-1 or



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(For legend see next page.)

2 [37]. Similarly, plasmid-based reporter assays demonstrated that the epithelial cell-specific regulatory activity of the +22.6-kb site was dependent upon binding of the epithelial cell-specific transcription factor Elf5 [58]. In fibroblasts not expressing GATA-1 or 2, or Elf5, it is plausible that absence of cell-specific regulatory activity at these sites leads to contributes to lack of *ABO* expression (shown in Fig. 1a). Therefore, it is likely that cell type-specific expression of *ABO* is dependent upon expression of cell-specific transcription factors that bind to the +5.8-kb or +22.6-kb site.

Molecular Basis for Cell Differentiation-Specific Expression of ABO

Although the in vitro differentiation of CD34+ cells into erythroid cells indicated that the downregulation of *ABO* expression might be attributed to a decline of RUNX1 and GATA-2 in the later phase of erythroid differentiation [56], the mechanism of *ABO* expression at an early stage of erythroid differentiation remains to be explored.

Molecular Basis for Weak Blood Group Phenotypes That Lack Variants in the Coding Region and Splicing Site

Many variants found in the regulatory regions for *ABO* transcription appeared to be responsible for weak phenotypes. However, there are rare individuals with weak phenotypes in whom no variants have been found in the coding region, splicing sites, or regulatory regions for *ABO* transcription [65]. Therefore, more research is required to elucidate the complete mechanism of transcriptional regulation of *ABO*.

The prevalence of weak ABO phenotypes was reported to be 0.048% among transfusion donors in Tokyo, Japan, while the total occurrence of the B_m and A_1B_m subgroups was 0.024% [29]. Genetic analysis using serological procedures and sequence-specific PCR targeting *B*^{m}*5.8* demonstrated *B*^{m}*5.8* in 1,300 individuals, c.28+5861T>G in two, and *B*^{m}*3.0* in one individual among 1,303 Japanese with B_m and A_1B_m [94]. Thus, it is plausible that *B*^{m}*5.8* might have been inherited over a long period of time and spread throughout the Japanese population. Since *B*^{m}*5.8* has not been reported in Korea and China, from where ancient people migrated to Japan, it is possible that *B*^{m}*5.8* could be specific to the Japanese population [95].

Molecular Basis for A- or B-Antigen Reduction in Malignant Cells

As previously described, absence of A- or B-antigens in cancer cells was attributed to gene deletion, promoter methylation, and unknown causes. Additionally, reduction of A- or B-antigens on RBCs in patients with leukemia may be caused by *ABO* promoter methylation as well as mutations in RUNX1 and GATA-2. Further research is needed to delineate the comprehensive molecular basis for A- or B-antigen reduction in malignant cells.

Prospects

Although numerous studies have characterized the relationship between ABO blood group antigens and diseases, the physiological significance of ABO blood groups remains vague. In addition, it is unclear why *ABO* has been conserved throughout evolution, although the *O*-allele encodes a protein that lacks transferase activity. One hypothesis is that the protein encoded by the *O*-allele could possess a critical function within organisms that haves not yet been discovered. Alternatively, the regions surrounding *ABO* could be critical for life or survival of a species. As described above, the +22.6-kb site might regulate *ABO* and *OBP2B* in a coordinated manner. OBP2B, expressed in the prostate and mammary gland [96] is part of the lipocalin protein which is known to bind to small molecules such as odorants and fatty acids [97]. Although its physiological role has not been delineated, it seems likely that OBP2B plays an important physiological role in reproduction and lactation. Based on this crucial proposed biological role of OBP2B in organisms, Sano et al. [87] suggested that the gene and its regulatory regions may have been conserved during evolution and that *ABO* near the regulatory region may also have been

Fig. 6. Homology of nucleotide sequences from the upstream to downstream regions of *ABO* or *FUT1* between human and non-human primates. **a** Human *FUT1*. **b** Human *ABO*. In the diagrams, upper panel shows positions relative to the transcription start site of *FUT1* or *ABO*. The second panel from the top indicates the acetylation at lysine 27 of histone 3 in *FUT1* or *ABO*, often found near active regulatory elements, which was demonstrated by ENCODE Regulation Tracks. The third panel from the top denotes repeating elements including SINE, LINE, and LTR, which were revealed by RepeatMasker over the genomic structure of *FUT1* or *ABO*. The fourth panel shows the genomic structure from the upstream region through *FUT1* or *ABO* to the downstream region, including exons, as well as regulatory regions such as the +5.8- and +22.6-sites for erythroid cells and epithelial cells, respectively, on the basis of the human genome draft GRCh38/hg38. Exons are denoted by filled boxes, and the regulatory regions are indicated by empty boxes. The fifth panel shows a comparison of the human genome sequences with their reference sequences in non-human primates including chimpanzee, gorilla, gibbon, crab-eating macaque, rhesus macaque, and marmoset using Primate Genomes, Chain and Net alignments [93].

conserved. However, elucidating the physiological significance of ABO blood groups and the reason that *ABO* has been conserved remain challenges for future research.

Conflict of Interest Statement

The authors have no conflicts of interest to declare.

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Author Contributions

K.O., R.S., and Y.K. conceived, designed, and wrote the paper.

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