GATA2 regulates differentiation of bone marrow-derived mesenchymal stem cells

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ABSTRACT

The bone marrow microenvironment comprises multiple cell niches derived from bone marrow mesenchymal stem cells. However, the molecular mechanism of bone marrow mesenchymal stem cell differentiation is poorly understood. The transcription factor GATA2 is indispensable for hematopoietic stem cell function as well as other hematopoietic lineages, suggesting that it may maintain bone marrow mesenchymal stem cells in an immature state and also contribute to their differentiation. To explore this possibility, we established bone marrow mesenchymal stem cells from GATA2 conditional knockout mice. Differentiation of GATA2-deficient bone marrow mesenchymal stem cells into adipocytes induced accelerated oil-drop formation. Further, GATA2 loss- and gain-of-function analyses based on human bone marrow mesenchymal stem cells confirmed that decreased and increased GATA2 expression accelerated and suppressed bone marrow mesenchymal stem cell differentiation to adipocytes, respectively. Microarray analysis of GATA2 knockdowned human bone marrow mesenchymal stem cells revealed that 90 and 189 genes were upregulated or downregulated by a factor of 2, respectively. Moreover, gene ontology analysis revealed significant enrichment of genes involved in cell cycle regulation, and the number of G1/G0 cells increased after GATA2 knockdown. Concomitantly, cell proliferation was decreased by GATA2 knockdown. When GATA2 knockdowned bone marrow mesenchymal stem cells as well as adipocytes were co-cultured with CD34-positive cells, hematopoietic stem cell frequency and colony formation decreased. We confirmed the existence of pathological signals that decrease and increase hematopoietic cell and adipocyte numbers, respectively, characteristic of aplastic anemia, and that suppress GATA2 expression in hematopoietic stem cells and bone marrow mesenchymal stem cells.

Introduction

Bone marrow mesenchymal stem cells (BM-MSC) are self-renewing precursor cells that differentiate into bone, fat, cartilage, and stromal cells of the bone marrow, thereby forming a microenvironment that maintains hematopoietic stem cells.[1] Accumulating evidence indicates the importance of the bone marrow microenvironment during hematopoietic cell development. Increased adipogenesis in the bone marrow negatively affects hematopoietic activity,[2,3] whereas the osteoblastic niche supports hematopoietic stem cell function by activating Notch signaling.[4] Therefore, precise regulation of BM-MSC differentiation into various lineages maintains hematopoiesis.

Preadipocytes derived from MSC mature into adipocytes through a complex process involving numerous extracellular factors as well as transcription factors.[1,5] Studies conducted on preadipocyte cell lines, such as mouse 3T3-L1 and 3T3-F442A, have uncovered the CCAAT/enhancer binding protein (C/EBP) family of transcription factors and the peroxisome proliferator-activated receptor γ (PPARγ) as key proadipogenic regulators.[6,7] During preadipocyte–adipocyte differentiation, the expression of C/EBPβ and C/EBPδ initially increases, which subsequently activates the expression of C/EBPα and PPARγ, leading to the induction of genes involved in adipocyte function.[8,9] However, the mechanism of differentiation of BM-MSC into adipogenic progenitors and ultimately into mature adipocytes in the bone marrow remains to be elucidated.

GATA2, a transcription factor critically required in the genesis and/or function of hematopoietic stem cells (HSC),[10-13] is expressed in various hematopoietic and non-hematopoietic tissues, including HSC, multipotent hematopoietic progenitors, erythroid precursors, megakaryocytes, eosinophils, mast cells, endothelial cells, and specific neurons.[11,12,14-16] GATA2 is expressed by preadipocytes and BM-MSC and plays a central role in the control of adipogenesis.[13,16,17] GATA2 overexpression in a mouse preadipocytic stromal cell line induces resistance to adipocyte differentiation, whereas GATA2 knockdown accelerates adipocyte differentiation,[17] implying that GATA2 functions to arrest preadipocyte differentiation. Although GATA2 may suppress transcription of *C/EBP* and *PPARγ* in preadipocytes,[16,18] the molecular mechanism by which GATA2 controls adipocyte differentiation remains unclear.

Aplastic anemia is characterized by decreased HSC and fatty marrow replacement. Moreover, GATA2 expression is decreased in CD34-positive cells in aplastic anemia,[19,20] Because BM-MSC express GATA2, it is possible that the signal that downregulates GATA2 expression in HSC may also suppress its expression in BM-MSC in aplastic anemia, thereby resulting in fewer HSC and an impaired microenvironment, which could support hematopoiesis. To test this hypothesis, we assessed the role of GATA2 during differentiation from BM-MSC.

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Methods

Generation of bone marrow mesenchymal stem cells

To generate mouse BM-MSC, bone marrow cells from GATA2 conditional knockout mice were cultured in MesenCult MSC Basal Medium supplemented with 20% MSC stimulatory supplements (Stem Cell Technologies). The BM-MSC were transfected with the retroviruses expressing iCre to delete the DNA binding domain of GATA2 by inducing the Cre-loxP system.[21,22]

To generate human BM-MSC, bone marrow mononuclear cells from healthy donors were cultured with Dulbecco's modified Eagle's medium (Life Technologies) supplemented with 20% fetal bovine serum (Life Technologies), 10 ng/mL basic fibroblast growth factor (PeproTech), 10 mM HEPES (Life Technologies), and 100 μg/mL penicillin/streptomycin (Invitrogen).[23-25] Established BM-MSC were used until the seventh generation.

The study was approved by the ethical committee of Tohoku University Graduate School of Medicine. Clinical samples were collected after obtaining written informed consent. The ethics policies of the Declaration of Helsinki were followed.

Characterization of bone marrow mesenchymal stem cells

BM-MSC immunophenotypes were determined using a FACSAria II (BD). To induce differentiation into adipocytes, human Mesenchymal Stem Cell Adipogenic Differentiation Medium (Lonza) was used. After 12–16 days, morphological changes were assessed using an inverted microscope. Typical adipocytes were stained with Oil Red O.[2] The area of mature adipocytes was determined using HistoQuest software (Novel Science).

Quantitative reverse transcriptase polymerase chain reaction analysis and transcription profiling

Quantitative reverse transcriptase polymerase chain reaction analysis (RT-PCR) was performed as previously described.[26] Primer sequences are available upon request.

For transcription profiling, the Human Genome U133 Plus 2.0 Array was used (Affymetrix). Gene ontology analysis was conducted using the DAVID bioinformatics program (*http://david.abcc.ncifcrf.gov/*).

Short interfering RNA-mediated knockdown

Anti-GATA2 and control short interfering RNA (siRNA)[26] were transfected into human BM-MSC with Lipofectamine™ RNAiMAX reagent (Life Technologies). Cells were analyzed 48 h after transfection.

Viral vectors and cell transduction

Retroviral overexpression of GATA2 was performed using the MSCV retrovirus vector, which co-expresses green fluorescent protein (GFP) by internal ribosome entry sites (IRES), transfecting into Platinum Retroviral Packaging Cell Lines (PLAT-F)[27] with FuGENE HD (Roche). Human BM-MSC were pretreated with Retronectin (TAKARA BIO.), and GFP-positive cells were sorted using FACSAria II (BD Biosciences).

Co-culture of CD34-positive-enriched cells with a mesenchymal stem cell feeder layer

BM-MSC were transfected with control or GATA2-siRNA. On day 3, control and GATA2 knockdowned BM-MSC, respectively, were replaced with serum-free medium containing CD34-positive-enriched cells (RIKEN). Serum-free medium (StemPro-34 SFM: Life Technologies) contained 100 ng/mL stem cell factor, 100 ng/mL interleukin (IL)-3, and 25 ng/mL granulocyte-monocyte colony-stimulating factor (Peprotech). The cells were co-cultured for 7 days, and subsequently harvested and analyzed with FACSAria II (BD).

Colony-forming cell assay

CD34 positive-enriched cells, co-cultured with BM-MSC for 7 days, were seeded into semisolid culture (MethoCult™ H4435, Stem Cell Technologies). After 14 days, colony-forming units were counted.

Cell proliferation and cell cycle analysis

The total number of viable cells was determined by a colorimetric method using MTS (3-4,5-dimethylthiazol-2-yl-5-3-carboxymethoxyphenyl-2-4-sulfophenyl-2H-tetrazolium, inner salt; CellTiter 96). Absorbance at 490 nm was measured with an iMark microplate reader (Bio-rad). For cell cycle analysis, cells were fixed in ice-cold 70% ethanol and stained with 20 μg/mL propidium iodide (Sigma), 0.2 mg/mL RNase (Sigma), and 0.1% Triton X-100 (Sigma). DNA content was determined using FACSAria II and FlowJo software (*http://www.flowjo.com/*).

Statistical analysis

Statistical significance was assessed using a two-sided Student *t*-test.

Results

Acceleration of adipocyte differentiation in mesenchymal stem cells from GATA2 knockout mice

We first generated BM-MSC from bone marrow cells of conditional GATA2 knockout mice (GATA2^{fl}), in which the DNA binding domain of GATA2 (exon 5 encoding the C-terminal zinc-finger motif) could be deleted by inducing the Cre-loxP system (GATA2–) (*Online Supplementary Figure S1A*). We confirmed that GATA2^{fl} BM-MSC retained the potential to differentiate into adipogenic lineages (*Online Supplementary Figure S1B*). Flow cytometric analysis confirmed the characteristic immunophenotype,[28] showing that GATA2^{fl} BM-MSC expressed CD29, CD44 and Sca-1 but not markers such as CD11b, CD34 and CD45 (*Online Supplementary Figure S1C*).

To determine whether the loss of GATA2 influenced the BM-MSC phenotype, the DNA-binding domain of GATA2 was deleted using the Cre-loxP system, and GATA2 knockout–BM-MSC (GATA2^{-} BM-MSC) were generated. Quantitative RT-PCR analysis revealed that *Gata2* expression was significantly decreased in the GATA2^{-} MSC, implying that iCre-mediated deletion of the GATA2 C-finger resulted in decreased GATA2 autoregulation (Figure 1A). When GATA2^{fl} and GATA2– BM-MSC were exposed to adipogenic differentiation stimuli, we observed an overall increase in the expression of *Cebpa* (CEBPα), *Pparg* (PPARγ), and *Fabp4* (aP2) in GATA2^{-} BM-MSC (Figure 1B). Moreover, the expression of these genes peaked during days 8–12 of differentiation and then dropped to levels similar to those of control cells (Figure 1B), whereas the expression level of *Cebpb* (CEBPβ) was slightly higher in GATA2^{-} BM-MSC at the early (day 4) and last (day 16) stages of differentiation (Figure 1B). Furthermore, oil drop formation was markedly increased in GATA2^{-} MSC (Figure 1C). These results suggest that loss of GATA2 function induces the expression of adipogenic factors and adipocyte differentiation of BM-MSC.

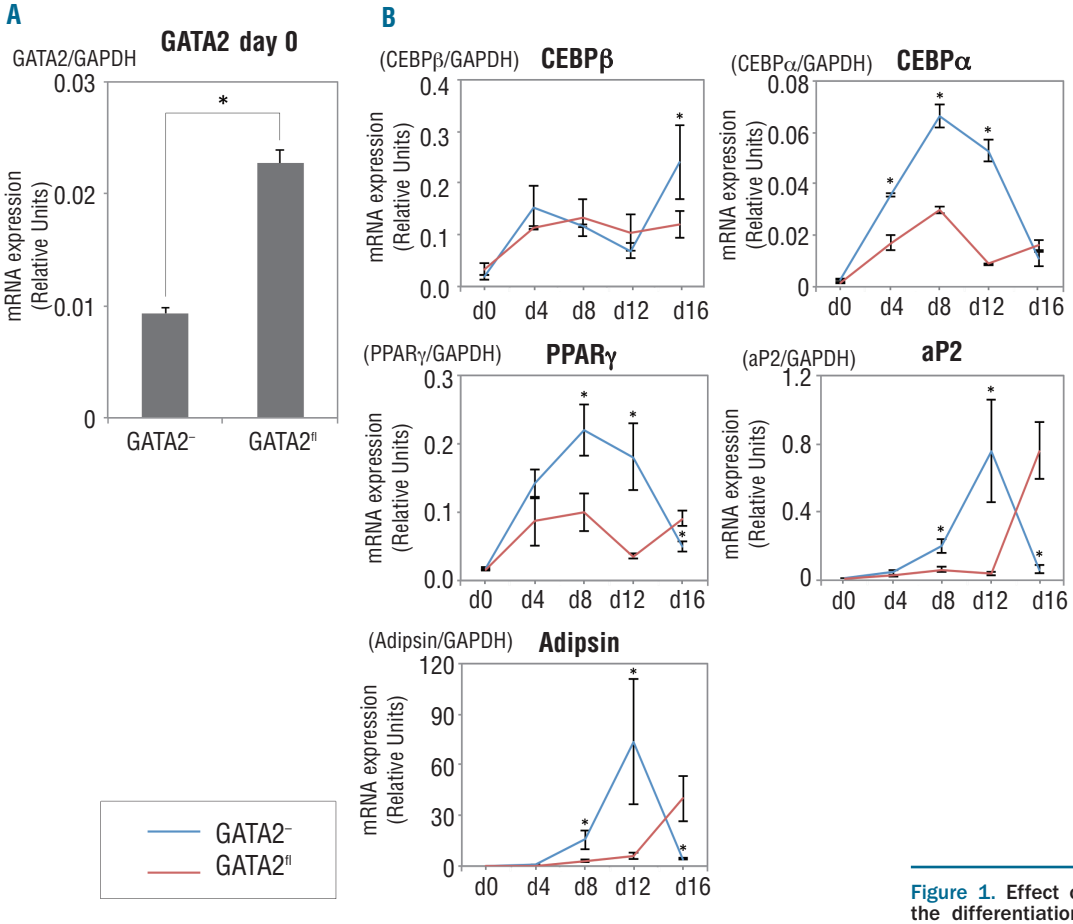
Generation and characterization of human bone marrow mesenchymal stem cells

Next, to elucidate the role of GATA2 in the context of

human BM-MSC differentiation, we generated BM-MSC from human mononuclear cells derived from bone marrow samples. We confirmed that BM-MSC differentiated into the adipogenic lineage (*Online Supplementary Figure S2A*). Flow cytometric analysis further confirmed the characteristic immunophenotype,[24,29,30] showing that the BM-MSC expressed CD29, CD44, CD90 and CD105 but not CD14, CD34, and CD45 (*Online Supplementary Figure S2B*).

Short interfering RNA-mediated GATA2 knockdown promotes differentiation of human bone marrow mesenchymal stem cells into adipocytes

To determine whether GATA2 regulates adipocyte differentiation in human BM-MSC, we suppressed GATA2 expression using a specific siRNA. Control or GATA2-siRNA were transfected into human BM-MSC 48 h before inducing adipocyte differentiation. We demonstrated that GATA2 mRNA levels were significantly decreased on day 0 and during adipocyte differentiation until day 8 (Figure 2A-B). Thereafter, we analyzed the expression of key adipocyte-specific genes at various time-points during adipocyte differentiation. The levels of expression of *C/EBPα*, *PPARγ* and *aP2* were significantly increased in the GATA2-knockdowned cells (Figure 2B). Furthermore, oil drop formation on day 12 was significantly increased in the GATA2 knockdown cells, as determined based on the Oil Red O staining-positive area (Figure 2C-D). These findings were consistent with the results for GATA2-deficient murine BM-MSC (Figure 1).



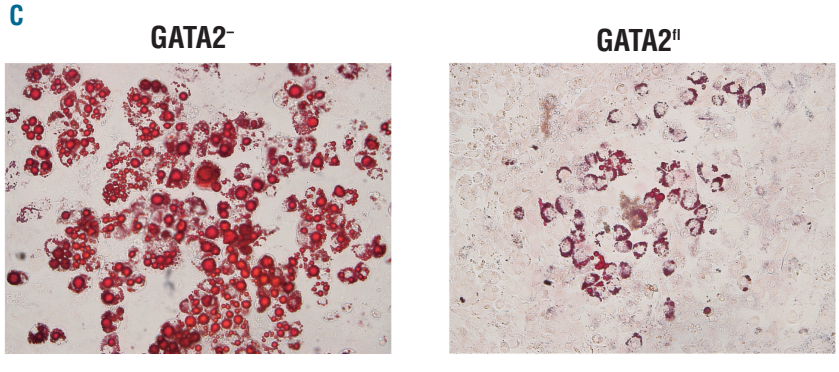


Figure 1. Effect of GATA2 depletion on the differentiation of murine MSC into adipocytes. GATA2-depleted BM-MSC (GATA2⁻) were generated by inducing iCre into the BM-MSC, established from GATA2 conditional knockout mice (GATA2fl). Each BM-MSC was then differentiated into the adipocyte lineage for up to 16 days. The data are representative of two independent BM-MSC lines. (A, B) Quantitative RT-PCR analysis for Gata2 expression on day 0 (A) and adipocyte-specific genes during adipocyte differentiation (B). The expression of each target gene relative to that of *Gapdh* was calculated. The data are expressed as mean ± standard deviation, SD (n = 3). **P* < 0.05. (C) Oil Red O staining on day 16. The picture is a representative example of at least three independent experiments.

GATA2 overexpression suppresses differentiation of human bone marrow mesenchymal stem cells into adipocytes

We overexpressed GATA2 in human BM-MSC using MSCV-GFP-IRES. After transfecting GATA2-expressing or control retroviruses, GFP-positive cells were sorted. Quantitative RT-PCR assay confirmed GATA2 overexpression (Figure 3A, B). When these cells were differentiated into adipocytes, the levels of expression of *C/EBPα*, *PPARγ*, *aP2* and *Adipsin* were significantly diminished by GATA2 overexpression (Figure 3B). Concomitantly, oil drop formation on day 12 was also significantly decreased in cells overexpressing GATA2 (Figure 3C,D).

Taken together, our data suggest that decreased GATA2 expression by human BM-MSC accelerates adipocyte differentiation, whereas GATA2 overexpression suppresses adipocyte differentiation.

Enrichment of cell cycle regulatory genes based on transcriptional profiling to identify GATA2-regulated genes in human bone marrow mesenchymal stem cells

To identify GATA2-target genes in BM-MSC, we conducted comprehensive expression profiling of BM-MSC transfected with control or GATA2-siRNA. Inhibition of *GATA2* expression was confirmed based on the profiling data as well as quantitative RT-PCR analysis (0.000104 ± 0.000008 and 0.000198 ± 0.000022, for GATA2 siRNA and control siRNA, respectively, $P<0.05$) (Table 1, *Online Supplementary Table S1*, *Online Supplementary Figure S3*). Based on the average of two independent datasets, we demonstrated that GATA2 knockdown activated and repressed 90 and 189 genes (> 2-fold), respectively (Table 1, *Online Supplementary Table S1*). The analysis revealed the differential expression of cell-cycle regulators (*CHEK1*, *CCNB1*, *CCNB2*, *GTSE1*, and *CDC20*), adhesion molecules (*LAMP1* and *CD44*), as well as

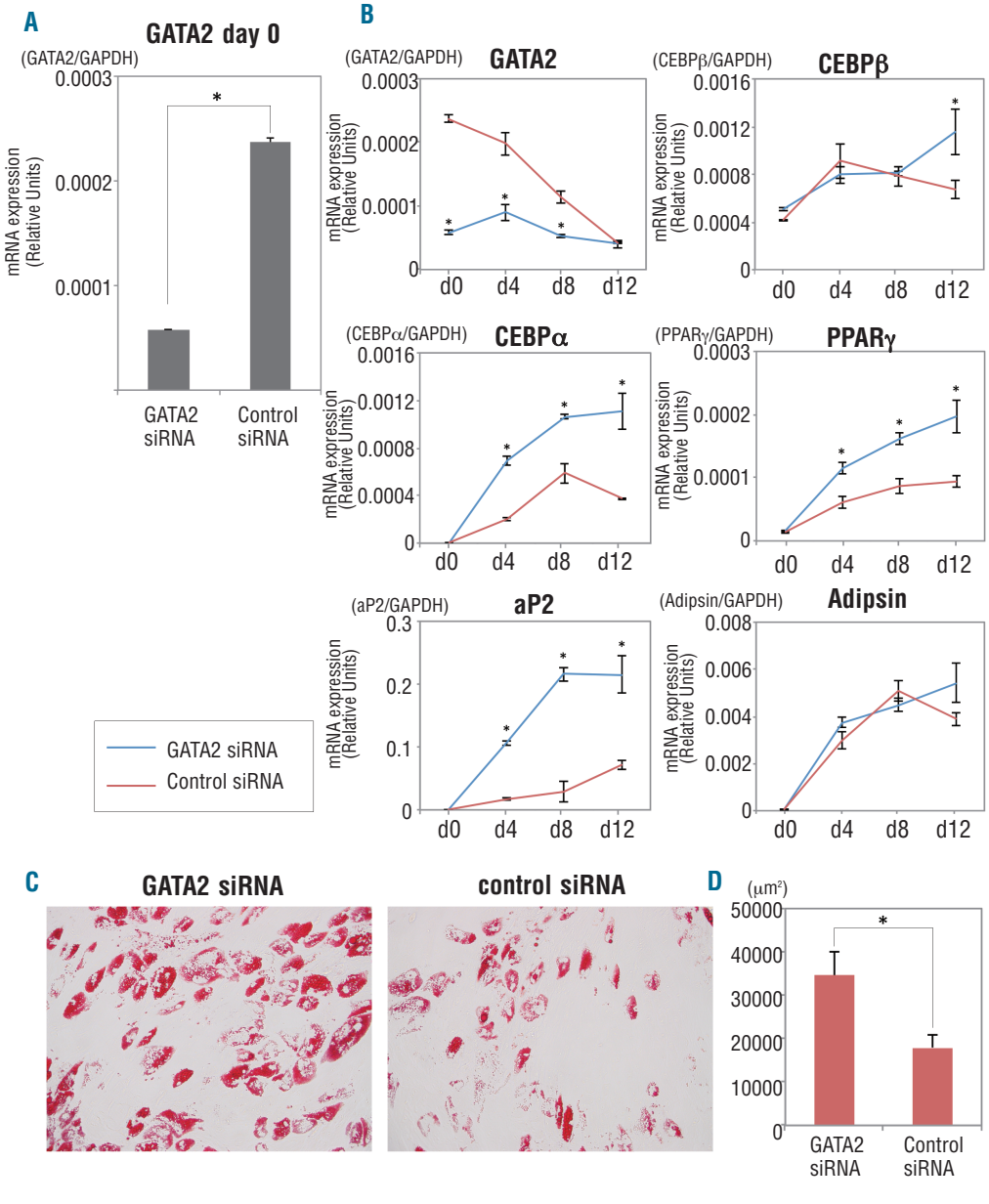


Figure 2. Effects of GATA2 knockdown on the differentiation of human MSC into adipocytes. GATA2 expression was suppressed in human BM-MSC by transfecting them with a human GATA2-siRNA, and the cells were induced to differentiate into the adipocyte lineage for up to 12 days. The data are representative of three independent BM-MSC lines. (A, B) Quantitative RT-PCR analysis for GATA2 expression on day 0 (A) and adipocyte-specific genes during adipocyte differentiation (B). The expression of each target gene relative to that of *GAPDH* was calculated. (C, D) Oil Red O staining (C) and the area of mature adipocytes (D). The data are expressed as mean ± SD (n=3). *$P<0.05$.

ENPP1, which regulate osteoblastic differentiation (Table 1).[31] In contrast, and unexpectedly, adipocyte-related genes were not detected. Gene ontology analysis revealed significant enrichment of genes related to "cell cycle" (P=8.6x10^{-11}) and "protein modification" (P=2.6 x10^{-3}; Table 2).

As described above, we identified decreased expression of various cell cycle regulatory genes after GATA2 knockdown. Previous studies of hematopoietic cells have suggested that GATA2 expression varies during the cell cycle and that GATA2 regulates cell-cycle regulators.[32,33] We, therefore, evaluated whether the cell cycle was altered in BM-MSC in which GATA2 expression was inhibited. The number of cells present in G1/G0 was significantly increased when GATA2 expression was decreased (Figure 4), which was due to the significantly increased proportion of cells in the G1 phase (*Online Supplementary Figure S4*). We further confirmed that cell proliferation was decreased by decreased GATA2 expression (Figure 5). These results suggest that GATA2 has an important role in BM-MSC proliferation by regulating cell-cycle regulators.

Reduced hematopoietic support of human bone marrow mesenchymal stem cells by GATA2 knockdown

Although BM-MSC differentiate into various cell types that form the hematopoietic microenvironment, BM-MSC themselves can support HSC.[34] To determine whether the ability of BM-MSC to support HSC was compromised by decreasing GATA2 expression levels, we co-cultured cord blood-derived CD34-positive cells with BM-MSC that were transfected with control or GATA2 siRNA. After co-culture of CD34-positive cells with the BM-MSC, the HSC fraction was isolated using the gating strategy of the International Society of Hematotherapy and Graft Engineering (ISHAGE) (Figure 6A).[35,36] The frequency of CD34-positive cells on day

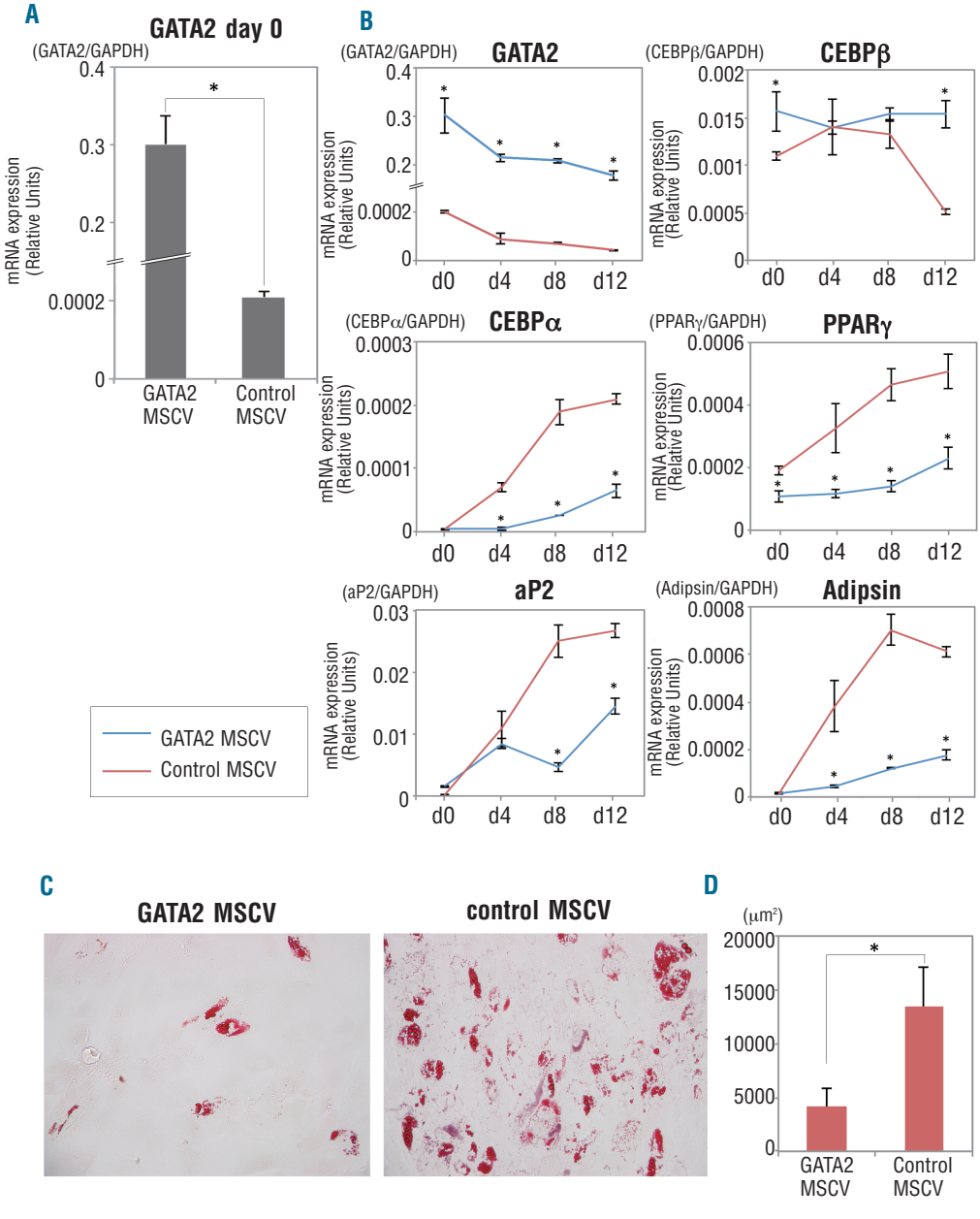


Figure 3. Effect of GATA2 overexpression on the differentiation of human MSC into adipocytes. GATA2 was overexpressed in human MSC using the MSCV retroviral vector system, and the cells were induced to differentiate into the adipocyte lineage for up to 12 days. The data are representative of three independent BM-MSC lines. (A, B) Quantitative RT-PCR analysis for GATA2 expression on day 0 (A) and adipocyte-specific genes during adipocyte differentiation (B). The expression of each target gene relative to that of *GAPDH* was calculated. *P<0.05. (C, D) Oil Red O staining (C) and the area of mature adipocytes (D). The data are expressed as mean ± SD (n=3). *P<0.05.

Table 1. List of GATA2-regulated genes in human BM-MSC.

Upregulated		Downregulated			
Fold change	Gene symbol	Fold change	Gene symbol	Fold change	Gene symbol
5.57	*SLC40A1*	0.19	*PTMA*	0.39	*LOC100506248*
5.09	*CACNA2D3*	0.22	*PGK1*	0.39	*KIF14*
4.58	*SLC16A6*	0.23	*ENPP1*	0.39	*LRRFIP1*
4.32	*IGF1*	0.24	*HJURP*	0.39	*MGAT1*
4.25	*CRYGS*	0.25	*STC1*	0.39	*TERF2IP*
3.83	*LOC100506119*	0.26	*CCNA2*	0.39	*MLF1IP*
3.74	*EGR3*	0.27	*CEP55*	0.39	*WHSC1*
3.61	*GPX3*	0.27	*AURKA*	0.39	*SQRDL*
3.41	*BEX1*	0.28	*MCM10*	0.40	*TMOD2*
3.39	*IL8*	0.28	*TOP2A*	0.40	*PRC1*
3.25	*MEGF10*	0.28	*ACAT1*	0.40	*BIRC5*
3.23	*IGFBP5*	0.29	*NSF*	0.40	*ARL5A*
3.18	*CHIC1*	0.29	*CDC6*	0.40	*CCNB1*
3.10	*GRIA1*	0.29	*CRIPT*	0.40	*RPL36*
3.04	*PTBP2*	0.29	*CNIH4*	0.40	*FANCI*
3.03	*TECPR2*	0.29	*AP2M1*	0.40	*CD44*
3.01	*NRG1*	0.29	*DTL*	0.40	*CCNE2*
2.99	*SLC44A1*	0.29	*ARG2*	0.41	*PFKFB4*
2.98	*FLRT3*	0.29	*KIF3B*	0.41	*LAMB1*
2.96	*PRL*	0.30	*ZHX1*	0.41	*PRIM1*
2.94	*LPAR6*	0.30	*C11orf82*	0.41	*PTBP1*
2.92	*C3AR1*	0.30	*PRELID1*	0.41	*DYNLT3*
2.85	*PTPRD*	0.30	*CPS1*	0.41	*SLC30A5*
2.84	*MAMDC2*	0.30	*DEPDC1*	0.42	*PRKAR2A*
2.76	*CHST15*	0.31	*TPX2*	0.42	*PRPS1*
2.75	*ZNF302*	0.31	*SPC25*	0.42	*TMPO*
2.74	*NKX3-2*	0.31	*HIST1H4A*	0.42	*MON1B*
2.69	*TPM1*	0.31	*SOCS2*	0.42	*NUF2*
2.69	*GRIA3*	0.32	*CDC20*	0.42	*OIP5*
2.67	*NOV*	0.32	*PLK4*	0.42	*DKFZP434I0714*
2.65	*EPHA5*	0.32	*GINS2*	0.42	*TGOLN2*
2.63	*TPM2*	0.32	*KIF11*	0.42	*KIF15*
2.62	*LCTL*	0.32	*C19orf10*	0.42	*RACGAP1*
2.62	*CDKN1C*	0.33	*CENPI*	0.42	*NADK*
2.60	*ADRA2A*	0.33	*HELLS*	0.42	*GCNT1*
2.60	*PDGFD*	0.33	*ROCK2*	0.43	*MTMR4*
2.60	*FNDC4*	0.33	*TMED10*	0.43	*CEBPG*
2.59	*TNPO1*	0.33	*CEP128*	0.43	*CDCA7L*
2.58	*CNTNAP3*	0.33	*CAND1*	0.43	*GTSE1*
2.58	*DEPTOR*	0.33	*SHCBP1*	0.43	*KIF4A*
2.56	*LINC00086*	0.33	*HSPA9*	0.43	*GGCX*
2.55	*SFRP1*	0.33	*C2orf18*	0.43	*TUG1*
2.55	*C12orf76*	0.33	*PXDN*	0.43	*CDT1*
2.54	*AOX1*	0.33	*ASPM*	0.43	*RNF41*
2.53	*EFHC2*	0.33	*PBK*	0.43	*CENPF*
2.52	*GDPD1*	0.33	*APOBEC3G*	0.43	*RPA1*
2.51	*LOC100653132*	0.34	*STX12*	0.43	*FOXM1*
2.51	*FAM135A*	0.34	*LRRFIP1*	0.44	*RBM45*
2.50	*SGCD*	0.34	*HMMR*	0.44	*SLC39A6*
2.48	*MTHFD2L*	0.34	*NCAPG*	0.44	*FGFR1OP2*
2.48	*ABCA8*	0.34	*RRM2*	0.44	*LEPREL1*
2.47	*KIAA0226L*	0.34	*NEK2*	0.44	*MKI67*
2.47	*NR2F1*	0.34	*FAM176B*	0.44	*HMGB2*
2.47	*CEP19*	0.35	*PRR11*	0.44	*PDE8A*
2.44	*PTPN22*	0.35	*BUB1*	0.44	*IRAK1*
2.43	*PDLIM7*	0.35	*CDCA3*	0.44	*PLEKHA3*
2.42	*PDK4*	0.35	*CDCA2*	0.44	*RFC3*
2.41	*EGR1*	0.36	*DYNC1LI2*	0.44	*CCNB2*
2.39	*SPON1*	0.36	*RAD51AP1*	0.44	*ZNF395*
2.38	*SIPA1L2*	0.36	*SPTLC1*	0.44	*CD164*
2.38	*HLA-DRA*	0.36	*UBE2C*	0.44	*TTK*
2.36	*COL24A1*	0.36	*ATG3*	0.44	*TK1*
2.34	*NME5*	0.36	*G3BP2*	0.44	*XYLT1*
2.34	*TTN*	0.36	*BUB1B*	0.44	*UHRF1*
2.33	*ACVR2A*	0.36	*CCBE1*	0.44	*NCOR1*
2.33	*LOC100505971*	0.36	*DPM2*	0.45	*CCDC50*
2.29	*ABCC3*	0.36	*FBN2*	0.45	*ATL3*
2.28	*MYL12A*	0.36	*MAD2L1*	0.45	*CIB1*
2.25	*KIAA0895*	0.37	*DNAJC30*	0.45	*PRDX6*
2.25	*EIF5A2*	0.37	*CASC5*	0.45	*SGTB*
2.24	*NCALD*	0.37	*WDR76*	0.46	*NT5E*
2.22	*HS2ST1*	0.37	*NUSAP1*	0.46	*ADAMTS5*
2.22	*MMP16*	0.37	*ELL2*	0.46	*CDKN3*
2.21	*CTSC*	0.37	*RAB30*	0.46	*AMIGO3*
2.20	*MED28*	0.37	*FAM54A*	0.46	*SNX12*
2.20	*SEPP1*	0.37	*KIF20A*	0.46	*UBLCP1*
2.17	*AR*	0.37	*SYDE1*	0.47	*VAPA*
2.17	*AKR1C3*	0.37	*SCOC*	0.47	*DPY19L3*
2.16	*LINC00340*	0.37	*KIF2C*	0.47	*LOC153546*
2.15	*ANKRD46*	0.37	*DLGAP5*	0.47	*DHX40*
2.14	*ANKRD1*	0.38	*CENPE*	0.47	*CD47*
2.14	*VPS36*	0.38	*PRICKLE4*	0.47	*BARD1*
2.13	*TRIB1*	0.38	*BCAP29*	0.47	*TRAF3*
2.13	*TMEM100*	0.38	*ARNTL2*	0.47	*P2RX4*
2.12	*JUN*	0.38	*EIF4EBP1*	0.48	*PARD6G*
2.11	*GBP1*	0.38	*NDC80*	0.48	*CKAP4*
2.11	*TBC1D19*	0.38	*C14orf1*	0.48	*RAP1GDS1*
2.07	*QPCT*	0.39	*ANLN*	0.48	*LMTK2*
2.05	*DRAM1*	0.39	*CDC25C*	0.48	*GPR107*
2.03	*CAPN3*	0.39	*ARL6IP1*	0.48	*IDS*
		0.39	*SGOL2*	0.49	*GATA2*
		0.39	*DEPDC1B*	0.49	*PRKD1*
		0.39	*WISP1*	0.49	*ABHD6*
		0.39	*CDK1*	0.49	*CHEK1*
		0.39	*MPV17L2*		

Genes showing >2-fold differentiation based on the average of two independent profiling analyses are listed.

7 tended to decrease upon co-culturing with BM-MSC with GATA2 knockdown, but this was not statistically significant (Figure 6B). Subsequently, we assessed the colony-forming capacity of CD34-positive cells after culture with each siRNA-treated BM-MSC. As shown in Figure 6C-E, the total number of colonies was significantly lower when the CD34-positive cells were cultured with BM-MSC transfected with the GATA2-siRNA. To compare the effects with a more advanced stage of adipocyte differentiation, we conducted the same series of analyses based on BM-MSC-derived adipocytes, demonstrating that HSC frequency and colony formation were decreased by GATA2 knockdown (Figure 7). In brief, our data suggest that the decrease of GATA2 expression in BM-MSC decreased the cells' ability to support the hematopoietic microenvironment.

As noted, GATA2 expression in CD34-positive cells is significantly decreased in patients with aplastic anemia by an unknown mechanism.[19,20] Because cytokines such as transforming growth factor-β,[37] interferon-γ,[37] tumor necrosis factor-α,[38,39] IL-6,[39] IL-17A,[39] and IL-1β[40,41] may be involved in the pathogenesis of aplastic anemia, we evaluated whether the addition of these cytokines may accelerate adipocyte differentiation. Unexpectedly, adipocyte differentiation was suppressed by these cytokines, except for IL-6 (*Online Supplementary Figure S5A-F*). In addition, the suppression of adipocyte differentiation did not always correlate with the changes of GATA2 expression level, nor *AP2* expression (i.e. tumor necrosis factor-α and IL-6), possibly because these cytokines might affect *AP2* expression level and adipocyte differentiation independently of GATA2. Next, we assessed the effect of bone morphogenic protein (BMP)-4, because a previous study demonstrated that BMP4 regulates GATA2 expression in embryonic stem cells.[42] As shown in *Online Supplementary Figure S5G*, BMP4 suppressed adipocyte differentiation and significantly induced GATA2, suggesting that BMP4 could be one of the factors involved in the regulation of GATA2 in BM-MSC.

Table 2. Gene ontology analysis of GATA2-regulated genes in human BM-MSC.

Biological process	Count	*P* value
Upregulated		
Signal transduction	24	2.50E-02
Developmental processes	18	1.30E-02
Cell communication	11	4.40E-02
Cell proliferation and differentiation	9	8.90E-02
Mesoderm development	7	4.20E-02
Muscle contraction	5	1.10E-02
Muscle development	4	2.80E-02
Extracellular matrix protein-mediated signaling	3	3.50E-02
Downregulated		
Cell cycle	40	1.40E-15
Protein modification	21	4.90E-03
Cell proliferation and differentiation	17	2.90E-02
Mitosis	17	2.60E-07
Protein phosphorylation	14	8.90E-03
Cell cycle control	12	1.80E-03
DNA metabolism	8	3.70E-02
General vesicle transport	7	3.50E-02
DNA replication	7	1.60E-03
Chromosome segregation	7	6.60E-04

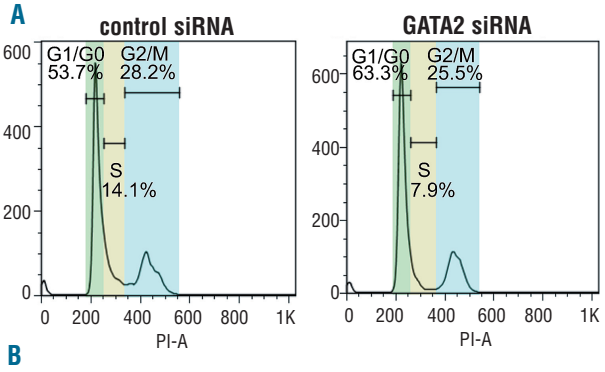
Molecular function	Count	*P* value
Upregulated		
Signaling molecule	8	5.20E-02
Extracellular matrix	5	7.80E-02
Growth factor	4	1.60E-02
Extracellular matrix glycoprotein	3	6.90E-02
Glutamate receptor	2	8.80E-02
Downregulated		
Kinase	16	1.10E-03
Cytoskeletal protein	14	3.50E-02
Protein kinase	12	8.00E-03
Microtubule family cytoskeletal protein	12	6.50E-06
Microtubule binding motor protein	8	1.60E-06
Non-receptor serine/threonine protein kinase	7	5.40E-02
Kinase modulator	5	6.30E-02
Transcription cofactor	5	6.00E-02
Kinase activator	4	1.80E-02

Genes showing >2-fold differentiation after GATA2 knockdown were analyzed.

Discussion

The balance between proliferation and differentiation of MSC may be tightly regulated by BMP, Hedgehog, and Wnt signaling pathways, among others.[3,43-54] BMP2 and BMP4 promote adipocyte differentiation.[47] Noggin inhibits BMP signaling and promotes osteogenic differentiation.[43] In addition, Hedgehog and Wnt signaling pathways inhibit adipocyte differentiation but promote osteoblastic differentiation.[44-48] Efforts to identify the regulatory mechanisms that control the differentiation of BM-MSC into adipocytes/osteoblasts may lead to the development of new clinical applications in the fields of regenerative medicine and tissue engineering and enhance our understanding of hematopoiesis, since BM-MSC are the primary sources of the hematopoietic microenvironment.[1]

Although GATA2 may play an important role in regulating adipocyte differentiation from a mouse preadipocytic cell line,[16-18] its role in regulating human BM-MSC differentiation is unknown. In the present study, we revealed that knockdown and overexpression of GATA2 accelerated and



B

	G0/G1 stage (%)	S stage (%)	G2/M stage (%)
control siRNA	58.3 ± 7.4	10.9 ± 3.9	26.4 ± 4.8
GATA2 siRNA	65.0 ± 5.4	6.8 ± 2.2	25.3 ± 3.5

Figure 4. Increase of G1/G0 phase cells with decreased GATA2 expression in BM-MSC. (A) Representative cell-cycle profile in human BM-MSC treated with control or anti-GATA-2 siRNA, based on propidium iodide staining. (B) Percentage of cells in each stage of the cell cycle (expressed as mean ± SD, n=3).

inhibited adipocyte differentiation, respectively (Figures 2 and 3). We assumed that BM-MSC fate might be determined by the balances of multiple transcription factors, rather than solely by GATA2. For example, GATA1 and PU.1, master regulators in erythroid and granulocyte differentiation, respectively, act mutually antagonistically.[55,56] Similar antagonism has also been reported between C/EBPα and PU.1 during neutrophil differentiation.[57,58] Furthermore, in murine MSC, the propensity for differentiation toward osteoblasts or adipocytes was affected by various factors including *Maf*, *Runx2*, *Cebpb* and *Pparg*.[59,60] Nevertheless, our data clearly demonstrate that GATA2 could be one of the important factors that determine immaturity and differentiation toward adipocytes in BM-MSC.

We have demonstrated that GATA2 knockdown in BM-MSC increased the number of cells in G0/G1 (Figure 4, *Online Supplementary Figure S4*), with significant downregulation of cell cycle regulators such as *CHEK1*, *CCNB1*, *CCNB2*, *GTSE1*, and *CDC20* (Table 1). GATA2 expression oscillates during the cell cycle such that expression is high in the S phase but low in G1/S and M phases.[32] Moreover,

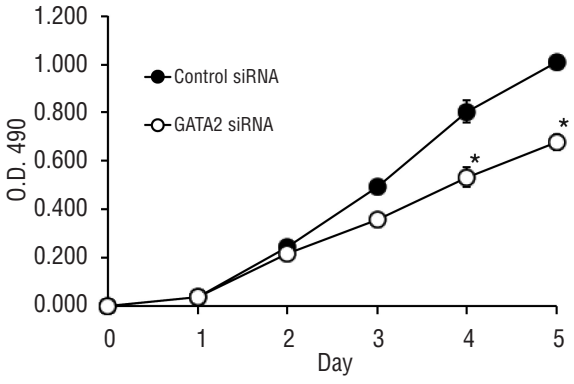
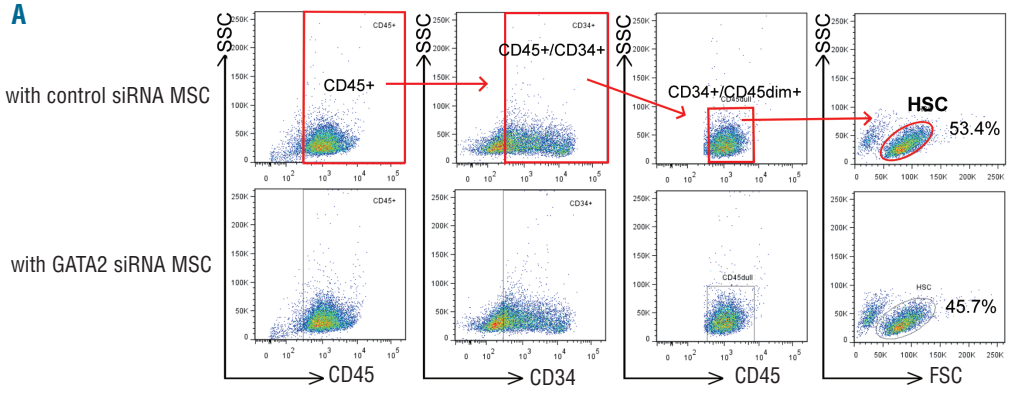


Figure 5. Effect of GATA2 knockdown on the proliferation of human BM-MSC. GATA2 expression was suppressed in human BM-MSC by transfecting them with a human GATA2-siRNA, and the cells were further cultured in the medium for 5 days. The number of viable cells was determined every 24 h by a colorimetric method. The data are expressed as mean ± SD (n=4). **P*<0.05.

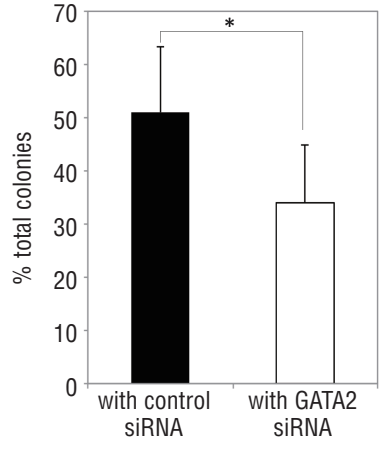
A



B

	with control siRNA MSC	with GATA2 siRNA MSC
HSC (%)	38.7±13.1	33.4±11.9

C



D

	BFU-E	CFU-G	CFU-M	CFU-GM	CFU-GEMM
with control siRNA MSC	14.8 ± 3.1	16.8 ± 4.3	12.0 ± 3.3	6.7 ± 5.0	1.0 ± 0.9
with GATA2 siRNA MSC	9.7 ± 4.5*	15.0 ± 2.0	8.8 ± 4.0	4.5 ± 3.7	0.2 ± 0.4

E

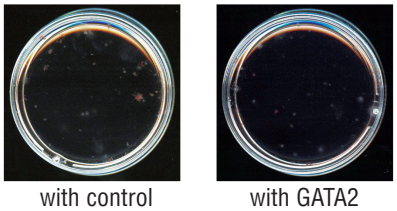
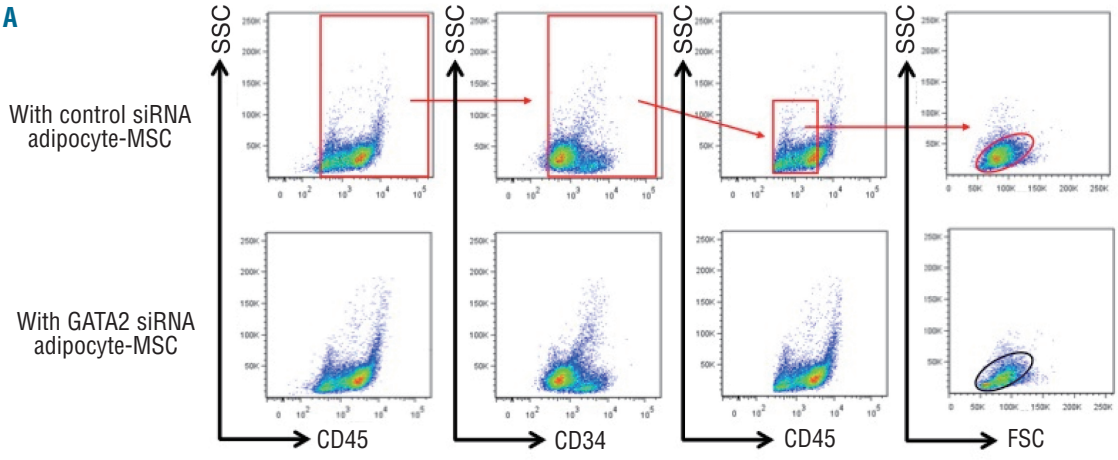


Figure 6. Reduced hematopoietic support of human BM-MSC by GATA2 knockdown. (**A**) Representative flow cytometry data to demonstrate the strategy for isolating the HSC fraction based on the ISHAGE gating strategy.[35,36] (**B**) The average frequency of the HSC fraction after co-culture with control or GATA2-knockdowned BM-MSC (mean ± SD, n=3). (**C-E**) The number of total colonies (**C**), each colony type (**D**) and representative image of colony formation (**E**) are shown (mean ± SD, n=3, for **C** and **D**). **P*<0.05.

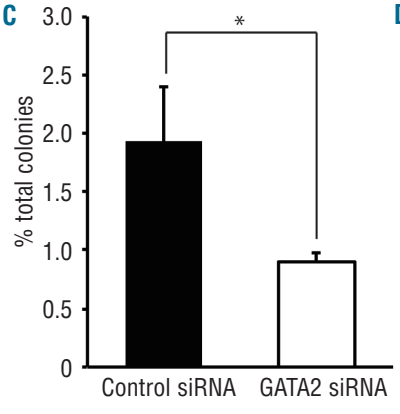
A



B

	with control siRNA adipocyte MSC	with GATA2 siRNA adipocyte MSC
HSC (%)	67.1 ± 4.8	37.3 ± 4.8

C



D

	BFU-E	CFU-G	CFU-M	CFU-GM	CFU-GEMM
with control siRNA	4.7 ± 4.1	4.0 ± 1.4	6.7 ± 1.2	3.3 ± 0.5	0.7 ± 0.9
with GATA2 siRNA	2.0 ± 1.6	1.7 ± 0.9	3.0 ± 0.8	1.7 ± 0.9	0.7 ± 0.5

Figure 7. Reduced hematopoietic support of human BM-MSC by GATA2 knockdown. Human BM-MSC were differentiated into adipocytes (6 days) on the day of adipocyte differentiation and subsequently, co-cultured with CD34-positive cells. (A) Representative flow cytometry data for isolating the HSC fraction based on the ISHAGE gating strategy.[35,36] (B) The average frequency of the HSC fraction after co-culture with control or GATA2-knockdowned BM-MSC differentiated into adipocytes (adipocyte-MSC) (mean ± SD, n=3). (C-D) The number of total colonies (C) and each colony type (D) are shown (mean ± SD, n=3, for C and D). *P<0.05.

GATA2 regulates cell cycle regulators, including CCND3, CDK4, and CDK6,[33] suggesting that GATA2 contributes to the regulation of the HSC pool within the bone marrow. We, therefore, conclude that GATA2-mediated cell cycle regulation occurs in BM-MSC. Recent evidence suggests that adipocyte differentiation is triggered during the G1 phase when progenitor cells are exposed to adipogenic stimuli such as insulin, insulin-like growth factor 1, dexamethasone, and cyclic AMP.[61,62] Furthermore, considering our finding that GATA2 knockdown in BM-MSC increased the number of G1 phase cells (Figure 4, *Online Supplementary Figure S4*), the susceptibility to adipogenic stimuli may have been augmented by GATA2 knockdown, leading to accelerated adipocyte differentiation.

We have also demonstrated that GATA2 expression in HSC is lower in patients with aplastic anemia and leads to decreased *HOXB4* expression,[19,20] which would contribute to the reduction in the size of the HSC pool.[26] Furthermore, GATA2 expression is also lower in BM-MSC derived from patients with aplastic anemia.[13] In the present study, we demonstrated that decreased GATA2 expression in BM-MSC accelerated adipocyte differentiation. Thus, decreased GATA2 expression by HSC and BM-MSC may lead to decreased number of HSC as well as fatty marrow change, which is a characteristic feature of aplastic anemia.

In addition to the accelerated adipocyte differentiation from BM-MSC by GATA2 knockdown (Figure 2), we further demonstrated that GATA2-knockdowned BM-MSC compromised colony-forming capacity based on co-culture with CD34-positive cells (Figure 6). Although the molecular mechanism responsible for this effect is unknown, decreased expression of cell adhesion molecules such as *LAMB1*, *CD44*, and *FBN2* (Table 1) may be involved in impaired HSC maintenance. *LAMB1* encodes laminin β1, which is expressed in the hematopoietic microenvironment, and contributes to the regulation of hematopoiesis.[63] Furthermore, CD44 promotes the homing process between HSC and the hematopoietic niche through hyaluronic acid, which serves as a ligand.[64,65] Thus, GATA2 downregulation in patients with aplastic anemia may result in a decrease of extracellular matrix, similar to the functions of LAMB1 and CD44, resulting in impaired HSC support.

Immunosuppressive therapy is effective in 75% of cases of aplastic anemia, suggesting that immunological injury plays a role in the pathogenesis of aplastic anemia.[66] However, in our study, the addition of various cytokines did not accelerate adipocyte differentiation or decrease GATA2 expression (*Online Supplementary Figure S5*), suggesting that

transforming growth factor-β, interferon-γ, tumor necrosis factor-α, IL-6 and IL-17A and IL-1β might not be involved in the regulation of GATA2 expression in BM-MSC. We included BMP4 in our analysis because this protein regulates GATA2 expression.[42] As expected, we demonstrated that BMP4 induced GATA2 expression, suggesting that BMP4 may affect GATA2 expression in BM-MSC (*Online Supplementary Figure S5G*). However, we observed that the addition of BMP4 suppressed adipocyte differentiation, unlike the results of another study on preadipocytic C3H10T1/2 cell lines.[47] We suggest that this difference may be attributed, in part, to the concentration of BMP4 used here. Nevertheless, in addition to BMP signaling, several factors, such as the Wnt signaling pathway, regulate GATA2 expression.[67] Further analyses are, therefore, required to elucidate the pathogenesis of aplastic anemia.

In conclusion, our findings support the hypothesis that GATA2 plays an important role in regulating the differentiation potential of BM-MSC and contributes to hematopoietic supporting capacity. Therefore, in bone marrow, GATA2 is not only involved in the generation and/or maintenance of HSC, but also in regulating the hematopoietic microenvironment. Identifying the regulatory mechanism of GATA2 in HSC and BM-MSC may lead to the development of novel therapeutic approaches for bone marrow failure syndromes.

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Authorship and Disclosures

Information on authorship, contributions, and financial & other disclosures was provided by the authors and is available with the online version of this article at www.haematologica.org.

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