Infants with Down syndrome (DS) are at a high risk of developing transient abnormal myelopoiesis (TAM). A GATA1 mutation leading to the production of N-terminally truncated GATA1 (GATA1s) in early megakaryocyte/erythroid progenitors is linked to the onset of TAM and cooperated with the effect of trisomy 21 (Ts21). To gain insights into the underlying mechanisms of the progression to TAM in DS patients, we generated human pluripotent stem cells harbouring Ts21 and/or GATA1s by combining microcell-mediated chromosome transfer and genome editing technologies. In vitro haematopoietic differentiation assays showed that the GATA1s mutation blocked erythropoiesis irrespective of an extra chromosome 21, while Ts21 and the GATA1s mutation independently perturbed megakaryopoiesis and the combination of Ts21 and the GATA1s mutation synergistically contributed to an aberrant accumulation of skewed megakaryocytes. Thus, the DS model cells generated by these two technologies are useful in assessing how GATA1s mutation is involved in the onset of TAM in patients with DS.

**Subjects:**
- Genetic Engineering
- Genetic Vectors
Results and Discussion

A human chromosome 21 (hChr.21) was transferred to human ES cells via microcell-mediated chromosome transfer (MMCT). We previously generated a monochromosomal hybrid library in mouse A9 cells, which contained a single human chromosome. DS model mice were generated by transferring an extra hChr.21 into mouse ES cells using the A9 library via MMCT. Similarly, we generated human ES cells containing an extra hChr.21, creating Ts21. A pSTneo-tagged hChr.21 was transferred to human ES (KhES-1)-derived subclones (designated as WT-ES) via MMCT. Twelve G418-resistant clones from 3 independent experiments were obtained. Six clones contained an additional hChr.21 (Ts21), and 6 clones contained 2 additional copies of hChr.21 (tetrasomy 21) (Supplementary Fig. 1). Multicolour fluorescence in situ hybridisation (mFISH) analysis indicated that the hChr.21 was successfully transferred into wild-type (WT)-ES cells and that the karyotype was 47,XX, +21 (Fig. 1b, c). FISH analysis of the exogenous hChr.21 showed that the pSTneo-derived signal was in a single hChr.21 (Supplementary Fig. 2). To determine whether Ts21-ES cells could differentiate into all 3 embryonic germ layers, Ts21-ES lines were injected into testes of severe combined immunodeficiency (SCID) mice. Histological analyses revealed all 3 embryonic germ layers in all teratomas (Fig. 1d). Microarray analyses revealed that genes on hChr.21 in Ts21-ES cells were globally overexpressed, but gene expression from hChr.18 was comparable with that in WT-ES cells (Fig. 1e). These data suggest that the exogenous hChr.21 was successfully transferred to WT-ES cells and that the Ts21-ES cells have differentiation potential.

The GATA1 mutation was generated via one of the genome editing technologies, zinc-finger nucleases (ZFNs), which were used previously to modify the endogenous genome of several species. mRNAs or plasmids encoding a ZFN targeting exon 2 of GATA1 DNA were transfected into WT-ES cells. A mutation detection assay (Cell assay) showed that 5 of 384 clones and 2 of 96 clones using the mRNAs and plasmids, respectively, were positive for the mutation (Supplementary Fig. 3). The mutation-positive mRNA-transfected clones were subcloned to reduce the possibility of heterogeneous populations. A restriction fragment length polymorphism (RFLP) assay using BsiHKAI enzyme showed that 1 (pZ7) of 19 clones (17 mRNA-transfected subclones and 2 plasmid-transfected clones) contained the different deletions in both alleles of exon 2 of GATA1 (Supplementary Fig. 4); sequence analyses revealed that 2 clones (pZ19-2 and pZ28-5) contained heterozygous insertion/deletion (or deletion) in the GATA1 gene and 1 clone (pZ7) contained different deletions (8 bp and 17bp) in both alleles, resulting in a premature TGA stop codon in exon 2 (Supplementary Figs. 5 and 6 and Fig. 2a). The clones with the premature stop codon in exon 2 had normal karyotypes (46,XX) and differentiation potential to 3
hChr.21, BACH123, was overexpressed in the Ts21-ES and GATA1s/embryonic germ layers in the teratoma were randomly selected for clones with Ts21 and GATA1s with differentiation potential to 3 embryonic germ layers (Supplementary Figs. 7 and 8). The pZ7 clone (designated GATA1s-ES) with the deletions in both alleles of exon 2 was used for further analyses.

An additional hChr.21 was transferred to GATA1s-ES cells via MMCT. Cytogenetic and histological analyses showed that the clones in the GATA1s genetic background contained Ts21, and 2 clones with Ts21 and GATA1s with differentiation potential to 3 embryonic germ layers in the teratoma were randomly selected for further analyses (designated GATA1s/Ts21-ES) (Supplementary Fig. 9). Microarray analyses revealed that genes on hChr.21 in GATA1s/Ts21-ES cells were globally overexpressed, but gene expression from hChr.18 was comparable with that in GATA1s-ES cells (Supplementary Fig. 10).

ES-sac–mediated in vitro haematopoietic differentiation analyses were performed (Supplementary Fig. 11). Western blot analyses of the erythroid lineage showed that a representative protein from hChr.21, BACH123, was overexpressed in the Ts21-ES and GATA1s/Ts21-ES lines compared with the WT-ES and GATA1s-ES cells (Supplementary Fig. 12 and Fig. 2b). Western blot analyses using a GATA1 antibody to recognise the C-terminus of GATA1/GATA1s protein showed that full-length GATA1 protein was expressed in WT-ES and Ts21-ES lines, but not in GATA1s-ES and GATA1s/Ts21-ES lines (Supplementary Fig. 12 and Fig. 2b). Additionally, GATA1s protein was remarkably increased in the GATA1s-ES and GATA1s/Ts21-ES lines. These results were also confirmed in the megakaryocytic differentiation stage (ES-sac (day 14), megakaryocyte (day 20), and erythroid (day 20)) (c).

Statistical analyses were performed by comparison with WT-ES cells (WT-ES1, WT-ES1-1 and WT-ES1-2). The percentage of CD34+/CD41a−, CD34−/CD41a+ and CD71+/CD235+ cells are shown in each differentiation stage (ES-sac (day 14), megakaryocyte (day 20), and erythroid (day 20)) (c). Statistical analyses were performed by comparison with WT-ES cells (WT-ES1, WT-ES1-1 and WT-ES1-2). *p < 0.05, **p < 0.01 by two-tailed Student’s t test. The percentage of CD34−/CD41a+, CD34+/CD41a+ and CD34−/CD41a+ cells are shown in the megakaryocyte stage (d).
Curiously, we found that the population of ES-sac–mediated megakaryocytic (CD41a+/CD42b+) cells from GATA1s-ES cells was higher than that from WT-ES cells, although the further introduction of an additional hChr.21 into each cell line (WT-ES and GATA1s-ES cells) resulted in a slightly reduced frequency. (Supplementary Fig. 14 and Fig. 2c). Further analysis of the megakaryocytic differentiation showed that the ratio of immature (CD34+/CD41a+) to mature (CD34+/CD41a+) megakaryocytic cells derived from GATA1s-ES and GATA1s/Ts21-ES cells was significantly higher than that from WT-ES and Ts21-ES cells, respectively, suggesting that GATA1s disturbs the maturation of megakaryocytes and/or enhances the proliferation of immature megakaryocytes (Supplementary Fig. 15 and Fig. 2d). Furthermore, CD34+/CD41a− cells accumulated in Ts21-ES cell cultures with a reduced frequency of CD34+/CD41a+ cells, and the additional GATA1s mutation worsened the phenotype of Ts21-ES (Supplementary Fig. 15 and Fig. 2d). Consequently, the efficiency of CD41a+/CD42b+ cells in GATA1s/Ts21-ES cells was seemingly comparable to that in WT-ES cells. We concluded that Ts21 and GATA1s mutation differentially affect the megakaryocyte differentiation, and the combination of Ts21 and GATA1s mutations synergistically influences the process of megakaryocyte differentiation. Our in vitro differentiation system revealed for the first time that Ts21 disturbs the differentiation of megakaryocytes and further GATA1s mutation intrinsically perturbs the process of megakaryopoiesis in combination with increasing the dosage of genes located on hChr.21.

Taken together, our novel system combined MMCT and ZFN technologies to generate DS model cells. The combination of chromosome transfer and genome editing technologies could therefore enable the generation of in vitro chromosome abnormality syndrome models with multiple genetic alterations. Progression from TAM to DS-AMKL requires additional mutations in genes including oeshin/CTCF, EHZ2, other epigenetic regulators, and RAS/signal transducing molecules. However, the function or mechanism of each class of mutation on the leukemiaogenesis remains uncertain. Our methods can aid in resolving these questions, because the desired mutations in addition to the GATA1s can be inserted to the GATA1s/Ts21-ES cells using genome editing technologies. Importantly, all of the developed ES cell lines were isogenic and genetically defined. MMCT using other chromosome donor A9 cells will enable the generation of pluripotent stem cell-derived models for different trisomy syndromes including Ts18 and Ts13 in the same genetic background. Human chromosomes can be efficiently modified in the homologous recombination-proficient chicken DT40 cells, which can be used as a shuttle system to transfer the modified chromosome to other cells. Defined genomic regions can be also cloned into human artificial chromosomes. MMCT using other chromosome donor A9 cells will enable the generation of pluripotent stem cell-derived models for different trisomy syndromes including Ts18 and Ts13 in the same genetic background. Human chromosomes can be efficiently modified in the homologous recombination-proficient chicken DT40 cells, which can be used as a shuttle system to transfer the modified chromosome to other cells. Defined genomic regions can be also cloned into human artificial chromosomes.

Methods

Cell culture. Mouse A9 cells containing hChr.21 (A9(21-16)) that were used as fusion donors for chromosome transfer were established as described previously. The A9(21-16) cells were maintained in Dulbecco’s modified Eagle’s medium plus 10% foetal bovine serum (FBS) with 800 µg/mL G418 (Promega, Tokyo, Japan). A human ES line, a KhES-1-derived subline, was used following the human ES cell research guidelines of the Japanese government. Because the subline contained chromosomal abnormalities in chromosome 1q, the subline was further subcloned (designated as WT-ES1). WT-ES1 was used for MMCT and ZFN transfection experiments. WT-ES1 was further subcloned for the control cell lines (designated as WT-ES1-1 and WT-ES1-2). The parental human ES cell line and micr0cell hybrid clone were cultured in mTeSR1 (CELLJEN, Tokyo, Japan) in a humidified incubator at 37°C, 5% CO2, 85% relative humidity. mTeSR1 medium was Iscove’s modified Dulbecco’s medium (Sigma-Aldrich) supplemented with 10% FBS and 2 mM L-glutamine, 0.45 mM α-monomethylcysteine (Sigma-Aldrich), 50 µg/mL ascorbic acid (Sigma-Aldrich), and 15% FBS.

MMCT. MMCT was performed as described previously. A9 cells containing hChr.21 were used as donor microcell hybrids. Briefly, WT-ES1 and GATA1s-ES (p27) cells were fused with microcells prepared from donor hybrid A9 (21-16) cells and selected with G418 (50 µg/mL). The transferred hChr.21 in each line was characterized by cytogenetic analyses.

Microarray analyses. Total RNA from WT-ES, Ts21-ES, GATA1s-ES and GATA1s/Ts21-ES cells was prepared using RNeasy (Qagen, Hilden, Germany) according to the manufacturer instructions. Microarray analyses were performed using a 3D-Gene Human Oligo chip 25k (Toray Industries Inc., Tokyo, Japan). Microarray slides were scanned using a 3D-Gene Scanner (Toray Industries) and processed by 3D-Gene Extraction software (Toray Industries).

Cytogenetic analyses. Slides of microcell hybrids and ZFN-transfected clones were stained with quinacrine mustard and Hoechst 33258 to enumerate chromosomes. Images were captured using an AxioImagerZ2 fluorescence microscope (Carl Zeiss GmbH, Jena, Germany). FISH analyses were performed using fixed metaphases of microcell hybrids using digoxigenin-labelled (Roche, Basel, Switzerland) human Cot-1 DNA (Life Technologies) and biotin-labelled (Roche) pSvneo plasmid DNA essentially as described previously. Chromosomal DNA was counterstained with DAPI (Sigma-Aldrich). Images were captured using the NIS-Elements system (Nikon, Tokyo, Japan). mFISH analyses were performed in accordance with the manufacturer instructions (MetaSystems, Altlussheim, Germany). Human mFISH probes were purchased from MetaSystems GmbH. Metaphase images were captured using a Coolsnap CCD camera and the ISIS mFISH software (MetaSystems).

Teratoma formation and histology. To produce teratomas, 1 × 10^6 WT-ES, Ts21-ES, GATA1s-ES, and GATA1s/Ts21-ES cells were subcutaneously injected into testes of SCID mice (Charles River, Yokohama, Japan). After 8 weeks, resected teratomas were fixed in 20% formalin, processed for paraffin sectioning, and then stained with haematoxylin and eosin. All animal experiments were approved by the Institutional Animal Care and Use Committee of Tottori University.

Transfection. Custom-designed ZFN plasmids and ZFN-encoding mRNA for targeted modification of the human GATA1 gene were generously provided by Sigma-Aldrich. The design, cloning, and validation of the ZFNs were performed by Sigma-Aldrich. Human ES cells (2 × 10^5) were collected in 100 µL Nucleofector solution (Lonza, Tokyo, Japan) with 2.5 µg of each ZFN plasmid and 2.5 µg of pCX-EGFP (Qiagen), 2 µg of pCMV-GFP (Qiagen), (Transgenomic, Omaha, NE, USA) in accordance with the manufacturer instructions. PCR products were purified by QIAquick PCR Purification Kit (Qiagen), digested with the enzyme, electrophoresed on a 2% agarose gel, and stained with ethidium bromide. Furthermore, the PCR products were subcloned into the pCR-TOPO vector (Life Technologies), and the vector DNA was sequenced by a 3130XL Genetic Analyzer (Life Technologies) sequencer.

Genomic PCR and mutation analyses. Genomic DNA was extracted from ZFN plasmids or ZFN-encoding mRNAs using a genomic extraction kit (Genra System, Minneapolis, MN, USA), and PCR was performed using primers as follows. Primer pairs for the SURVEYOR mutation detection assay (Cell assay) and RFLP analyses using BsiHKAI restriction enzyme to detect the mutation in the GATA1 region were GATA1-F/GATA1-R (347 bp), 5’-TTTCATGCTGCTGAGACCCC-3’ and 5’-GACTTACGCAAGGATCCTACA-3’.

The Cell assay was performed using SURVEYOR Mutation Detection kit (Transgenomic, Omaha, NE, USA) in accordance with the manufacturer instructions. PCR products were purified by QIAquick PCR Purification Kit (Qiagen), digested with the enzyme, electrophoresed on a 2% agarose gel, and stained with ethidium bromide. Furthermore, the PCR products were subcloned into the pCR-TOPO vector (Life Technologies), and the vector DNA was sequenced by a 3130XL Genetic Analyzer (Life Technologies) sequencer.

Haematopoietic differentiation of human ES cells. The differentiation of human ES cells into haematopoietic cells was performed as described previously. In brief, small clumps of human ES cells were transferred onto mitomycin C-treated CHST/ST2 cells and further cultivated in differentiation medium supplemented with 20 ng/mL human vascular endothelial growth factor (R&D Systems) and combinations of other cytokines/mediators (human stem cell factor (p83), interleukin-3, granulocyte-macrophage colony stimulating factor (p83), and combinations of other cytokines/mediators (human stem cell factor (p83), interleukin-3, granulocyte-macrophage colony stimulating factor) and human erythropoietin (Prospec-Tany TechnoGene, East Brunswick, NJ, USA)).

Protein extracted from the differentiated ES cells was transferred onto fresh mitomycin C-treated C3H10T1/2 cells and further cultivated in haematopoietic cell differentiation medium supplemented with 20 ng/mL human vascular endothelial growth factor (R&D Systems) and combinations of other cytokines/mediators (human stem cell factor (p83), interleukin-3, granulocyte-macrophage colony stimulating factor) and human erythropoietin (Prospec-Tany TechnoGene, East Brunswick, NJ, USA)).

Western blot analyses. Protein extracted from the differentiated ES cells was separated by sodium dodecyl sulphate polyacrylamide gel electrophoresis on an 8%
polycrylamide gel and transferred to a polyvinylidene difluoride membrane. The membrane was blocked with 5% dry milk and probed with a mouse monoclonal antibody against BACH1 (F-9; sc-271211); Santa Cruz Biotechnology, Santa Cruz, CA, USA) or a goat polyclonal antibody against the C-terminus of GATA1 (C20; sc-1233; Santa Cruz Biotechnology). The membrane was then incubated with a horseradish-peroxidase–conjugated secondary antibody and developed with enhanced chemiluminescence reagents (Pierce Western Blotting Substrate; Thermo, Yokohama, Japan). To confirm that the amount of protein in each lane was comparable, the membrane was stripped and probed with a monoclonal antibody against α-tubulin (DM-1A; ICN Biomedicals, Santa Ana, CA, USA). HEL 92.1.7 whole cell lysate and K562 nuclear extract were used as positive controls (sc-2130 and sc-2277, respectively; Santa Cruz Biotechnology). CSH1H1T1/2 whole cell lysate was used as a negative control.

Flow cytometry analyses. The expression of cell surface molecules was analysed by flow cytometry (FACSAria; Becton Dickinson, Franklin Lakes, NJ, USA). On day 14, a fraction of the HPCs within the ES-sacs were stained with CD34 antibody for 30 minutes on ice. Nonadherent cells on day 20 of culture were prepared in PBS containing 3% FBS (staining medium) and stained with combinations of antibodies for 30 minutes on ice. All samples were then washed with staining medium and analysed by flow cytometry. The following antibodies were used: allophycocyanin (APC)-conjugated anti-CD34 (Biolegend, San Diego, CA, USA), APC-conjugated anti-CD41a (integrin IIb subunit, Biolegend), phycoerythrin (PE)-conjugated anti-CD41a (Biolegend), PE-conjugated anti-CD42b (Glycoprotein Ib, Biolegend), PE-conjugated anti-CD71 (BD Pharmingen, San Diego, CA, USA), and APC-conjugated anti-CD235 (Glycophorin A, Biolegend).

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