Panel of human cell lines with human/mouse artificial chromosomes

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Human artificial chromosomes (HACs) and mouse artificial chromosomes (MACs) are non-integrating chromosomal gene delivery vectors for molecular biology research. Recently, microcell-mediated chromosome transfer (MMCT) of HACs/MACs has been achieved in various human cells that include human immortalised mesenchymal stem cells (hiMSCs) and human induced pluripotent stem cells (hiPSCs). However, the conventional strategy of gene introduction with HACs/MACs requires laborious and time-consuming stepwise isolation of clones for gene loading into HACs/MACs in donor cell lines (CHO and A9) and then transferring the HAC/MAC into cells via MMCT. To overcome these limitations and accelerate chromosome vector-based functional assays in human cells, we established various human cell lines (HEK293, HT1080, hiMSCs, and hiPSCs) with HACs/MACs that harbour a gene-loading site via MMCT. Model genes, such as tdTomato, TagBFP2, and ELuc, were introduced into these preprepared HAC/MAC-introduced cell lines via the Cre-loxP system or simultaneous insertion of multiple gene-loading vectors. The model genes on the HACs/MACs were stably expressed and the HACs/MACs were stably maintained in the cell lines. Thus, our strategy using this HAC/MAC-containing cell line panel has dramatically simplified and accelerated gene introduction via HACs/MACs.

Abbreviations
HAC  Human artificial chromosome
MAC  Mouse artificial chromosome
MMCT  Microcell-mediated chromosome transfer
hiMSC  Human induced immortalscell mesenchymal stem cell
hiPSC  Human induced pluripotent stem cell
Tc  Trans-chromosomic
SIM  Simultaneous or sequential insertion of multiple genes-loading vectors
HPRT  Hypoxanthine-guanine phosphoribosyltransferase
MI  Multiple integrase
CRISPR  Clustered regularly interspaced short palindromic repeats
Cas9  CRISPR-associated protein 9
MV  Measles virus

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ELuc  Emerald luciferase  
FCM  Flow cytometry  
FISH  Fluorescence in situ hybridisation  
6TG  6-Thioguanine  
PDL  Population doubling level  
FBS  Fetal bovine serum  
HAT  Hypoxanthine-aminopterin-thymidine  

Figure 1. Schematic diagram of gene delivery via HACs/MACs. (a) Schematic diagram of the conventional gene-introducing strategy using HACs/MACs. In the conventional strategy, HACs/MACs for gene expression are constructed by transfection and insertion of the target gene(s) into the HAC/MAC in CHO or A9 cells that contain HACs/MACs as step 1. The constructed HACs/MACs are then introduced into target cells by MMCT as step 2. (b) Schematic diagram of the other gene-introducing strategy using the preprepared human cell panel with HACs/MACs. The HAC or MAC was transferred into representative human cell lines. The human cell line panel included HEK293 cells, HT1080 cells, human immortalised mesenchymal stem cells (hiMSCs), and human induced pluripotent stem cells (hiPSCs, 201B7). The human cell lines were directly available for gene loading into HACs/MACs with site-specific recombination. Simultaneous insertion of multiple gene-loading vectors into HEK293 cells, HT1080 cells, and hiPSCs (201B7) that contained HACs/MACs was tested by the SIM system. Each gene-loading vector of the SIM system contained Emerald luciferase (ELuc), a red fluorescent protein (tdTomato), or blue fluorescent protein (TagBFP2-N). hiMSCs that contained 21HAC2 were used to attempt insertion of three types of plasmid vectors that contained a red fluorescent protein (mCherry) driven by a different promoter with the Cre-loxP system. Each mCherry was expressed by a general constitutive promoter, e.g., PGK, CAG, or EF1α.

Human artificial chromosomes (HACs) and mouse artificial chromosome (MACs) have unique characteristics as vectors for gene delivery, which include stable and independent maintenance without disruption of the host genome and the capacity to carry numerous genes and megabase-sized genomic loci with physiological regulatory elements. HAC/MAC technologies have been used for gene and cell therapies of Duchenne muscular dystrophy and to generate trans-chromosomic (Tc) animals that include a mouse model of Down syndrome and humanised drug metabolism. Furthermore, several types of HACs have been used in cancer research and drug development for cancer therapy, centromere and telomere function elucidation, a system of quantitatively tracking epigenetic memory in the field of synthetic biology, and protein production.
platform for simple and stable gene expression. To increase HAC/MAC applicability, we prepared HPRT-deficient human cell lines that contained HACs/MACs, which are applicable to gene loading by SIM and Cre-loxP systems in this study. The detailed structures of each HAC/MAC are shown in Supplementary Fig. S1. Hence, we report the generation of a human cell line panel for simple gene loading of genes of interest (GOI) using HACs/MACs (Fig. 1b). As representative human cell lines, we used HEK293, which is an immortalised human cell line, and HT1080, which is a cancer cell line, as well as a human immortalised mesenchymal stem cell (hiMSC) line38,39 and human induced pluripotent stem cell (hiPSC; 201B7) line40. We further attempted to adapt the Cre-loxP system (Supplementary Fig. S2a) and SIM system for multiple gene loadings (Supplementary Fig. S2b) in the generated human cell lines with HACs/MACs.

Results and discussion

Establishment of HAC/MAC-retaining human cell lines. Five types of mammalian artificial chromosomes, which included HACs and MACs, were used in this study (Supplementary Fig. S1). HACs were derived from human chromosome 21, such as 21HAC1 without EGFP and 21HAC2 with EGFP41, and MACs were derived from mouse chromosome 11, such as MAC2 without EGFP, MAC4 with EGFP37,42,43, and MAC6 with EGFP. These HACs/MACs had a loxP site and partial HPRT gene as an acceptor site for the SIM system, which enabled simultaneous insertion of three circular plasmids. However, conventional gene introduction via HACs/MACs requires transfer of the HACs/MACs with desired gene(s) by MMCT into target cells and there is a major technical difficulty. In this study, we established a human somatic/stem cell line panel (HEK293 cell, HT1080 cell, hiMSC, and hiPSC lines) that contained HACs/MACs (Fig. 1) (Table 1).

Table 1. List of human cell lines that contained HACs/MACs. Cell lines, HACs/MACs, EGFP marker, and drug resistance are summarised. Regarding drug resistance, R indicates cells resistant to antibiotics, i.e., Hyg hygromycin, Puro puromycin, BS blasticidin S, and S indicates that cells are selectable by drugs, i.e., HAT hypoxanthine-aminopterin-thymidine medium and Ganc ganciclovir.

| Cell line | Component | EGFP | Drug resistance |
|-----------|-----------|------|-----------------|
| HEK293   | 21HAC1    | Absent | HygR, PuroR, HATS, and GancS |
|          | 21HAC2    | Present | HygR, PuroR, BSR, HATS, and GancS |
|          | MAC2      | Absent | HygR, PuroR, and HATS |
|          | MAC4      | Present | HygR, PuroR, and HATS |
| HT1080   | 21HAC2    | Present | HygR, PuroR, BSR, HATS, and GancS |
|          | MAC4      | Present | HygR, PuroR, and HATS |
| hiMSC    | 21HAC2    | Present | HygR, PuroR, BSR, HATS, and GancS |
| hiPSC    | MAC6 (ΔNeoR) | Present | PuroR and HATS |

Then, plasmid vector(s) with a GOI were inserted into the HAC/MAC via the SIM system for HEK293 cells, HT1080 cells, and hiPSCs or the Cre-loxP system for hiMSCs. Fluorescence in situ hybridisation (FISH) analyses revealed that a single additional HAC or MAC was maintained independently from the host chromosome in each somatic/stem cell line. Specifically, HEK293 cells contained 21HAC1 (Fig. 2a), 21HAC2 (Fig. 2b), MAC2 (Fig. 2c), or MAC4 (Fig. 2d), HT1080 cells contained 21HAC2 (Fig. 2e) or MAC4 (Fig. 2f), hiMSCs contained 21HAC2 (Fig. 2g), and hiPSCs (201B7) contained MAC6 (Fig. 2h). A summary of the cell line panel is described in Table 1. Regarding hiPSCs (201B7) with MAC6, the Neo resistance gene on MAC6 was disrupted (MAC6-ΔNeoR) for further gene insertion and drug selection. Although promoters for overexpression of transgenes often undergo gene silencing in human pluripotent stem cells44, HACs/MACs maintained the desired gene expression level in long-term cell culture, which differed from gene transduction with plasmid DNA via random insertion. Therefore, the insertion of the NeoR gene driven by the PGK promoter on MAC6 would be applicable to obtain a clone with an inserted circular plasmid vector for drug selection of hiPSCs. Thus, the NeoR gene on MAC6 in hiPSCs was knocked out to establish 201B7/MAC6-ΔNeoR cells (Supplementary Fig. S1e).

Demonstration of gene loading with three vectors by simultaneous introduction using the SIM system into HACs/MACs in HEK293 and HT1080 cells. We demonstrated that somatic/stem cell lines that contained HACs/MACs accepted three plasmid vectors by simultaneous introduction using the SIM system (Supplementary Fig. S2b). As model genes for demonstration, a luminescent protein, Emerald luciferase (ELuc), and two fluorescent proteins, tdTomato and TagBFP2-N, were selected (hereafter collectively called EITB). Specifically, we evaluated HEK293 cells that contained 21HAC1, 21HAC2, MAC2, or MAC4, HT1080 cells that contained 21HAC2 or MAC4, and hiPSCs that contained MAC6-ΔNeoR by introducing the three vectors with each model gene using the SIM system. Numerous drug-resistant clones were observed and an arbitrary number of clones were analysed by PCR to detect the correct insertion of the plasmid into the HAC/MAC. A summary of the PCR analysis is shown in Supplementary Table S1. The obtained drug-resistant clones were analysed by fluorescence microscopy, flow cytometry (FCM), and luciferase activity assays. These assays showed fluorescent proteins and luciferase expression in each cell line of representative HEK293 and HT1080 clones as expected. The results of HEK293/21HAC2-EITB cells are shown in Fig. 3a,b,e. HEK293/MAC4-EITB cells are shown in Fig. 3c–e. HT1080/21HAC2-EITB cells are shown in Fig. 3f,g,j, and HT1080/MAC4-EITB cells are shown in Fig. 3h–j. Representative results of the expression frequency of the introduced fluorescent protein gene and
Luciferase gene expression level were similar for each parental clone used for gene transfer. Therefore, to further expand the cell line panel, clones that show excellent gene expression should be selected. FISH analyses showed stable maintenance of HACs/MACs that contained the transgenes independently from host chromosomes. Fluorescence imaging of HEK293/21HAC1 and HEK293/MAC2 also showed expression of tdTomato and TagBFP2-N (Supplementary Fig. S3a,b). FISH analyses were also performed in HEK293/21HAC1 and HEK293/MAC2 cells that contained the vectors using the ELuc plasmid probe. The results showed that one HAC/MAC was maintained in each cell and that the transfected plasmid was inserted into the HAC/MAC as expected (Supplementary Fig. S3c,d). These results showed that multiple gene loadings into the HAC/MAC via the SIM system was achieved in our established human cell line panel (Fig. 2a–f), which enables seamless application of our HAC/MAC technology for gene function assays in human cells in the future.

**Characterisation of hiMSCs that contain HAC and demonstration of gene loading by the Cre-loxP system.** hiMSCs with a transferred 21HAC2 (Fig. 2g), which were clones A03 and D11, stably expressed EGFP (Fig. 4a). Various MSC markers were also analysed by RT-PCR in these clones (Fig. 4b). Expression level of CXCL1 and IL8 is higher in resultant clones than in parental hiMSCs. CD90 is a typical MSC marker and the others including CXCL1 and IL8 are linked to therapeutic functionality. For the recipient hiMSC cell lines employed for HAC introduction, 6-thioguanine (6TG)-resistant clones (#3 and #6) were used to obtain HAT-resistant cell lines by HPRT gene reconstruction. The 21HAC2-carrying cell lines (A03 and D11) showed comparable or higher expression levels of the various MSC markers compared with the original hiMSC cell line. These results indicated that the hiMSC clones that contained 21HAC2 maintained the characteristics of MSCs (Fig. 4b). To evaluate the HAC retention ratio after long-term cell culture with or without an antibiotic (blasticidin; Bsd), FISH analysis was performed and results showed stable maintenance of the HAC at population doubling levels (PDLs) of 24 and 39, even without Bsd (Fig. 4c). These results indicated that the two hiMSC clones that contained 21HAC2 (hiMSC/21HAC2 A03 and D11) could be used as a platform for gene loading. We validated whether 21HAC2 functioned as a safe harbour for gene expression in these established clones. As an example of functional analysis, we attempted to evaluate the expression level of the transgene promoted by three types of constitutive promoters (PGK, EF1a, and CAG) on 21HAC2 in hiMSCs with a defined (single in
Characterisation of hiPSCs that contained MAC and demonstration of gene loading by the SIM system. hiPSCs (201B7) that contained MAC6-ΔNeoR expressed EGFP (Fig. 5a). We performed long-term cell culture without an antibiotic (G418) and quinacrine-Hoechst karyotyping to evaluate the HAC retention ratio. Karyotyping of hiPSCs showed an ideal karyotype that included a MAC, 47, XX, + MAC (Fig. 5b, left panel). The long-term culture of hiPSCs revealed that the karyotype and MAC were stable at a PDL of 20 (Fig. 5b, right panel). Next, we attempted gene loading with the SIM system into 201B7/MAC6-ΔNeoR cells. The obtained hiPSC clone [(201B7)/MAC6-ΔNeoR-EITB] expressed tdTomato, TagBFP2-N, and EGFP (Fig. 5c). FISH analysis showed that the transgenes were integrated into the MAC and that the MAC was independently maintained in the hiPSCs (Fig. 5d). The expression level of each fluorescent marker and ELuc in 201B7/MAC6-ΔNeoR-EITB cells was evaluated by FCM analysis and luciferase assays, respectively (Fig. 5e). Expression of all introduced genes was detectable among the representative two clones. Furthermore, 201B7/MAC6-ΔNeoR-EITB cells showed a normal karyotype and stable maintenance of the MAC as well as 201B7/MAC6-ΔNeoR. In vitro, the expression of pluripotency markers was verified. The iPSC clones 201B7/MAC6-ΔNeoR-EITB showed activity of alkaline phosphatase and expression of OCT 3/4 as well as 201B7 (Fig. 5f,g). Quantitative RT-PCR analysis confirmed expression of OCT 3/4 in each clone (Fig. 5h). To verify the pluripotency of these iPSC lines, we tested their ability to differentiate into all three germ layers in vivo using the teratoma method. 201B7/MAC6-ΔNeoR and 201B7/MAC6-ΔNeoR-EITB cells showed maintenance of pluripotency to differentiate into the three germ layers after gene loading and cloning (Fig. 5i,j). These results showed that 201B7/MAC6-ΔNeoR cells were applicable to gene loading with MAC technology.

Conclusion
We generated a novel human cell line panel with HEK293 cell, HT1080 cell, hiMSC, and hiPSC (201B7) lines that contained HACs/MACs, which enabled rapid and precise insertion of GOIs at a defined site on HACs/MACs by a simple transfection method. The GOIs were stably expressed in each cell line, which indicated that the integration site can act as a “safe harbour” to support transgene expression. Thus, our new preprepared cell panel with HACs/MACs may dramatically simplify the construction of HACs/MACs with desired genes and constructed HACs/MACs can be used immediately and directly for functional analyses of genes in desired cell lines.

Material and methods
Ethics statement. This study was approved by the Institutional Animal Care and Use Committee of Tottori University (Permit numbers: 20-Y-14, 17-Y-27, and 16-Y-19) and the Recombinant DNA Experiment Safety Committee of Tottori University to perform recombinant DNA experiments. All experiments were carried out in compliance with the ARRIVE guidelines. All methods were performed in accordance with the relevant guidelines and regulations.

Cell culture. CHO cells derived from a HPRT-deficient cell line (ICRBO218) (NIBIOHN, Osaka, Japan), which contained 21HAC15, MAC24, or MAC446, were cultured in Ham’s F-12 medium (FUJIFILM Wako, Osaka, Japan) with 10% FBS, 1% penicillin/streptomycin (FUJIFILM Wako), and 800 µg/ml hygromycin B (FUJIFILM Wako). CHO cells that contained 21HAC2 were cultured in Ham’s F-12 medium with basicidin S (FUJIFILM Wako). HEK293 cells were purchased from the ATCC (ATCC® CRL-1573™) and cultured in Eagle’s minimum essential medium (Sigma-Aldrich, St. Louis, MO, USA) with 10% fetal bovine serum (FBS, Biowest, Vieux Bourg, Nuaillé, France), 1% non-essential amino acids (Sigma-Aldrich), 1% L-glutamine (Sigma-Aldrich), and 1% penicillin/streptomycin (FUJIFILM Wako). HT1080 cells47 obtained from the ATCC (ATCC® CCL-121™) were cultured in Dulbecco’s modified Eagle’s medium (FUJIFILM Wako) with 10% FBS and 1% penicillin/streptomycin. The human immortalised mesenchymal stem cell (hiMSC)48 line was kindly provided by Dr. J. Toguchida and was cultured in Dulbecco’s modified Eagle’s medium (FUJIFILM Wako) with 10% FBS and 1% penicillin/streptomycin. HEK293 clones that contained each HAC/MAC were selected with 200 µg/mL hygromycin B (FUJIFILM Wako). HT1080 cell and hiMSC clones that contained HAC2 were selected with 8 and 4 µg/mL basicidin S, respectively. HT1080 clones that contained MAC2 were selected with 200 µg/mL hygromycin B. For drug selection after transfection using the SIM system, HACs/MACs expressed the HPRT gene for hypoxanthine-aminoantoiner-thymidine (HAT) resistance following gene insertion. HEK293 and HT1080 clones were selected after transfection in HAT medium (Sigma-Aldrich). hiPSC cell line 201B7 (HPS0063) was provided by the RIKEN BRC and cultured in StemFit AK02N (Takara Bio, Kusatsu, Japan) with Laminin-511 (Nippi, Aichi- chiku, Japan). hiPSCs that contained MAC6 with a neomycin resistance gene were selected with 90 µg/ml G418.
Microcell-mediated chromosome donor. To prepare microcells that contained 21HAC1, MAC2, or MAC4, 1 × 10^7 chromosome donor CHO cells that contained each artificial chromosome were cotransfected with 12 μg pTNH6-H-αCD9 for HEK293 cells and hiPSCs or pTNH6-H for HT1080 cells and 12 μg pCAG-T7-F for each recipient cell line by Lipofectamine 2000 (Thermo Fisher Scientific, Waltham, MA, USA) in accordance with the manufacturer’s instructions. CHO 4H6.1 M cells stably expressed MV-H and F, which provided microcells that contained 21HAC2 as reported previously. Twelve flasks of CHO cells were prepared and micronuclei were induced by treatment with 0.1 μg/mL colcemid. The detailed MMCT protocol has been described previously. The collected microcells were cocultured and fused with 2 × 10^6 cells of each recipient cell line for 24 h in a 6-cm dish (Corning, Corning, NY, USA). Then, the fused recipient cells were subcultured into three 10-cm dishes. Drug selection was started with optimal selectable antibiotics after a further 24 h of incubation. After 14–21 days, drug-resistant colonies were picked up and expanded for the following analyses.

Plasmid construction. To construct pBG-V0b1-ins-ELuc-ins, an EcoRV-digested fragment, which included an ELuc expression unit from CAG-ELuc, was ligated into pBG-V0b1 linearised with EcoRV. To construct pBG2-V1a-ins-tdtmt-ins and pBG2-V2a-ins-BFP-ins, each fragment of pCMV-tdTomato (Takara Bio) and pTagBFP2-N (Evrogen) was amplified by PCR with the following primers, F: 5′-AAACCGGTGTGCTTGCTGACACTGACATTTT-3′, R: 5′-AAAAACGCGCCTCCTCGGTTAT-3′, R: 5′-AGCGGCGTTGGACGCCTTGGAGTCTT-3′.

To construct GLV3-pEF1a-BFP, tDNA-EF1a-BGHpA, a synthesised DNA was digested by EcoRI and ligated into pBG2-V1a for pBG2-V1a-ins-tdtmt-ins and pBG2-V2a for TagBFP2-N via MluI sites in each vector. To construct GLV2-EF1a-tdTomato, tDNA-pEF1a-BGHpA, a synthesised DNA was digested by EcoRI digestion and ligated into pBG2-V1a for pBG2-V1a-ins-tdtmt-ins and pBG2-V2a for pBG2-Vla using primers BFP_cl_EcoRI_F: 5′-AAT TCT GAC TGT CTA GAC AGTCA -3′, R: 5′-CGT CTG CGG CCG GAG CAC-3′, and ligated to a PCR product amplified from pCMV-tdTomato digested by NheI and AvrII. tdTomato was digested by AscI and NheI, and ligated to a PCR product amplified from pBG2-V2a by primers PhiC31 attB_AvrII-NeoII F: 5′-AAA ACC TAG GCC CTC GGC TTG GCC ACC ATG AGG CAG ATG-3′ and ligated to a PCR product amplified by PCR from pBG2-V1a using primers BFP_cl_EcoRI_F: 5′-AAT TCT GAC TGT CTA GAC AGTCA -3′, R: 5′-CGT CTG CGG CCG GAG CAC-3′.

Analyses of HEK293 and HT1080 cells that contained HACs/MACs that harboured tdTomato, BFP, and ELuc via the SIM system. (a) Representative images of fluorescent protein expression in HEK293 cells that contained 21HAC2 inserted with tdTomato, BFP, and ELuc via the SIM system. Brightfield (top, left), tdTomato (bottom, left), EGFP (top, right), and BFP (bottom, right). Bar = 200 μm. (b) Representative image of FISH analysis of HEK293 cells that contained 21HAC2 and the three plasmids. Red: alpha satellite probe (p11-4) staining the centromere of Chr.13, 21 and 21HAC2; green: pBG2-V0b-ins-ELuc-ins. (c) Representative images of fluorescent protein expression in HEK293 cells that contained MAC4 inserted with tdTomato, BFP, and ELuc via the SIM system. Bar = 200 μm. (d) Representative image of FISH analysis of HEK293 cells that contained 21HAC2 and the three plasmids. Green: pBG2-V0b-ins-ELuc-ins. (e) Two Y-axis graph showing the results of FCM analysis with 10,000 cells and luciferase assays of two clones of HEK293 cells that contained 21HAC1, 21HAC2, MAC2, and MAC4 inserted with the three plasmids. Left Y-axis shows the ratio of cells expressing each fluorescent protein in FCM analysis. Right Y-axis shows the photon counts (counts/second) in luciferase assays. Green, red, and blue bars show the ratio of cells expressing each fluorescent protein, and the yellow bar shows the photon counts of the luciferase assay of the clone. Two representative clones are shown for each experiment. (f) Representative images of fluorescent protein expression in HT1080 cells that contained 21HAC2 inserted with tdTomato, BFP, and ELuc via the SIM system. Bar = 200 μm. (g) Representative image of FISH analysis of HT1080 cells that contained 21HAC2 and the three plasmids. Red: alpha satellite probe (p11-4) staining the centromere of Chr.13, 21 and 21HAC2; green: pBG2-V0b-ins-ELuc-ins. (h) Representative images of fluorescent protein expression in HT1080 cells that contained MAC4 inserted with tdTomato, BFP, and ELuc via the SIM system. Bar = 200 μm. (i) Representative image of FISH analysis of HT1080 cells that contained MAC4 and the three plasmids. Red: mouse Cot-1 staining MAC4; green: pBG2-V0b-ins-ELuc-ins. (j) Two Y-axis graph showing the results of FCM analysis and luciferase assays of two clones of HT1080 cells that contained 21HAC2 and MAC4 inserted with the three plasmids. Two representative clones are shown for each experiment.
removed EGFP. The HS4 insulator on X3.1-I-EGFP-I was amplified with primers 5′-ATCATGGATGACTCTAGAGGACAGCC-3′ and 5′-ATAACTAGTCGACCGGCGGCTCCTGACTCCGGTCTGGA-3′, and ligated using NcoI and SpeI sites. mCherry expression vectors with three types of constitutive promoters (PGK, EF1α, and CAG) were purchased from VectorBuilder (Chicago, IL, USA). The vector information is available from the database of VectorBuilder with the following vector IDs: pRP-[Exp]-hPGK > mCherry, pRP-[Exp]-EF1A > mCherry, and pRP-[Exp]-CAG > mCherry. These mCherry expression cassettes were prepared by NotI digestion of the mCherry expression vector. Then, each mCherry expression cassette was inserted into the modified X3.1 without EGFP.

Transfection and gene loading into each HAC/MAC by Cre-loxP and SIM systems. HEK293 and HT1080 cells that contained HACs/MACs were prepared at 5 × 10^6 cells per 10-cm dish. HEK293 and HT1080 cells were transfected using a previously described method. Transfected plasmids were 3.5 µg pBG2-V0b-ins-ELuc-ins, 7 µg pBG2-V1a-ins-tOMF4-ins, 10.5 µg pBG2-V2a-ins-BFP-ins, 3 µg pBS185 (pCMV-Cre), 3 µg pCMV-β-gal, and 3 µg pCMV-PhiC31 integrase. These plasmids were mixed and transfected with Lipofectamine 2000 (Invitrogen), following the manufacturer's instructions. Then, the transfected cells were selected in 2% HAT medium. HiPSCs that contained HACs/MACs were prepared at 2 × 10^6 cells per 10-cm dish.
Figure 5. Characterisation of hiPSCs (201B7) that contained MAC6-ΔNeoR and 201B7/MAC6-ΔNeoR inserted with the three vectors by the SIM system. (a) Representative images of fluorescent protein expression in 201B7/MAC6-ΔNeoR cells. The left panel shows phase contrast and the right panel shows EGFP expression. Bar = 200 µm. (b) Karyotypes of hiPSCs (201B7)/MAC6-ΔNeoR during establishment (left, PDL 0) and in long-term cell culture (right, PDL 20). (c) Representative images of fluorescent protein expression in 201B7/MAC6-ΔNeoR cells inserted with tdTomato, BFP, and ELuc via the SIM system. Bar = 200 µm. (d) Representative image of FISH analysis of 201B7/MAC6-ΔNeoR cells with the three plasmids. Red: mouse cot-1 staining MAC4; green: pBG2-V0b-ins-ELuc-ins. (e) Two Y-axis graph showing the results of FCM analysis and luciferase assays of two clones of 201B7/MAC6-ΔNeoR cells inserted with the three plasmids. (f) Representative image of alkaline phosphatase staining of 201B7 and 201B7/MAC6-ΔNeoR-ElTB#2 cells. Bar = 50 µm. Alkaline phosphatase activity was detected by deposition of purple pigment. (g) Representative image of immunofluorescence staining for OCT3/4 in 201B7 and 201B7/MAC6-ΔNeoR cells. Bar = 50 µm. (h) Relative mRNA expression levels of OCT 3/4 in each cell line or clone. (n = 3). N.D. means not detected. (i) Teratoma formation analysis of 201B7/ MAC6-ΔNeoR cells. Left, middle, and right panels show ectodermal, mesodermal, and endodermal tissues, respectively. (j) Teratoma formation analysis of 201B7/MAC6-ΔNeoR-ElTB#2. Left, middle, and right panels show ectodermal, mesodermal, and endodermal tissues, respectively.
The introduced plasmids were 3.5 µg pBG2-V0b1-ins-ELuc-ins, 7 µg GLV2-tdTomato, 10.5 µg GLV3-NeoR-BFP, 3 µg pEF1a-Cre, 3 µg pCAG-Bbx1 integrase, and 3 µg pCAG-PhiC31 integrase. The mixture of plasmids was introduced into hiPSCs by a NEPA21 electroporator (NEPAGENE, Ichikawa, Japan) with the following conditions: pouring pulse, 135 or 175 V; pulse length, 2.5 milliseconds; pulse interval 50 ms; number of pulses, 2; decay rate, 10% and polarity +, and then transfer pulse, 20 V; pulse length, 50 milliseconds; pulse interval, 50 milliseconds, number of pulses, 2, decay rate, 40% and polarity +/−. Then, the electroporated cells were expanded in the 10-cm dish and 90 µg/ml G418 was added to the culture medium at 48 h after electroporation. We also used another method of electroporation by a Nucleofector 4D (Lonza, Basel, Switzerland). The introduced plasmids were 3.5 µg pBG2-V0b1-ins-ELuc-ins, 7 µg GLV2-tdTomato, 10.5 µg GLV3-NeoR-BFP, 3 µg pEF1a-Cre, 3 µg pEF1a-Bbx1 integrase, and 3 µg pEF1a-PhiC31 integrase. A total of 1 × 10⁶ hiPSCs and plasmids were mixed with P3 Primary Cell 4D-Nucleofector™ X Kit L (Lonza), following the manufacturer’s instructions, and pulsed with the CA-137 program. Eight micrograms of X3.1 that contained mCherry with each constitutive promoter and 2 µg of a Cre expression vector (pBS185) were mixed and introduced into hiMSCs by the NEPA21 electroporator with the following conditions: pouring pulse, 175 V; pulse length, 2.5 milliseconds; pulse interval, 50 milliseconds; the number of pulses, 2; decay rate, 10% and polarity +, and then transfer pulse, 20 V; pulse length, 50 milliseconds, pulse interval, 50 milliseconds, number of pulses, 2; decay rate 40% and polarity +/-.

Then, hiMSCs were selected in 2% HAT medium. pBS185 CMV-Cre was a gift from Brian Sauer (Addgene plasmid # 11,916; http://n2t.net/addingen:11916; RRID: Addgene_11916)⁴⁹.

**Gene knockout by CRISPR/Cas9.** Gene knockout of HPRT1 was performed in HEK293 cells, hiMSCs, and hiPSCs with the multiplex CRISPR/FokI-dCas9 vector system⁹⁶,⁹⁷. The multiplex CRISPR/FokI-dCas9 vector system targeted six sequences for knockout of the HPRT1 gene: HPRT T1: 5’- TAAACGAGCCGGCGGGCGC -3’, HPRT T2: 5’- TGGGCTGCTGTAGACGCTGCG -3’; HPRT T3: 5’- AAATCTGGACCA TTAATGATTAG -3’, HPRT T4: 5’- CTCATGACCTATATTTATACGGAG -3’, HPRT T5: 5’- CACAGAGGG CTACAAATGTATGG -3’ and HPRT T6: 5’- TAAATCTCTTGTGACCTGCCTG -3’. The vector system was transfected into HEK293 cells and then, the HPRT knockout cells were selected by 100 µM 6TG treatment. The vector system was transfected into hiPSCs and then, the HPRT knockout cells were selected by 100 µM 6TG treatment; and the electroporator with the following conditions: pouring pulse, 135 or 175 V; pulse length, 2.5 milliseconds; pulse interval, 50 milliseconds, number of pulses, 2, decay rate 40% and polarity +/−. We also used another method of electroporation by a Nucleofector 4D (Lonza, Basel, Switzerland). The introduced plasmids were 3.5 µg pBG2-V0b1-ins-ELuc-ins, 7 µg GLV2-tdTomato, 10.5 µg GLV3-NeoR-BFP, 3 µg pEF1a-Cre, 3 µg pEF1a-Bbx1 integrase, and 3 µg pEF1a-PhiC31 integrase. A total of 1 × 10⁶ hiPSCs and plasmids were mixed with P3 Primary Cell 4D-Nucleofector™ X Kit L (Lonza), following the manufacturer’s instructions, and pulsed with the CA-137 program. Eight micrograms of X3.1 that contained mCherry with each constitutive promoter and 2 µg of a Cre expression vector (pBS185) were mixed and introduced into hiMSCs by the NEPA21 electroporator with the following conditions: pouring pulse, 175 V; pulse length, 2.5 milliseconds; pulse interval, 50 milliseconds; the number of pulses, 2; decay rate, 10% and polarity +, and then transfer pulse, 20 V; pulse length, 50 milliseconds, pulse interval, 50 milliseconds, number of pulses, 2; decay rate 40% and polarity +/−. Then, hiMSCs were selected in 2% HAT medium. pBS185 CMV-Cre was a gift from Brian Sauer (Addgene plasmid # 11,916; http://n2t.net/addingen:11916; RRID: Addgene_11916)⁴⁹.

**PCR analysis.** PCR analyses were performed with KOD Fx (TOYOBO, Osaka, Japan), following the manufacturer’s instructions. The primers to detect gene insertion in HAGs/MACs via the SIM system were HPRT junc sp F 5’- CGGGTCTTCTCCTCTGGAACAA -3’ and HPRT junc sp R 5’- TCCATAAGACAGATGCTATGCA CC -3’. The primer 5’- HPRT EX1-2 and HPRT EX3-9 reconstitution in HEK293, HT1080 and hiMSCs, and TRANS L1 ⁵

**FISH analysis.** Cells were treated with colcemid to induce metaphase arrest, treated with 0.075 M KCl, and then fixed with methanol/acetate (3:1) (FUJIFILM Wako). FISH was performed with the p11-4 alpha satellite probe⁴⁴ to stain the alpha satellite of hChr.13, 21 and HAC, and mouse Cot-1 DNA to stain the MAC. The probes were labelled with digoxigenin (Roche, Basel, Schweiz) and the inserted plasmid vector targeted to the chromosome fragment was labelled with biotin (Roche). The DNA probes were labelled with a nick translation kit (Roche), following the manufacturer’s instructions. The detailed protocol has been described previously⁵⁸.

**Teratoma formation and histological analysis.** Mice were maintained under specific pathogen-free conditions with a 12-h light−dark cycle. Human iPSCs (1 × 10⁶) were subcutaneously transplanted into a testis of anaesthetised severe combined immunodeficiency mice (Charles River, Yokohama, Japan). A mixed anaesthetic agent prepared with 0.3 mg/kg medetomidine hydrochloride, 4 mg/kg midazolam, and 5 mg/kg butorphanol tartrate was administered intraperitoneally to the mice. Teratomas appeared after ~ 8 weeks. The anaesthetised mice were sacrificed and the teratomas were collected. Then, the teratomas were fixed with 20% neutral formalin/PBS and processed for paraffin sectioning. The sections were stained with haematoxylin and eosin.

**FCM analysis.** To evaluate the ratio of cells that expressed fluorescent proteins, the cells were analysed by FCM using a BD LSRFortessa X-20 flow cytometer (Beckton Dickinson) equipped with 488, 405, and 561 nm lasers to detect EGFP, BFP2, and tdTomato, respectively. The ratio of fluorescence-positive cells of each fluorescent protein was determined by measuring 10,000 cells in each experiment.

**Luciferase assay.** The assay was performed with 6 × 10⁴ cells in each well of a 96-well plate. Luciferase activity was measured with Emerald Luc Luciferase Assay Reagent Neo (TOYOBO), following the manufacturer’s instructions. Bioluminescence was measured for 1 s by an EnVision (PerkinElmer, Waltham, MA, USA)⁶⁰.

**RT-PCR.** Total RNA was isolated with a Nucleospin RNA plus kit (Takara Bio), following the manufacturer’s instructions. cDNA was synthesised with a PrimeScript™ II 1st strand cDNA Synthesis Kit (Takara Bio), following the manufacturer’s instructions. RT-PCR analyses were performed with KOD one, cDNA, and the following primer sets: CXCL1 F 5’- TGTGGAGGCGGAGGGAATGTA -3’ and R 5’- GCCGTCTTCTGCTGAAGTAGG -3’, CD90 F 5’- ATGAACCTGGCCATCACGA -3’ and R 5’- GTGTGCTCAAGGCACCCC -3’, IL-8 F 5’- ACCGGA
AGAAACATCTCAC -3' and R 5' - ATTTGGGGTGGAAGGTTTG -3', and CCL2 F 5' - GCAGCAAGTCG CCAAAGAA -3' and R 5' - AACAGGGGTCGCTGGGAAAG -3'.

**Quantitative RT-PCR.** qRT-PCR analysis was performed with TB Green® Fast qPCR Mix (Takara Bio), following the manufacturer's instructions. The following primer sets were used: mCherry F 5' - AAGGGCGAG GAGATAAACAT -3' and R 5' - ACATGAACCTGAGGCGAGGCGAGT -3', GAPDHIF 5' - AGGAGATCTGCCTAG ACAC -3' and R 5' - GCAGCTACAGCAAATTC -3', ORC3F 5' - AGAAGATGTTGTCGGAGTGTG -3', R 5' - CCACCTTTTGGTTTGCCCATTC -3'. The enzyme reactions and measurements were performed with the Applied Biosystems 7300 Fast Real-Time PCR System (Thermo Fisher Scientific), following the manufacturer's instructions. Fold changes in the gene expression level of mCherry were calculated using the ΔΔCt method and normalised to GAPDH gene expression.

**Immunofluorescence staining.** iPS cells were fixed with 4% paraformaldehyde/PBS (Sigma Aldrich) in a freezer overnight. The samples were blocked with PBS that contained 5% dry skim milk and 0.1% NP-40 (FUJI-FILM Wako) for 15 min at RT. Then, the samples were stained for 1 h at RT with mouse IgG2b against OCT3/4 (1:50) (Santa Cruz Biotechnology, sc-5279, Dallas, TX, USA) as the primary antibody and then anti-mouse IgG2b-Alexa 555 (1:500) (Thermo Fisher Scientific, A-21147) for detection. Image were obtained under a Keyence BZ-X800 (Keyence, Osaka, Japan).

**Alkaline phosphatase staining.** The iPS cells were prepared in confluent and fixed with 4% paraformaldehyde/PBS (Sigma Aldrich) in a freezer overnight. The staining was performed with Alkaline Phosphatase Staining Kit (Cosmo Bio Co., LTD., Tokyo, Japan). The image were obtained by microscopy for cell culture (OLYMPUS CORPORATION, Tokyo, Japan) and the attached CCD camera DP22 and Software.

**Data availability**

The datasets of vector sequences and cell lines generated during and/or analysed during the current study are available from the corresponding author on reasonable request.

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Author contributions
N.U. conceived and designed the experiments. S.T., S.K., T.Suzuki, T.Sakuma., T.Y., and R.M. constructed plasmid vectors for Cre-loxP and SIM systems to load the genes of interest and CRISPR/Cas9 to disrupt the HPRT1 gene. S.T., S.K., H.M., R.M., N.M., and Y.N. transferred the HACs/MACs to recipient cell lines. S.T., S.K., Y.N., M.O., R.M., and C.H. analysed the cell lines by PCR, FISH, luciferase activity, flow cytometry, teratoma formation, RT-PCR, and qRT-PCR. N.U., S.T., S.A., M.O., K.T., and Y.K. wrote the manuscript. All authors reviewed the manuscript.

Competing interests
Dr. Mitsuo Oshimura is CEO and a shareholder of Trans Chromosomics Inc., Dr. Satoshi Abe is a member of Trans Chromosomics Inc., and the other authors declare no conflicts of interest.

Additional information
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