Human Cell Line Panel With Human/Mouse Artificial Chromosomes for Functional Analyses of Desired Genes

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Research Article

**Keywords:** Human artificial chromosome, Mouse artificial chromosome, SIM system, Microcell-mediated chromosome transfer

**DOI:** [https://doi.org/10.21203/rs.3.rs-699365/v1](https://doi.org/10.21203/rs.3.rs-699365/v1)

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Human cell line panel with human/mouse artificial chromosomes for functional analyses of desired genes

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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 of HACs/MACs has been achieved into various human cells including human immortalised mesenchymal stem cells (hiMSCs) and human induced pluripotent stem cells (hiPSCs). However, the conventional strategy of gene-introduction with HAC/MAC required 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 microcell-mediated chromosome transfer (MMCT). To overcome these limitations and accelerate chromosome vector based functional assay 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 the premade HAC/MAC-introduced cell lines via the Cre-loxP system or simultaneous insertion of multiple gene-loading vectors (SIM system). 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 the HAC/MAC-containing cell line panel has dramatically simplified and accelerated gene introduction via HACs/MACs, thereby facilitating functional analyses of introduced genes.

Introduction
Human artificial chromosomes (HACs) and mouse artificial chromosome (MACs) have unique characteristics as vectors for gene delivery, which include stably and independently maintenance without disruption of the host
genome and the capacity to carry numerous genes and megabase-sized genomic loci with physiological regulatory elements \(^1\text{-}^4\). HAC/MAC technologies have been used for gene and cell therapies of Duchene muscular dystrophy \(^5\text{-}^9\) and to generate trans-chromosomal (Tc) animals including a mouse model of Down syndrome \(^10,11\) and humanised drug metabolism \(^12\text{-}^17\). Furthermore, several types of HACs have been used in cancer research and drug screening for cancer therapy \(^18,19\), centromere and telomere function elucidation \(^20\text{-}^20\), a system of quantitatively tracking epigenetic memory in the field of synthetic biology \(^21\), and protein production \(^22\). To accelerate the gene-loading of multiple genes into HACs/MACs, we developed several systems for multiple gene insertions, such as simultaneous or sequential integration of multiple gene-loading vectors (SIM system) \(^23,24\), a multi-integrase (MI) system \(^25\text{-}^28\), and homologous recombination with clustered regularly interspaced short palindromic repeats (CRISPR)/CRISPR-associated protein 9 (Cas9) \(^29\). HACs/MACs are transferrable into desired cells by microcell-mediated chromosome transfer (MMCT) \(^30\). Although MMCT traditionally employs polyethylene glycol \(^31\), we developed a novel microcell membrane fusion method with the envelope proteins of measles virus (MV) \(^32,33\), amphotropic virus, and ecotropic virus \(^34\), which improved the transfer efficiency (\(1\times10^{-4}\) to \(1\times10^{-5}\)). However, specialised equipment and a laborious and time-consuming process are required for MMCT of HACs/MACs, because in accordance with each experimental purpose, HACs/MACs with desired genes are constructed in CHO and A9 cells and individually transferred to a target cell line via MMCT, then isolated the clones containing the desired HACs/MACs (Fig. 1a). Therefore, we have previously employed mouse embryonic stem cells that contain a MAC with the MI system to facilitate the generation of Tc mice \(^35\). Under such circumstances, ready-made human cell lines containing HACs/MACs will be useful platform for simple and stable gene expression. The detailed structures of each HAC/MAC are shown in Supplementary Figure S1. Here, we report the generation of a human cell line panel to facilitate functional analyses of genes of interest using HACs/MACs (Fig. 1b). As representative human cell lines, we used HEK293 (ATCC® CRL-1573™), 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) line \(^36,37\) and human induced pluripotent stem cell (hiPSC; 201B7) line\(^38\). 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 EGFP\(^{39}\), and MACs were derived from mouse chromosome 11, such as MAC2 without EGFP, MAC4 with EGFP\(^{35,40,41}\), and MAC6 with EGFP. These HACs/MACs had a loxP site and partial HPRT gene as an acceptor site for the SIM system, which enable 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, HT1080, hiMSCs, and hiPSCs) that contained HACs/MACs (Fig. 1) (Table 1).

**Table 1**

| 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 gene of interest (GOI) were inserted into the HAC/MAC via the SIM system for HEK293, HT1080, 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 show gene silencing in human pluripotent stem cells, 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, NeoR gene on the MAC6 in hiPSCs was knocked out, namely 201B7/MAC6-ΔneoR (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 contain HACs/MACs accepted three plasmid vectors by simultaneous introduction using the SIM system (Supplementary Fig. S2b). As model genes for the demonstration, a luminescent protein, Emerald luciferase (ELuc), and two fluorescent proteins, tdTomato and TagBFP2-N, were selected (hereinafter collectively called EITB). Specifically, we evaluated HEK293 cells that contained 21HAC1, 21HAC2, MAC2, or MAC4, and 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. The obtained drug-resistant clones were analysed by fluorescence microscopy, flow cytometry (FCM), and luciferase activity. These assays showed fluorescent proteins and luciferase expression among each cell line of representative HEK293 and HT1080 clones as expected. The results of HEK293/21HAC2-EITB cells are shown in Fig. 3a, 3b, and 3e, HEK293/MAC4-EITB cells are shown in Fig. 3c–3e, HT1080/21HAC2-EITB cells are shown in Fig. 3f, 3g, and 3j, and HT1080/MAC4-EITB cells are shown in Fig. 3h–j. FISH analyses showed stable maintenance of HACs/MACs that contained the transgenes independently from host chromosomes. Fluorescence imaging of
HEK293/21HAC1-ElTB cells and HEK293/MAC2-ElTB also showed expression of tdTomato and TagBFP2-N (Supplementary Fig. S3a and S3b). 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 the transfected plasmid was inserted into the HAC/MAC as expected (Supplementary Fig. S3c and S3d). These results showed that multiple gene loading into HAC/MAC via SIM system was successfully achieved in our established human cell line panel (Fig. 2a–2f), enabling seamless application of our HAC/MAC technology for gene functional assay in human cells in future.

**Characterisation of hiMSCs that contain HACs/MACs and demonstration of gene loading by the Cre-loxP system**

As 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 analysis in these clones(Fig. 4b). For the recipient hiMSC cell lines for HAC introduction, 6-thioguanine (6TG)-resistant clones (#3 and #6) were used in order to obtain HAT-resistant 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 level (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 for a platform of gene loading. We validated whether the 21HAC2 could function 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, EF1α, and CAG) in 21HAC2 in hiMSCs with defined (single in this study) copy number. Among the drug-resistant clones obtained by transfection with a plasmid that carried each promoter, 10 clones were picked up in order of the fluorescence intensity of mCherry under a fluorescence microscope and used for subsequent analysis to measure each
promoter activity. Fluorescence imaging of mCherry-expressing cells with each promoter indicated that CAG and EF1α promoter activities were higher than the PGK promoter activity (Fig. 4d). qRT-PCR of the mRNA expression level of mCherry also indicated that CAG and EF1 promoter activities were higher at 22.4-fold (CAG) and 40.5-fold (EF1α) compared with the PGK promoter activity (n=10) (P < 0.01) (Fig. 4e). There was no significant difference between the activities of CAG and EF1 promoters. These results supported a previous study that compared promoter activity in MSCs with a viral vector system for gene expression.43 Because the Cre-loxP system had the same adaptor as the SIM system in 21HAC2, the SIM system would be applicable to hiMSCs/21HAC2. These results showed that hiMSCs that contained 21HAC2 were applicable to gene loading and gene functional analyses.

Characterisation of hiPSCs that contained HACs/MACs 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 (QH) 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). 201B7/MAC6-ΔNeoR cells formed teratomas with the three germ layers (Fig. 5c). 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. 5d). FISH analysis showed that the transgenes were integrated into the MAC and the MAC was independently maintained in the hiPSCs (Fig. 5e). 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. 5f). Expression of all introduced genes was detectable among the representative clones. Furthermore, 201B7/MAC6-ΔNeoR-EITB cells showed a normal karyotype and stable maintenance of the MAC as well as 201B7/MAC6-ΔNeoR. The hiPSC line 201B7/MAC6-ΔNeoR-EITB showed pluripotency to differentiate into the three germ layers after the gene-loading and cloning.
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: 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 acts as a “safe harbour” to support transgene expression. Thus, our new premade cell panel with HACs/MACs could dramatically simplify the construction of HACs/MACs with desired genes and constructed HACs/MACs can be used immediately and directly for the 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 Number: 20-Y-14, 17-Y-27, 16-Y-19) and the Recombinant DNA Experiment safety Committee of Tottori University for performing recombinant DNA experiments. All experiments were carried out in compliance with the ARRIVE guidelines. All methods were carried out in accordance with relevant guidelines and regulations.

Cell culture
CHO cells that derived from a hypoxanthine phosphoribosyl transferase (HPRT)-deficient cell lines (JCRB0218)(NIBIOHN, Osaka, Japan) contained 21HAC1, MAC2, or MAC4 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 blasticidin 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, Nuaille, France), 1% non-essential amino acids (Sigma-Aldrich), 1% L-glutamine (Sigma-Aldrich), and 1% penicillin/streptomycin (FUJIFILM Wako, Osaka, Japan). HT1080 cells
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) 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 blasticidin 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-aminopterin-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, Adachiku, Japan). hiPSCs that contained MAC6 with a neomycin resistance gene were selected with 90 µg/mL G418.

**Microcell-mediated chromosome transfer**

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 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 4H6.1M 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 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 hours 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 hours 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 includes 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’-GAACCTGCACTAGCCATCATGTTCTTTCCTGCGTTAT -3’, R: 5’-AAAAACGCGTGTCTATGCTAGCGATTTAAGATACATTGATGAGTT-3’. Then, each PCR product was digested with AflIII and ligated to a fragment of pinsB4ins prepared by HincII and AflIII digestion. Then, fragments from pinsB4ins that contained tdTomato or TagBFP2-N were prepared by EcoRI digestion 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 and ligated in an annealed double strand palindrome oligo DNA, 5’- AATTCTGACTGTCTAGACAGTCA -3’, including an XbaI site. Then, tDNA-pEF1a-BGHpA-XbaI was digested by XbaI and ligated to a fragment of pCMV-tdTomato digested by NheI and AvrII. tDNA-pEF1a-tdTomato was digested by Ascl and NheI, and ligated to a PCR product amplified by PCR from pBG2-Vla using the following primers: BxbI-PhiC31_CL_F: 5’- CGCATGGGCGCGCCTGCCCCTGCGCGCTGCCTCGTC -3’ and BxbI-PhiC31_CL_R: 5’- CTAGTCTAGGGACCCTACGCCCCCAACTGA -3’ and by digestion with Ascl and NheI. To construct GLV3-NeoR-pEF1a-BFP, tDNA-EF1a-BGHpA, a synthesised DNA, was digested by EcoRI and ligated to a PCR product amplified from pTagBFP2-N by primers BFP_cl_EcoRI_F: 5’- CGAGTCGAAATTCCGCCACCAGCTGCTTCAAGGCGAAGAGCTGA -3’ and BFP_cl_EcoRI_R: 5’- TGTAACGAAATCCTATTAAATTAGTTTTGTGCCTAGTTTGC -3’, and digested by EcoRI. Furthermore, tDNA-EF1a-BFP was digested by Ascl and NheI, and ligated to a PCR product amplified from pBG2-V2a by primers PhiC31 attB 3’HPRT AscI F: 5’- CGCATGGGCGCGCCTGCTAGGGATCAGGCTTCTCGGAAG-3’ and PhiC31 attB 3’HPRT cl R: 5’- CTAGTCTAGGGACTGCGGTCTTTCCTCCGCCT -3’, and digested by Ascl and AvrII (GLV3-pEF1a-BFP). Then, GLV3-pEF1a-BFP was digested by Ascl and Hind III, and ligated to three DNA fragments amplified
by PCR from GLV3-BFP with primers AscI-PhiC31 attB-AvrII F: 5’-GGCCGCATGGCGCGCCGATG -3’ and AscI-PhiC31 attB-AvrII R: 5’-AAAACCTAGGTCATCATGATGGACCAGATG -3’, pBG2-V1b1 with primers AvrII-NeoR-AgeI F: 5’- AAAACCTAGGTCATCATGATGGACCAGATG -3’ and AvrII-NeoR-AgeI R: 5’- AAAACCGGTCCCACAGCTGTTCTTTCCGC -3’, and an annealed double-stranded oligomer DNA with primers AgeI-HindIII oligo F: 5’- AGCTTGATTTCGGCCTATTGA -3’ and AgeI-HindIII oligo R: 5’- CCGGTCAATAGGCCAAATCA -3’. The mCherry expression vector with a Cre-loxP system was a modified X3.1-I-EGFP-I vector. X3.1-I-EGFP-I that contained the Cre-loxP system was digested with NcoI and SpeI, removing EGFP. The HS4 insulator on X3.1-I-EGFP-I was amplified with primers 5’- ATCCATGGATCGACTCTAGAGGGACAGCC -3’ and 5’-ATAACTAGTCGACGCGGCCGCCTCACTGACTCCGTCCTGGA -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 HACs/MACs 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-tdTomato-ins, 10.5 µg pBG2-V2a-ins-BFP-ins, 3 µg pBS185 (pCMV-Cre), 3 µg pCMV-Bxb1 integrase, 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. 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-Bxb1 integrase, and 3 µg pCAG-PhiC31 integrase. The mixture of the plasmids was
introduced into hiPSCs by NEPA21 electroporator (NEPAGENE, Ichikawa, Japan) with the following conditions: pouring pulse, 135 or 175 V; pulse length, 2.5 milliseconds; pulse interval 50 msec; the number of pulses, 2; decay rate, 10% and polarity +, and then transfer pulse, 20 V; pulse length, 50 msec; pulse interval, 50 msec; 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 hours after electroporation. We also used another method of electroporation by Nucleofector 4D (Lonza, Basel, Switzerland). The introduced plasmids were 3.5 µg pBG2-V0b-ins-ELuc-ins, 7 µg GLV2-tdTomato, 10.5 µg GLV3-NeoR-BFP, 3 µg pEF1a-Cre, 3 µg pEF1a-Bxb1 integrase, and 3 µg pEF1a-PhiC31 integrase. A total of 1×10^6 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 msec; pulse interval, 50 msec; the number of pulses, 2; decay rate, 10% and polarity +, and then transfer pulse, 20 V; pulse length, 50 msec; pulse interval, 50 msec; 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 # 11916 ; http://n2t.net/addgene:11916 ; RRID:Addgene_11916).

**Gene knockout by CRISPR/Cas9**

Gene knockout of HPRT1 was performed in HEK293 cells, hiMSCs, and hiPSCs with the multiplex CRISPR/FokI-dCas9 vector system. The multiplex CRISPR/FokI-dCas9 vector system targeted six sequences for knockout of the HPRT gene:

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- TAACGGAGCCGGCCGCGCGCGCG -3', HPRT T1: 5'-TAACGGAGCCGGCCGCGCGCGCG -3', HPRT T2: 5'-
- TGCCGTCTGGTTGAGCAGCCTCGGG -3', HPRT T3: 5'-AAATTCCTCAGCATATGATTAGG -3', HPRT T4: 5'-
- CTCATGGGACTAATTATGGACAGG -3', HPRT T5: 5'-CACAGAGGGCTACAATGTGATGG -3' and HPRT T6: 5'-
- TAAATTCTTTGCTGACCTGCTGG -3'.
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The vector system was transfected into HEK293 cells, and then HPRT knockout cells were screened by 100 µM...
6TG treatment. HT1080 clones with spontaneous mutation of the HPRT1 gene were selected by 6TG treatment. Gene knockout of the neomycin resistance gene was performed with CRISPR/Cas9 targeting 5′ - AGGCTATTCGGCTATGACTGGG -3′ 25.

**PCR analysis**
PCR analyses were performed with KOD Fx (TOYOBO, Osaka, Japan), following the manufacturer’s instructions. The primers to detect gene insertion in HACs/MACs via the SIM system were HPRT junc sp F 5′- CGGCTTCTCCTCCTGAAACAA -3′ and HPRT junc sp R 5′- TCCATAAGACAGAATGCTATGCAACC -3′ for HPRT EX1-2 and HPRT EX3-9 reconstitution in HEK293, HT1080 and hiMSCs, and TRANS L1 5′- TGGAGGCAATAAACAAGAAGAC -3′ and SIM Neo Rv 5′- CGCCTTGGCTGCTGGCGAACA -3′ for HPRT EX1-2 and Neo cDNA reconstitution in human iPSCs.

**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 probe50 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 previously24.

**Teratoma formation and histological analysis**
The mice were maintained under specific pathogen-free conditions with a 12-h light-dark cycle. Human iPSCs (1 × 10^6) were subcutaneously transplanted to a testis of anaesthetized severe combined immunodeficiency (SCID) mice (Charles River, Yokohama, Japan). A mixed anaesthetic agent prepared with 0.3 mg/kg of medetomidine hydrochloride, 4 mg/kg of midazolam, and 5 mg/kg of butorphanol tartrate was administered intraperitoneally for the mice. Teratomas appeared after ~8 weeks. The anaesthetized mice were sacrificed and the teratoma was explanted. Then, the teratoma was fixed with 20%
neutral formalin/PBS and processed for paraffin sectioning. The sections were stained with haematoxylin and eosin.

**Flowcytometry (FCM analysis)**
To evaluate the ratio of cells expressing fluorescent proteins, the cells were analysed by a FCM using Fortessa (BD) LSR X-20 flowcytometer (Beckton Dickinson) equipped with 488nm, 405nm, and 561 nm laser for detection of EGFP, BFP2, and tdTomato, respectively. The ratio of fluorescence-positive cell of each fluorescence protein was determined.

**Luciferase assay**
The assay was performed with $6 \times 10^4$ cells in each well of a 96-well plate. Luciferase activity was measured with Emerald Luc Luciferase Asssay Reagent Neo (TOYOBO) following the manufacturer’s instructions. Bioluminescence was measured for 1 sec by an EnVision (PerkinElmer, Waltham, MA, USA)²⁴.

**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'-TGTGAAGGCAAGGGGAATGTA -3' and R 5'- GCCCCTTTGTCTAAGCCAG -3', CD90 F 5'- ATGAACCTGGCCATCAGCA -3' and R 5'- GTGTGCTCAGGCACCC -3', IL-8 F 5'- ACCGGAAGGAACCATCTC -3' and R 5'- ATTTGGGTGGAAAGGT -3', and CCL2 F 5'- GCAGCAAGTGTCCTCCAAGAA -3' and R 5'- AACAGGTGTCTGGGGAAG -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'- AAGGGCGAGGAGAATCACAT -3' and R 5'- ACATGAACTGAGGACAGG -3', GAPDH1F 5'- AGCCACATCGCTCAGACAC -3' and R 5'- GCCCAATACGACCAATCC -3'.

The enzyme reactions and measurements were performed with the Applied Biosystems 7300 Fast Real-Time PCR System (Thermo Fisher), following the manufacturer’s instructions. Fold changes in the gene expression level of mCherry were calculated using the ∆∆Ct method and normalised to GAPDH gene expression.

**Figure legends**

**Fig. 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 ready-made human cell panel with HACs/MACs. The HAC or MAC was transferred to representative human cell lines, the human cell 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 HAC/MAC 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). 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.

**Table 1 List of human cell lines that contained HACs/MACs.** Cell lines, HAC/MAC, EGFP marker and drug resistance were summarized. Regarding drug resistance, R indicates cells resistant to antibiotics, i.e., Hyg=hygromycin, Puro=puromycin, BS=blasticidin S, and S shows that cells are selectable by drugs, i.e., HAT=hypoxanthine-aminopterin-thymidine medium and Ganc=Ganciclovir.
**Fig. 2** Representative images of FISH analyses of HEK293 cells, HT1080 cells, hiMSCs, and iPSCs that contained each HAC/MAC

(a) HEK293 cells that contained 21HAC1. Red: alpha satellite probe (p11-4) staining the centromere of Chr.13, 21, 21HAC1. (b) HEK293 cells that contained 21HAC2. Red: alpha satellite probe (p11-4) staining the centromere of Chr.13, 21 and 21HAC2; green: pCX-EGFP. (c) HEK293 cells that contained MAC2. Red: mouse cot-1 staining MAC2. (d) HEK293 cells that contained MAC4. Red: mouse cot-1 staining MAC4. (e) HT1080 cells that contained 21HAC2. Red: alpha satellite probe (p11-4) staining the centromere of Chr.13, 21, 21HAC1. (f) HT1080 cells that contained MAC4. Red: mouse cot-1 staining MAC4. (g) hiMSCs that contained 21HAC2. Red: alpha satellite probe (p11-4) staining the centromere of Chr.13, 21 and 21HAC2. (h) iPSCs (201B7) that contained MAC6. Red: mouse cot-1 staining MAC6. Inset: enlarged images of each HAC or MAC.

**Fig. 3** Analyses of HEK293 and HT1080 cells that contained HACs/MACs harbouring 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 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. (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.

Fig. 4 Characterisation of human immortalised mesenchymal stem cells (hiMSCs) that contained 21HAC2 and hiMSC/21HAC2 with the mCherry expression vector introduced by the Cre-loxP system
(a) Representative images of fluorescent protein expression in hiMSCs that contained 21HAC2. The left panel shows phase contrast and the right panel shows EGFP expression. Bar = 100 µm. (b) RT-PCR analysis of various MSC markers in hiMSCs that contained 21HAC2. The original gel-images of the RT-PCR were provided in Supplemental Figure S4. (c) Stability of 21HAC in each hiMSC clone (A03 and D11) after long-term cell culture with or without blasticidin S (BS). The measurement was performed at PDLs of 24 and 39 Bright blue bars show the retention ratio of 21HAC2 at PDL 24. Dark blue bars show the retention ratio at PDL 39 (d) Representative images of fluorescent protein expression in hiMSCs that contained 21HAC2. The left panel shows mCherry fluorescence under the control of the PGK promoter, the middle panel shows that under the control of the CAG promoter, and the right panel shows that under the control of the EF1α promoter. Bar = 200 µm. (e) Relative mRNA expression levels under the control of PGK, CAG, and EF1α promoters (n=10)(*P<0.01). P-values were calculated by the Student’s t-test.

Fig. 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) Teratoma formation analysis of 201B7/MAC6-ΔNeoR. The left, middle and right panels show ectodermal, mesodermal and endodermal tissues, respectively. (d) Representative images of fluorescent protein expression in 201B7/MAC6-ΔNeoR cells inserted with tdTomato, BFP, and ELuc via the SIM system. Bar = 200 µm. (e) 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. (f) 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. (g) Teratoma formation analysis of 201B7/MAC6-ΔNeoR expressing tdTomato, BFP, and ELuc. The left, middle and right panels show ectodermal, mesodermal and endodermal tissues, respectively.

Acknowledgements
We thank Drs. H. Kugoh, M. Hiratsuka, T. Moriwaki, K. Nakamura, T. Ohbayashi, H. Satofuka and T. Ohira for critical discussions. We thank T. Kurosaki, and Y. Sumida for performing the teratoma formation. We thank Dr. T. Nakamura for providing pTNH6-H and pCAG-T7-F plasmids. The hiMSCs were kindly provided by Dr. J. Toguchida. This work was supported in part by JSPS KAKENHI Grant Numbers 15K19615 (N.U.) and 18H06005 (K.T.), and JST CREST Grant Number JPMJCR18S4, Japan (Y.K.). We thank Edanz Group (https://www.jp.edanz.com/ac) for editing a draft of this manuscript. This research was partly performed at the Tottori Bio Frontier managed by Tottori Prefecture.

List of abbreviations
HAC: Human artificial chromosome
MAC: Mouse artificial chromosome
MMCT: Microcell-mediated chromosome transfer
hiMSC: human immortalised mesenchymal stem cell
hiPSC: human induced pluripotent stem cell
Tc: trans-chromosomic
SIM system: System for simultaneous or sequential insertion of multiple genes-loading vectors
MI: Multiple integrase
CRISPR: clustered regularly interspaced short palindromic repeats
Cas9: CRISPR-associated protein 9
HVJ: hemagglutinating virus of Japan
MV: Measles virus
Eco: Ecotropic
ELuc: emerald luciferase
FCM: flow cytometry
FISH: fluorescence in situ hybridisation
6TG: 6-thioguanine
PDL: Population doubling level
QH: quinacrine-Hoechst
FBS: Fetal bovine serum
HAT: Hypoxanthine-aminopterin-thymidine
SCID: severe combined immunodeficiency
MFI: mean fluorescence intensity

Additional information

Author's 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.Osaki, 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., T.S., S.A., M.Oshimura, K.T., and Y.K. wrote the manuscript. All authors reviewed the manuscript.

Declaration of competing interest
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.
Data availability
The datasets of vector sequence and cell lines, generated during and/or analysed during the current study are not publicly available due to no repository of the cell lines and plasmids in publicly but are available from the corresponding author on reasonable request.

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Figures

**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 ready-made human cell panel with HACs/MACs. The HAC or MAC was transferred to representative human cell lines, the human cell 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 HAC/MAC 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). 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.
Representative images of FISH analyses of HEK293 cells, HT1080 cells, hiMSCs, and iPSCs that contained each HAC/MAC (a) HEK293 cells that contained 21HAC1. Red: alpha satellite probe (p11-4) staining the centromere of Chr.13, 21, 21HAC1. (b) HEK293 cells that contained 21HAC2. Red: alpha satellite probe (p11-4) staining the centromere of Chr.13, 21 and 21HAC2; green: pCX-EGFP. (c) HEK293 cells that contained MAC2. Red: mouse cot-1 staining MAC2. (d) HEK293 cells that contained MAC4. Red: mouse cot-1 staining MAC4. (e) HT1080 cells that contained 21HAC2. Red: alpha satellite probe (p11-4) staining the centromere of Chr.13, 21, 21HAC1. (f) HT1080 cells that contained MAC4. Red: mouse cot-1 staining MAC4. (g) hiMSCs that contained 21HAC2. Red: alpha satellite probe (p11-4) staining the centromere of Chr.13, 21 and 21HAC2. (h) hiPSCs (201B7) that contained MAC6. Red; mouse cot-1 staining MAC6. Inset: enlarged images of each HAC or MAC.
Fig. 3

Analyses of HEK293 and HT1080 cells that contained HACs/MACs harbouring 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 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. (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.
Figure 4

Characterisation of human immortalised mesenchymal stem cells (hiMSCs) that contained 21HAC2 and hiMSC/21HAC2 with the mCherry expression vector introduced by the Cre-loxP system (a) Representative images of fluorescent protein expression in hiMSCs that contained 21HAC2. The left panel shows phase contrast and the right panel shows EGFP expression. Bar = 100 μm. (b) RT-PCR analysis of various MSC markers in hiMSCs that contained 21HAC2. The original gel-images of the RT-PCR were provided in Supplemental Figure S4. (c) Stability of 21HAC in each hiMSC clone (A03 and D11) after long-term cell culture with or without blasticidin S (BS). The measurement was performed at PDLs of 24 and 39 Bright blue bars show the retention ratio of 21HAC2 at PDL 24. Dark blue bars show the retention ratio at PDL 39 (d) Representative images of fluorescent protein expression in hiMSCs that contained 21HAC2. The left panel shows mCherry fluorescence under the control of the PGK promoter, the middle panel shows
that under the control of the CAG promoter, and the right panel shows that under the control of the EF1α promoter. Bar = 200 μm. (e) Relative mRNA expression levels under the control of PGK, CAG, and EF1α promoters (n=10) (*P<0.01). P-values were calculated by the Student’s t-test.

**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) Teratoma formation analysis of 201B7/MAC6-ΔNeoR. The left, middle and right panels show ectodermal, mesodermal and endodermal tissues, respectively. (d) Representative images of fluorescent protein expression in 201B7/MAC6-ΔNeoR cells inserted with tdTomato, BFP, and ELuc via the SIM system. Bar = 200 μm. (e) 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. (f) 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. (g) Teratoma formation analysis of 201B7/MAC6-ΔNeoR expressing tdTomato, BFP, and ELuc. The left, middle and right panels show ectodermal, mesodermal and endodermal tissues, respectively.

**Supplementary Files**

This is a list of supplementary files associated with this preprint. Click to download.

- supplementalfigure2.pdf
- supplementalfigure1.pdf
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