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Gene Targeting and Subsequent Site-Specific Transgenesis at the β-*actin* (*ACTB*) Locus in Common Marmoset Embryonic Stem Cells

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Nonhuman primate embryonic stem (ES) cells have vast promise for preclinical studies. Genetic modification in nonhuman primate ES cells is an essential technique for maximizing the potential of these cells. The common marmoset (*Callithrix jacchus*), a nonhuman primate, is expected to be a useful transgenic model for preclinical studies. However, genetic modification in common marmoset ES (cmES) cells has not yet been adequately developed. To establish efficient and stable genetic modifications in cmES cells, we inserted the enhanced green fluorescent protein (*EGFP*) gene with heterotypic *lox* sites into the β-*actin* (*ACTB*) locus of the cmES cells using gene targeting. The resulting knock-in ES cells expressed EGFP ubiquitously under the control of the endogenous *ACTB* promoter. Using inserted heterotypic *lox* sites, we demonstrated Cre recombinase-mediated cassette exchange (RMCE) and successfully established a monomeric red fluorescent protein (*mRFP*) knock-in cmES cell line. Further, a herpes simplex virus-thymidine kinase (*HSV-tk*) knock-in cmES cell line was established using RMCE. The growth of tumor cells originating from the cell line was significantly suppressed by the administration of ganciclovir. Therefore, the HSV-tk/ganciclovir system is promising as a safeguard for stem cell therapy. The stable and ubiquitous expression of EGFP before RMCE enables cell fate to be tracked when the cells are transplanted into an animal. Moreover, the creation of a transgene acceptor locus for site-specific transgenesis will be a powerful tool, similar to the *ROSA26* locus in mice.

Introduction

Embryonic stem (ES) cells are unique cells derived from early embryo that are capable of both pluripotency and self-renewal [1,2]. Genetic modification techniques, including gene targeting and transgenesis, in mouse ES cells have been used as important tools for many aspects of life science [3]. Recently, the establishment of both human ES cells and induced pluripotent stem (iPS) cells has gained public attention in terms of its application to stem cell therapy as a potential source of donor cells [4–6]. For the clinical use of these stem cells, however, thorough preclinical studies using nonhuman primate models are essential to evaluate efficacy and safety. Genetic modification techniques would be a powerful tool in such studies. For instance, the introduction of a reporter gene expression cassette would enable donor cells to be observed and tracked to estimate the utility of stem cell therapy in degenerative disease models such as spinal cord injury, Parkinson's disease, or myocardial infarction. Further, loss-of-function and gain-of-function studies would enable the functions of genes involved in tissue regeneration to be investigated.

However, transgenesis and gene targeting in primate ES cells, including human ES cells, remain difficult mainly because of the obscurity of cell cultures and the low transfection efficiency. Also, the fact that transgenes are often silenced in primate ES cells is another major problem [7,8].

The common marmoset (*Callithrix jacchus*), a type of new world monkey, has several advantages for use as an experimental primate [9,10]. These advantages include their small size (250–400 g), ease of breeding, and high reproductive efficiency (average of 4 pups/year from a breeding pair). Moreover, we have recently developed transgenic common marmosets, in which the transgene is transmitted through the germ line [11]. Therefore, the common marmoset is strongly expected to be useful as a nonhuman primate model for human diseases in preclinical studies. Our group previously reported the successful establishment of common marmoset ES (cmES) cell lines [12,13]. The cmES cells share

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biological characteristics with human and other primate ES cells such as marker gene expression, cytokine response, and differentiation ability, including teratoma formation. Nonetheless, genetic modification techniques in cmES cells have not yet been developed.

Recombinase-mediated cassette exchange (RMCE) is a transgenic strategy using recombinase activity [14–16]. RMCE enables the site-specific integration of a transgene efficiently by inserting a cassette flanked by a pair of heterospecific *lox* sites into a genomic locus in advance [17–20]. This method is highly advantageous relative to conventional transgenesis using nonhomologous end joining because of its predictable, reproducible, and stable expression from a single copy transgene. Therefore, this transgenic strategy is feasible for research using primate ES cells [21–24].

In this report, we demonstrated the insertion of a transgene cassette carrying enhanced green fluorescent protein (*EGFP*) flanked by *lox*P and *lox*PV into the β-*ACTIN* (*ACTB*) locus of cmES cells using gene targeting [25–27]. The resulting $ACTB^{+/EGFP}$ cmES cell line expressed EGFP ubiquitously under the control of the endogenous *ACTB* promoter before RMCE. Hence, this cell line was useful for transplantation experiments as a source of donor cells. We then performed RMCE in the $ACTB^{+/EGFP}$ cmES cells and showed the correct exchange of the *EGFP* transgene with the monomeric red fluorescent protein (*mRFP*) gene. Therefore, RMCE in this cell line is useful for site-specific transgenesis in cmES cells. Further, we also established a Herpes simplex virus-thymidine kinase (*HSV-tk*) knock-in cmES cell line using RMCE. The *HSV-tk* gene is a suicidal gene derived from herpes simplex virus 1, which converts ganciclovir (GCV), a harmless prodrug, into toxic metabolites and selectively eliminates *HSV-tk* gene-expressing cells [28]. The *HSV-tk* knock-in cmES cell line was capable of forming tumors, and tumor growth was significantly suppressed by GCV administration. Therefore, this HSV-tk/GCV system is a promising safeguard against possible tumorigenesis during stem cell therapy.

Materials and Methods

Cell culture

cmES cells (CMES40) were cultured on 50-Gy γ-irradiated mouse embryonic fibroblast feeder cells in ES medium (ESM) which consisted of Knockout Dulbecco's modified Eagle's medium (Invitrogen, Carlsbad CA) supplemented with 20% Knockout Serum Replacement (Invitrogen), 0.1 mM MEM Non-Essential Amino Acids Solution (Sigma-Aldrich, St. Louis, MO), 2 mM L-glutamine (Invitrogen), 0.1 mM β-mercaptoethanol (2-ME; Sigma-Aldrich), and 10 ng/mL basic fibroblast growth factor (bFGF; Wako Pure Chemical Industries Ltd., Osaka, Japan).

For cmES cell splitting, the cells were treated with 10 μM Y27632 (Merck, Darmstadt, Germany) in ESM at 37°C for 1 h, then washed in phosphate-buffered saline (PBS), and dissociated using 0.25% trypsin–ethylenediaminetetraacetic acid (Invitrogen). The dissociated cells were seeded onto new mouse embryonic fibroblast feeder cells.

Construction of targeting vector

A targeting vector was constructed to insert the exchangeable transgene cassette into the 3′ UTR of the marmoset *ACTB* gene. The genomic DNA sequence of the putative *C. jacchus ACTB* locus, which contains an exon–intron structure homologous to human and mouse *ACTB* loci, was identified from the *C. jacchus* genome database (Callithrix_jucchus-2.0.2: the Genome Sequencing Center at Washington University School of Medicine in St. Louis, http://genome.wustl.edu/pub/organism/Primates/Callithrix_jacchus/).

The 2.4-kb fragment containing exon 1–6 of the *C. jacchus ACTB* gene and the 5.4 kb fragment downstream of the stop codon were obtained using polymerase chain reaction (PCR) as the 5′ arm and the 3′ arm, respectively. PCR was performed using special primers and genomic DNA as templates, and then cloned using the Zero blunt TOPO PCR Cloning kit (Invitrogen). These fragments were ligated with the vector pPV-SE2N, which has an internal ribosome entry site (IRES), EGFP, Furin-2A (F2A) [29], and neomycin-resistant gene (Neo) flanked by *lox*P and *lox*PV.

Introduction of targeting vector

Forty micrograms of linearized targeting vector was introduced with 100 μL of Lipofectamine LTX into subconfluent cmES cells in a 10-cm^2 culture plate ($5–7\times10^6$ cells) according to the manufacturer's instructions (Invitrogen). At 16–24 h after transfection, the cells were dissociated and reseeded onto new feeder cells resistant to neomycin (day 1). On day 3, the medium was changed to ESM containing 25 μg/mL of G418 (Invitrogen). The next day, the concentration of G418 was increased to 50 μg/mL. At days 12–14, the G418-resistant colonies were selected and mechanically dissociated by pipetting. The dissociated cells were reseeded onto new feeder cells and partly onto a cell culture plate without feeder cells for DNA preparation.

To detect the homologous recombinants, genomic DNA was prepared using the DNeasy mini kit (Qiagen, Venlo, The Netherlands) and double-digested with *Bam*HI and *Eco*RI. Southern blotting was performed using a DIG High Prime DNA Labeling & Detection Starter Kit II (Roche Applied Science, Manheim, Germany). For the 5′ external probe, a 0.6-kb fragment was amplified using a PCR DIG Probe Synthesis Kit (Roche Applied Science) using the primer pair 5′-CATTCAGCAGCGTGGAGCTC and 5′-AACTTTCCCAG CCTGTCTAC. Single copy integration of the targeting vector was confirmed using Southern blotting with *Bam*HI-digested genomic DNA and the EGFP probe.

Karyotyping, alkaline phosphatase staining, and immunocytochemistry

The chromosome number and karyotype of the cmES cells (passage number, 55) were analyzed as reported previously [12,30]. For alkaline phosphatase staining, the cells were fixed with 4% paraformaldehyde for a minute at room temperature and washed twice with PBS(−), then stained with sigmaFAST BCIP/NBT kit (Sigma-Aldrich).

Immunocytochemistry for SSEA-4, TRA-1-60, and TRA-1-81 were performed as previously reported [12]. For the immunocytochemistry of NANOG, OCT3/4, SOX17, CD31, neural cell adhesion molecule (NCAM), and GFP, the cells were fixed with 100% ethanol for 10 min at room temperature. After antigen retrieval using heat only for NCAM, the cells were incubated with primary antibodies against NANOG

(RCAB0001P; Reprocell, Kanagawa, Japan), OCT3/4 (MAB4419; Millipore, Billerica, MA), SOX17 (AF1924; R&D Systems, Minneapolis, MN), CD31 (N1596; Dako, Carpinteria, CA), and NCAM (413331; Nichirei Biosciences, Tokyo, Japan) at dilutions of 1:200, 1:200, 1:20, 1:1, and 1:1, respectively, overnight at 4°C, followed by incubation with Alexa 594–conjugated anti-goat IgG for SOX17, Alexa 594–conjugated anti-rabbit IgG for NANOG, and Alexa 594–conjugated anti-mouse IgG for OCT3/4, CD31, and NCAM. After washing with PBS(−), the cells were incubated with Alexa 488–conjugated anti-GFP antibody (Invitrogen) for 1 h at room temperature, and washed with PBS(−) 3 times, and then examined under a fluorescence microscope.

Embryoid body formation and flow cytometry

For embryoid body (EB) formation, dissociated cmES cells were resuspended in ESM without bFGF and seeded onto low cell-binding dishes (Nalge Nunc, Rochester, NY). After 15 days of suspension culture, the EBs were dissociated and resuspended in PBS(−) with 2.5 μg/mL of propidium iodide and analyzed for GFP fluorescence using a FACSCalibur (Becton Dickinson, Franklin Lakes, NJ).

In vitro differentiation

For endoderm differentiation, undifferentiated cmES cells were cultured in ESM containing 100 ng/mL of Activin A (R&D Systems) for 5 days. For mesoderm differentiation, the EBs were formed for 7 days and transferred onto chamber slides in ESM containing 50 ng/mL of vascular endothelial growth factor-A (Peprotech, Rocky Hill, NJ) for the next 7 days. For ectoderm differentiation, cmES cells were cultured onto gelatin-coated chamber slide for 21 days in 10 ng/mL of bFGF and 0.5 μM A83-01 (Wako Pure Chemical Industries Ltd.) and N2 and B27 supplement (Invitrogen)-containing Knockout Dulbecco's modified Eagle's medium.

Teratoma formation

Teratoma formation was performed as previously described [12]. Briefly, 5–10×10^6 cmES cells were injected subcutaneously or into the kidney capsule of NOD/shi-scid, IL-2Rγnull (NOG) mice [31]. About 5 weeks after injection, the tumors were collected and fixed in neutral-buffered formalin and embedded in paraffin. The paraffin blocks were sectioned and subjected to immunohistochemical staining. Primary antibodies against NCAM (clone: 1B6; Leica Microsystems, Wetzlar, Germany), vimentin (clone: SP20; Nichirei Biosciences), α-fetoprotein (AFP, clone: C3; Santa Cruz Biotechnology, Santa Cruz, CA), and GFP (rabbit polyclonal anti-GFP antibody; Abcam, Cambridge, United Kingdom) were incubated with the paraffin sections at dilutions of 1:200, 1:100, 1:100, and 1:4,000, respectively. The localization of the bound monoclonal antibodies was detected using the universal immunoenzyme polymer system (Histofine Simple Stain MAX-PO; Nichirei Biosciences). The sections for NCAM and vimentin were pretreated in an autoclave for antigen retrieval. Suitable positive and negative controls were included.

Recombinase-mediated cassette exchange

For RMCE, a donor plasmid, pPV-BE-Red, consisting of BGH poly A, the EF1 α promoter, and *mRFP* cDNA flanked by *lox*P and *lox*PV was constructed [32]. Five micrograms of the donor plasmid and 5 μg of the Cre expression plasmid (pCAGGS-Cre) were cotransfected with Lipofectamine LTX into subconfluent cmES cells in a well of a 6-well plate. The next day, the cells were dissociated and reseeded onto new feeder cells. Resulting colonies that included mRFP-positive cells was selected, dissociated, and reseeded onto new feeder cells. A colony consisting of only mRFP-positive cells was isolated, and an mRFP-positive cell line was established. Genotyping was performed by genomic PCR using the following primer pair: *EGFP*, 5′-GCACAAGCTGGAGTA CAACTACAACAGC and 5′-TCACGAACTCCAGCAGGA CCATG; *mRFP*, 5′-CGAGGACGTCATCAAGGAGT and 5′-CTTGGCCATGTAGGTGGTCT; *Cre*, 5′-CGTACTGACGG TGGGAGAAT and 5′-CCCGGCAAAACAGGTAGTTA.

For the *HSV-tk* donor plasmid, the IRES-driven *HSV-tk* transgene was connected upstream of the IRES-driven Hygromycin-resistant gene and was flanked by *lox*P and *lox*PV (pPV-TK). The Cre expression plasmid and pPV-TK were introduced into BR29 cells, as described above, and the cells were reseeded onto hygromycin-resistant feeder cells. The cells were selected using 25 μg/mL of hygromycin (Wako Pure Chemical Industries Ltd.) for 2 weeks, and hygromycin-resistant colonies were selected. The IRES-*HSV-tk* transgene was detected using PCR with 5′-GTAATGACAAGCGC CCAGAT and 5′-ATGCTGCCCATAAGGTATCG.

GCV administration in vitro and in vivo

Cultured *HSV-tk* knock-in cmES cells and BR29 cells were dissociated and cultured with or without 1 μM GCV on feeder cells.

For the in vivo study, 5×10^5–10×10^5 *HSV-tk* knock-in cmES cells were injected into NOG mice subcutaneously. One and half months after injection, 18 out of 20 NOG mice developed tumors. The tumor diameter was measured (day 10), and the mice were divided into 2 groups: a GCV-treatment group and a control group. The GCV-treatment group was given 50 mg/kg of GCV intraperitoneally on days −2 and 0. The tumor diameter was measured 1 or 2 times per week. The tumor volume was calculated using the formula ($V = ab^2/2$), where a is the larger and b is the smaller axis of the tumor [33]. The tumor growth rate relative to the tumor volume on day 10 was calculated. On day 19, the tumors were collected and the specimens were prepared as described above. Immunostaining using anti-Ki-67 antibody (MIB-1; N1633; Dako) was performed, and Ki-67-positive and Ki-67-negative nuclei in 3 different areas of each slide were counted automatically using DynamicCellCount software (Keyence, Osaka, Japan).

Results

Cloning of the common marmoset ACTB gene

First, to determine the *ACTB* genomic DNA sequence of the common marmoset, we searched the Washington University GSC BLST server using the sequence for human *ACTB* cDNA as a probe. As a result, several sequences were hit, including obvious pseudogenes that did not have an exon–intron structure. From these sequences, we selected contig6614.25, which has a putative exon–intron structure

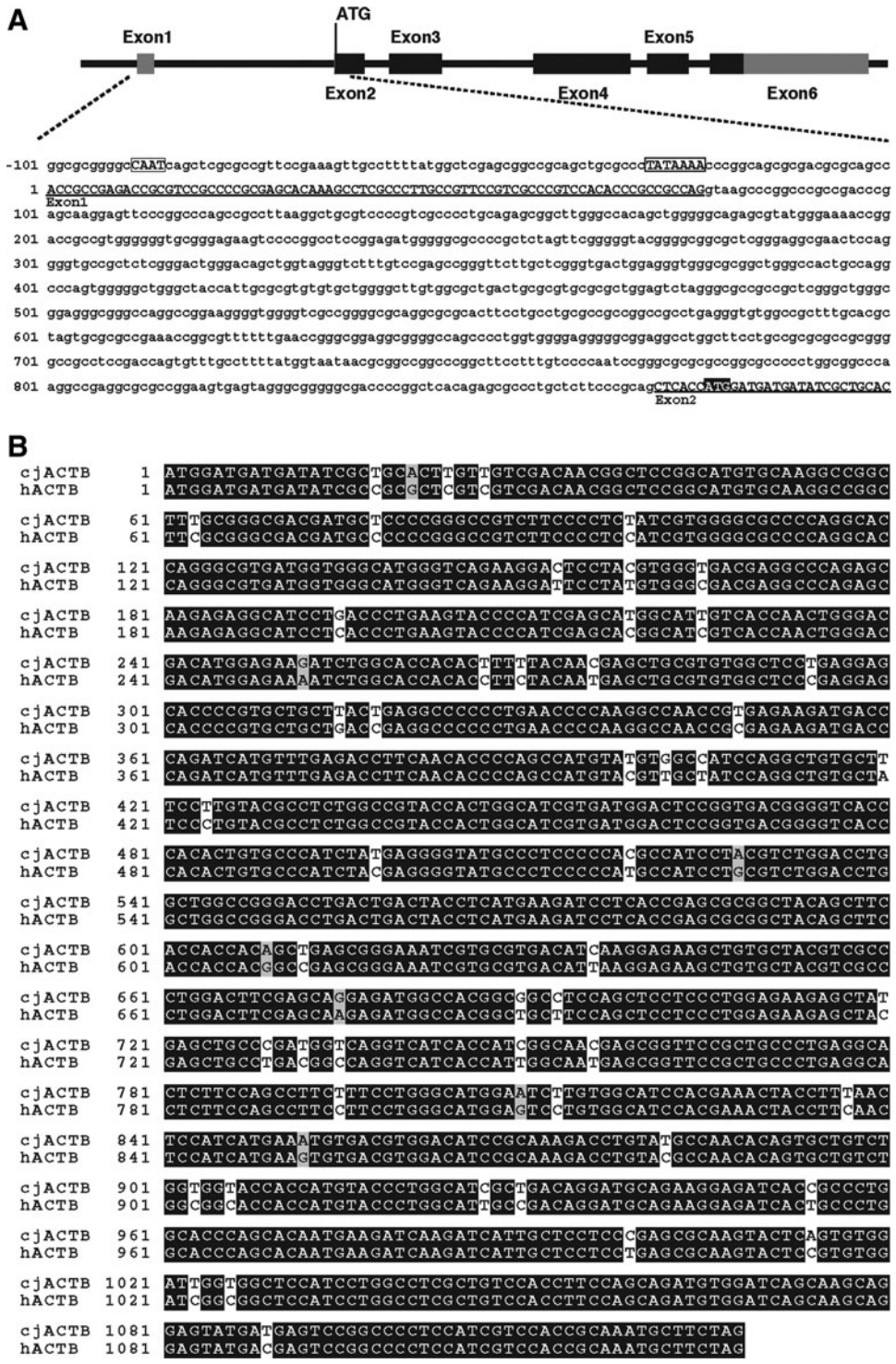

FIG. 1. Analysis of genomic locus for the common marmoset *ACTB* gene. **(A)** Schematic representation of the genomic locus for the common marmoset *ACTB* gene and the 5′ sequence. The UTRs and exons are represented by the *gray boxes* and the *black boxes*, respectively. The *underlined* sequences indicate the exons. The *open-boxed sequences* indicate the putative promoter and enhancer. The first ATG in exon 2 is *shaded*. **(B)** Comparison of the reconstructed open reading frame of the common marmoset *ACTB* gene (cjACTB) versus the human *ACTB* gene (hACTB) open reading frame.

and a TATA box and a CAAT box upstream of the putative ATG, similar to the human *ACTB* gene (Fig. 1A) [34]. The reconstructed open reading frame and amino acid sequence from the genomic sequence were 94% and 100% identical to those of the human *ACTB* gene, respectively (Fig. 1B). Thus, we defined the sequence as the common marmoset *ACTB* gene locus. Next, we designed the homology arms of the targeting vector to insert an exchangeable transgene cassette between the stop codon and the putative polyadenylation signal sequence (Fig. 2A). The designed homology arms were cloned from genomic DNA of the cmES cell line CMES40 using PCR. After confirmation of the sequence, the homology arms were ligated into a backbone plasmid consisting of a transgene cassette flanked by *lox*P and *lox*PV containing the F2A peptide-linked *EGFP* gene and the Neomycin (G418)-resistant gene (*Neo*) driven by IRES.

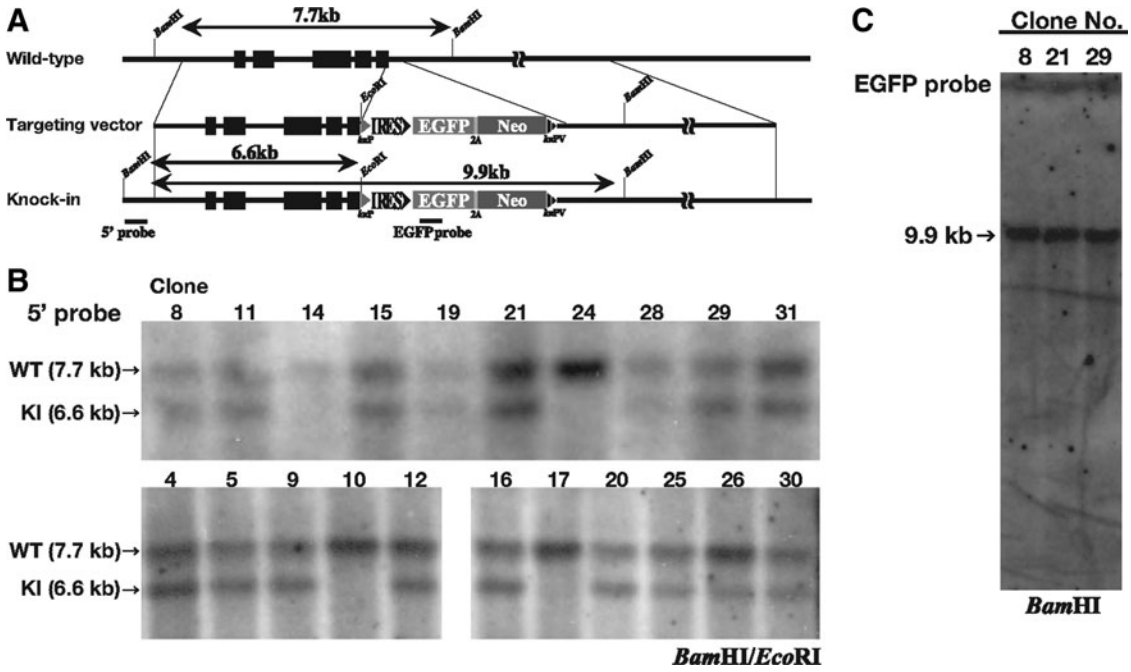

FIG. 2. Gene targeting of the common marmoset *ACTB* gene locus. **(A)** Schematic representations of the wild-type common marmoset *ACTB* gene locus, the targeting vector, and the targeted locus. **(B)** Southern blot analysis of *Bam*HI and *Eco*RI double-digested genomic DNA from G418-resistant clones using the 5′ probe. WT, wild-type; KI, Knock-in. **(C)** Southern blot analysis of the *Bam*HI-digested genomic DNA from clone No. 8, 21, and 29 using the enhanced green fluorescent protein (EGFP) probe shown in **(A)**.

ACTB gene targeting in cmES cells

For gene targeting, the targeting vector was linearized using *Aat*II digestion and introduced into cmES cell CMES40 using Lipofectamine LTX. After selection with G418, 31 resistant colonies from 2 transfections were obtained. These colonies were selected and reseeded onto new feeder cells and then expanded. Genomic DNAs were isolated from some of the cells and analyzed using a Southern blot analysis with the 5′ external genomic probe to confirm homologous recombination. As shown in Fig. 2B, 17 of the 21 clones that were analyzed were homologous recombinants. All the $ACTB^{+/EGFP}$ cmES cell clones were EGFP positive, whereas the other clones were EGFP negative except for one clone. Three $ACTB^{+/EGFP}$ cmES cell clones were used for the subsequent analyses (No. 8, 21, and 29). Single copy integration of the targeting vector was confirmed by detection of a single band of the expected size using a Southern blot analysis with the EGFP probe (Fig. 2C).

Characterization of $ACTB^{+/EGFP}$ cmES cells

Out of the $ACTB^{+/EGFP}$ cmES cell clones, clone No. 29 was used for the subsequent experiments; this cell line was named BR29. A karyotype analysis revealed the maintenance of a normal karyotype (46, XX) in the BR29 cells even after over 50 passages (Fig. 3A). This cell line forms typical ES cell colonies and constitutively expressed EGFP (Fig. 3B). Moreover, the cells were positive for alkaline phosphatase staining, which is characteristic of undifferentiated ES cells (Fig. 3C). To further confirm their undifferentiated state, the expression of primate ES cell-specific surface antigens (SSEA-4, TRA-1-60, and TRA-1-81) was examined using immunocytochemistry. As shown in Fig. 3C, all 3 antigens were expressed on the BR29 cells. Further, the expression of the ES cell-specific transcription factors OCT3/4 and NANOG was observed in the nuclei of the BR29 cells (Fig. 3D). Therefore, this cell line was thought to retain an undifferentiated state.

Differentiation ability of BR29 cells

To estimate their differentiation ability, undifferentiated BR29 cells were transferred into a suspension culture containing a differentiation medium. The BR29 cells spontaneously formed EBs, which expressed EGFP. No EGFP-negative cells were found in the EB (Fig. 4A). Further, a flow cytometry analysis confirmed ubiquitous EGFP expression in EBs (Fig. 4B).

BR29 cells also showed directed differentiation into derivatives of the three germ layers. As shown in Fig. 4C, differentiation into SOX17-positive endoderm cells, CD31-positive mesoderm cells, and NCAM-positive neuroectoderm cells was observed. EGFP expression was persistently observed in the differentiated state.

Next, a teratoma formation assay was performed to estimate their differentiation ability in vivo. When cmES cells were injected subcutaneously or into the kidney capsule of immunodeficient mice, the mice developed tumors (Fig. 5A). Immunohistochemistry of the tumors revealed that the tumor contained derivatives of all three germ layers (Fig. 5B–G). EGFP expression strictly marked the tumor tissue derived from the cmES cells (Fig. 5H, I).

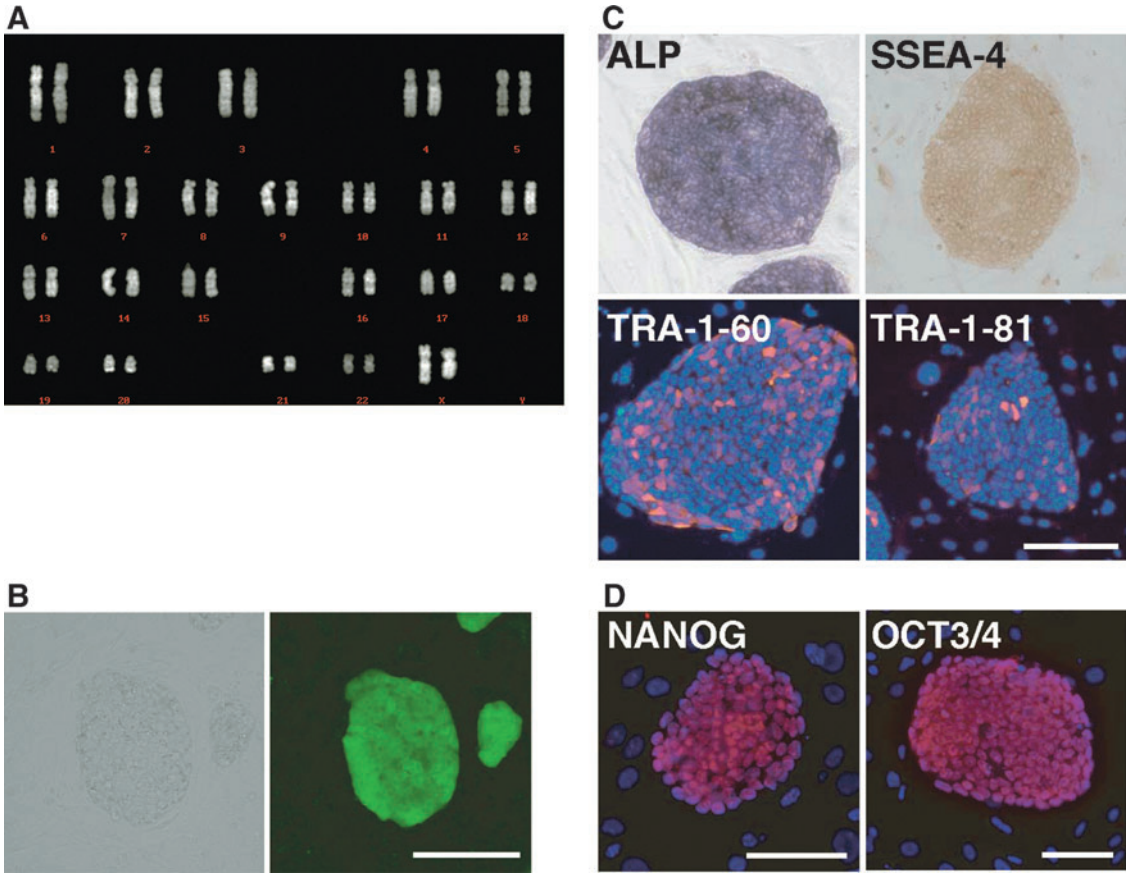

FIG. 3. BR29 cells retain a normal karyotype, an undifferentiated state, and express EGFP. **(A)** Karyotype analysis of the $ACTB^{+/EGFP}$ common marmoset embryonic stem (cmES) cell clone no. 29. **(B)** Phase-contrast microscopy (*left*) and fluorescence microscopy (*right*) of the $ACTB^{+/EGFP}$ cmES colonies. Bar, 200 μm. **(C)** Alkaline phosphatase (ALP) staining and immunocytochemistry for SSEA-4, TRA-1-60, and TRA-1-81 in $ACTB^{+/EGFP}$ cmES cell clone no. 29. Bar, 200 μm. **(D)** Detection of the ES cell-specific transcription factors, NANOG and OCT3/4, using immunocytochemistry. Bars, 100 μm.

Cre RMCE at the ACTB locus

The transgene inserted into the *ACTB* locus of the BR29 cells had 2 heterotypic *lox* sites, wild-type *lox*P and mutant *lox*P, also known as *lox*PV or *lox*2272. Recombination by Cre recombinase rarely occurs between these *lox* sites. Nonetheless, if a DNA fragment is flanked by the same set of *lox* sites, recombination occurs between each of the homologous *lox* sites. As a result of this recombination, the *EGFP* transgene cassette is replaced by the DNA fragment. To confirm the exchange reaction in the cell line, we constructed a donor plasmid EF1α promoter-driven *mRFP* expression cassette flanked by heterotypic *lox* sites (pPV-BE-Red). A poly A signal sequence was inserted upstream of the EF1α promoter to terminate transcription from the endogenous *ACTB* gene (Fig. 6A).

To examine the cassette replacement, the Cre expression plasmid and pPV-BE-Red were cotransfected into BR29 cells using lipofection. As a result, a colony including mRFP-positive cells was obtained. These mRFP-positive cells lost their EGFP expression, indicating that RMCE occurred properly (Fig. 6B). The colony including mRFP-positive and EGFP-negative cells was selected and mechanically dissociated by pipetting, and then reseeded onto new feeder cells. The mRFP-positive and EGFP-negative cmES cell line was established by subsequent serial subcloning based on mRFP expression (Fig. 6C). The cell line maintained the expression of ES cell-specific markers and showed a normal karyotype (Supplementary Fig. S1; Supplementary Data are available online at www.liebertonline.com/scd). Cassette exchange and the absence of Cre expression plasmid integration were confirmed using genomic PCR (Fig. 6D).

Establishment of $ACTB^{+/HSV\text{-}tk}$ cmES cells using RMCE

Next, we tried to establish *HSV-tk* knock-in cmES cell lines using RMCE (Fig. 7A). The cassette-replaced clones were selected using hygromycin resistance. As a result, 6 clones were obtained, all of which had lost their EGFP fluorescence. The genotype was confirmed using genomic PCR (Fig. 7B). The established clone No. 2, BR29-TK, showed an undifferentiated phenotype and a normal karyotype (Supplementary Fig. S1). To examine the sensitivity against GCV in BR29-TK, they were cultured in the growth medium with or without 1 μM of GCV for 7 days. Whereas the BR29 cells grew and formed colonies normally in the presence of GCV, the BR29-TK cells could not grow in the medium containing 1 μM of GCV (Fig. 7C).

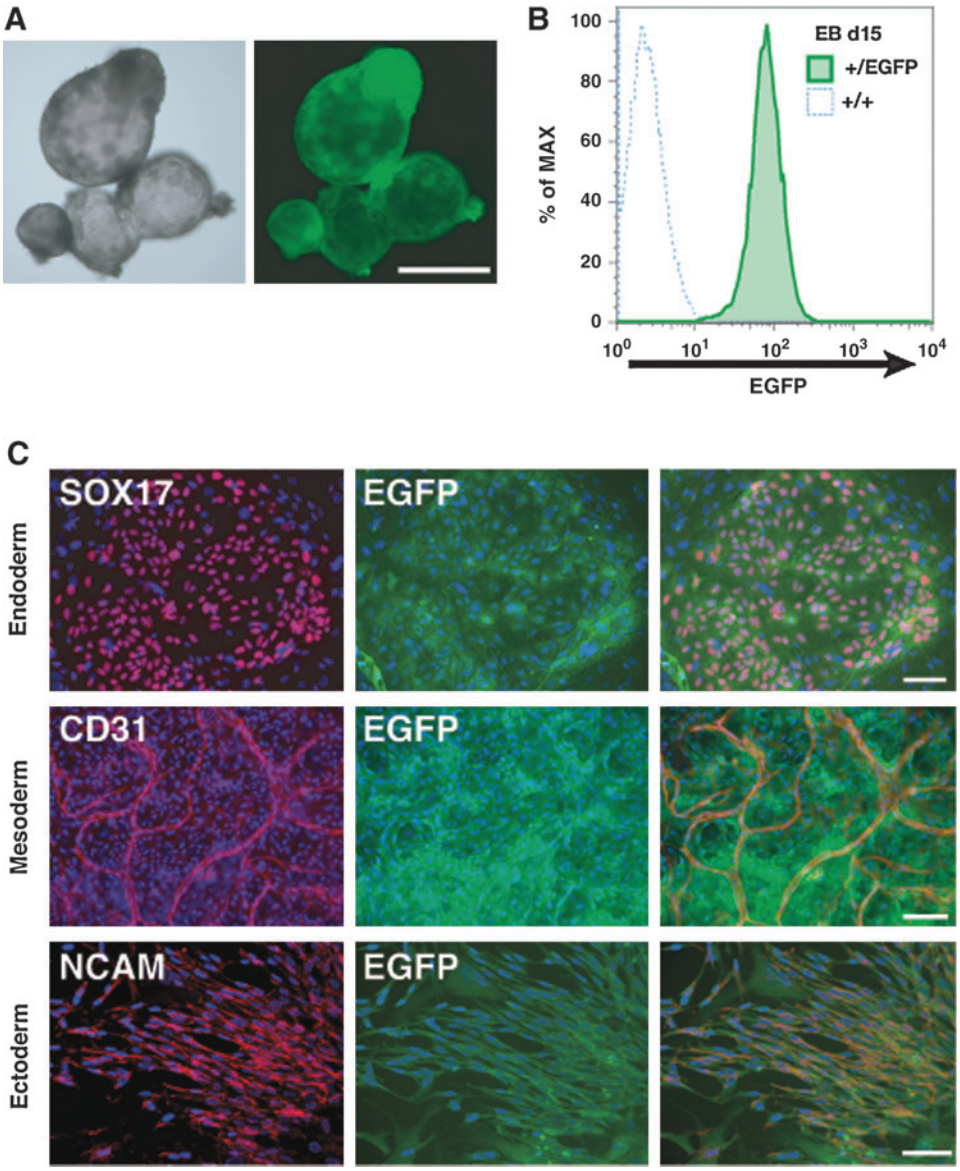

FIG. 4. BR29 cells can differentiate into three germ layers in vitro without the loss of EGFP expression. **(A)** Phase-contrast microscopy (*left*) and fluorescence microscopy (*right*) of day 21 embryoid bodies (EB). Bar, 500 μm. **(B)** Flow cytometric analysis of EGFP expression in day 15 EB. +/EGFP and +/+ indicate the BR29 cells and wild-type cmES cells, respectively. **(C)** Differentiation of BR29 cells into three germ layers. The *right column* shows overlapping images for each of the immunocytochemistry results (*left*, *top, to bottom*, SOX17, CD31, and NCAM; red) and the *center column* (EGFP; green) with DAPI staining (blue). Bars, 50 μm.

To test the effect of GCV treatment on the growth of HSV-tk-expressing tumors in vivo, BR29-TK cells were subcutaneously injected into immunodeficient mice. About 1–1.5 months after injection, 18 of the 20 immunodeficient mice developed a tumor. The tumor volume was measured at each time period (Fig. 7D). Half of the tumor-bearing mice were given 50 mg/kg of GCV on days −2 and 0 (Fig. 7D). As shown in Fig. 7E, the growth rate of the tumor was slightly decreased at 4 days after GCV administration. Although the growth rate gradually increased from days 7 to 19, a significant suppression of tumor growth in response to GCV treatment was observed. Subsequent immunohistochemistry of the tumor revealed that although Ki-67-positive growing tumor cells persisted within the GCV treatment group, the number of Ki-67-positive cells was less than that in the control group (Fig. 7F). Quantitative analysis of the proportion of Ki-67-positive nuclei against all the nuclei of these tumors confirmed a significant reduction in Ki-67-positive cells in the GCV treatment group (Fig. 7G).

Discussion

Primate, including human, and mouse ES cells have different biological properties in several aspects, such as gene expression and their responses to growth factors. Mouse epiblast stem cell (EpiSC) lines from early postimplantation embryos have been shown to share some features with primate ES cells [35,36]. Therefore, primate ES cells are likely to have mouse EpiSCs-like characteristics, rather than mouse ES cell-like ones. Mouse EpiSCs are known to be highly inefficient at producing chimeric mice upon aggregation or injection into host blastocysts, unlike mouse ES cells. Thus, well-developed genetic modification techniques in mouse ES cells were considered to be inapplicable to primate ES cells. Further, a high frequency of transgene silencing in primate ES cells has also been an obstacle for their use in basic research and preclinical research using transgenes [7,8]. However, a recent report has shown that the identity of mouse EpiSCs-like human ES cells can be rewired into a more immature state that extensively shares defining

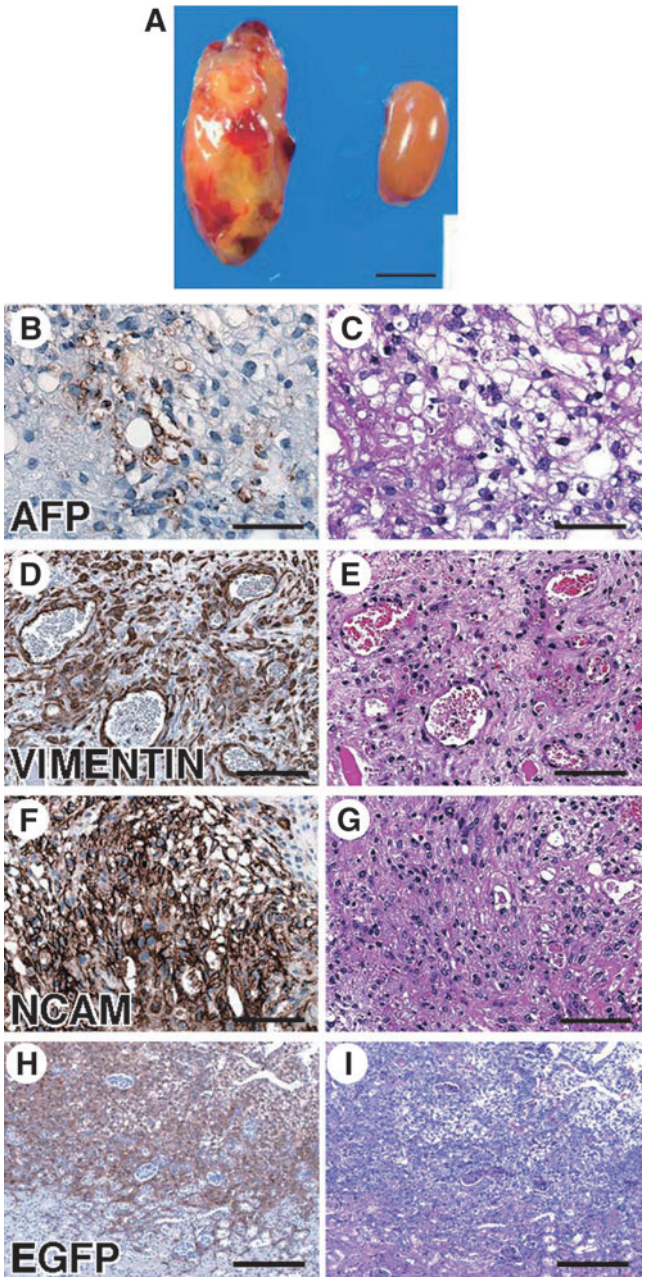

FIG. 5. Teratoma formation of the BR29 cells. **(A)** Tumors formed in the kidney capsule (*left side*) and normal kidney (*right side*). Bar, 1 cm. **(B–G)** Immunohistochemistry of the formed tumor. This tumor is comprised of α-fetoprotein (AFP)-positive cells **(B)**, vimentin-positive cells **(D)**, and neural cell adhesion molecule (NCAM)-positive cells **(F)**, indicating the endoderm, mesoderm, and ectoderm, respectively. Hematoxylin–eosin (HE) staining is shown in **(C, E, G)**. Bars in **(B)** and **(C)**, 50 μm. Bars in **(D–G)**, 100 μm. **(H)** EGFP expression at the border between the teratoma tissue and the normal kidney tissue. **(I)** HE staining of the serially sectioned specimen shown in **(H)**. Bars in **(H)** and **(I)**, 200 μm.

features with mouse ES cells [37]. Such a conversion of human ES cell characteristics was achieved through the ectopic induction of some pluripotent factors combined with leukemia inhibitory factor and the well-known 2i method [38], which was used for the production of the first germline-competent rat ES cells [39,40] using inhibitors of glycogen synthase kinase 3β and the mitogen-activated protein kinase (ERK1/2) pathway [37]. Thus, based on these technological improvements, an increasing need exists for an efficient site-specific transgenic system in primate ES cells for the future generation of knock-in and knock-out primate animals.

In the present study, we built a site-specific transgenic system using RMCE at the *ACTB* locus in cmES cells, enabling stable and ubiquitous transgene expression. For this purpose, we first established a gene targeting technique and inserted heterospecific *lox* sites with the *EGFP* transgene into the *ACTB* locus. The targeted cmES cells retained the undifferentiated ES cell characteristics, with a normal karyotype and a three germ layer differentiation ability after gene targeting, suggesting that our gene targeting method does not affect the undifferentiated state of cmES cells (Figs. 4 and 5). In addition, the inserted *EGFP* transgene could be replaced with another transgene cassette carrying the *mRFP* gene through Cre recombinase.

The instability of transgene expression, such as silencing or ectopic expression, mainly arises from a position effect of the integration site and has often been problematic in mouse transgenesis [41]. To circumvent this phenomenon, a site-specific transgenesis strategy has been developed using the *ROSA26* locus. Although Irion et al. recently reported a human *ROSA26* gene knock-in locus [42], whether an ubiquitous expression pattern from the fetal to adult stage is conserved in primates remains unclear. Another group also established a versatile human ES cell lines for RMCE using random integration with an exogenous promoter [23]. However, the expression pattern can never be predicted in vivo using this method. On the other hand, the *ACTB* gene is a typical housekeeping gene that is ubiquitously expressed and has been used as a positive control of gene expression in many aspects. Since the *ACTB* gene has a critical function in cell homeostasis, latent haploinsufficiency may occur if an allele is disrupted by the insertion of a transgene. Therefore, we designed a targeting vector that inserted an IRES-driven transgene into the 3′ UTR of this gene to avoid any loss of gene expression (Fig. 2A).

We adapted the lipofection method using Lipofectamine LTX to introduce the targeting vector. Electroporation is thought to be more effective for gene targeting in human ES cells than chemical methods [43,44], although we first examined the use of electroporation to introduce the targeting vector, the efficiency was quite low, and only one clone was a homologous recombinant (data not shown). On the other hand, 17 homologous recombinants out of 21 G418-resistant clones were obtained using lipofection (Fig. 2B). Recently, a notable improvement in viral vectors has made gene targeting in human and cynomolgus monkey ES cells possible through the use of helper-dependent adenoviral vector (HDAdV) [45]. HDAdV permits the insertion of a large DNA fragment with a high efficiency and has a low cytotoxicity, compared with common AdV [46–48]. However, the preparation of HDAdV requires an advanced technique, making this technique difficult to perform on a routine laboratory basis. By contrast, lipofection is a relatively simple method that requires neither specific, complicated techniques nor any specific instruments. Thus, the lipofection method used in this report is a promising tool for the targeting of other genes in cmES cells.

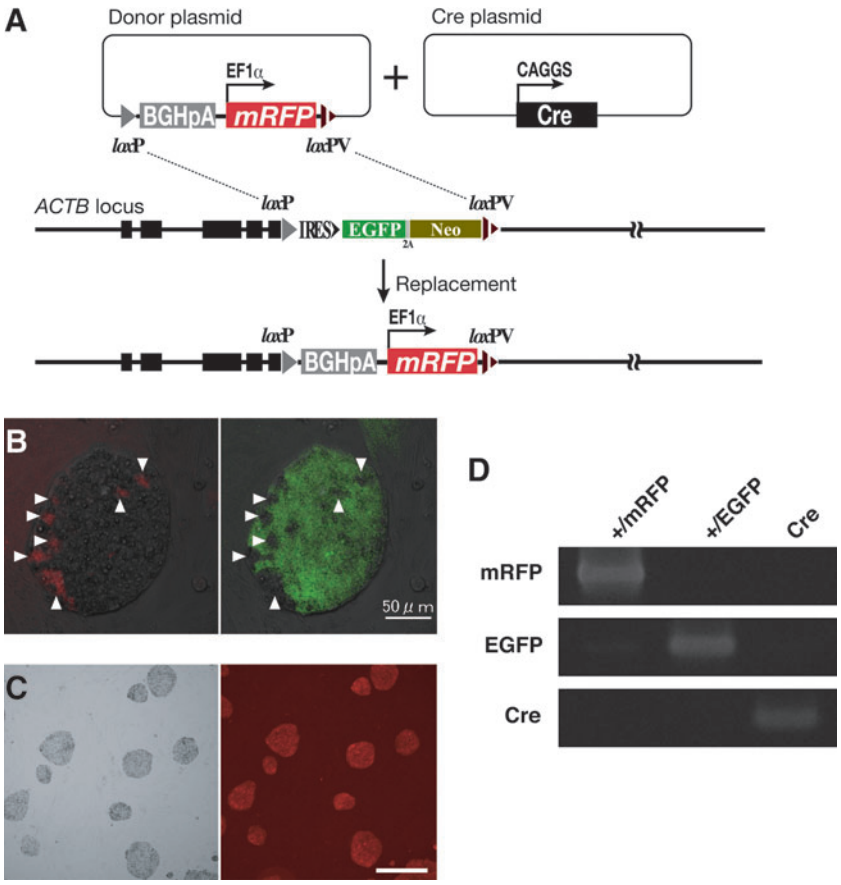

FIG. 6. Cre recombinase-mediated cassette exchange (RMCE) in BR29 cells. **(A)** Schematic representation of the strategy for RMCE at the *ACTB* locus. As a result of cassette replacement, EGFP fluorescence is lost instead of monomeric red fluorescent protein (mRFP) positivity. BGHpA, bovine growth hormone gene polyadenylation signal; EF1α, human elongation factor 1α gene promoter. **(B)** Fluorescence microscopy of mRFP (*left*) and EGFP (*right*) in a colony obtained by RMCE. The *arrowheads* indicate mRFP-positive and EGFP-negative cells. **(C)** Phase-contrast microscopy (*left*) and fluorescence microscopy (*right*) of the established *mRFP* knock-in cmES cells. Bar, 500 μm. **(D)** Polymerase chain reaction (PCR) analysis of the genomic DNA of the *mRFP* Knock-in cmES cells (+/mRFP), BR29 cells (+/EGFP). Cre indicates the Cre expression plasmid used as a positive control for PCR.

The cassette replacement experiment showed that the *EGFP* transgene was correctly replaced with the *mRFP* transgene by cotransfection with the Cre expression plasmid (Fig. 6B, D). The mRFP-positive cmES cell line was successfully established by serial subcloning without any drug selection (Fig. 6C). This technique should facilitate the establishment of various transgenic cmES cell lines with stable and ubiquitous expression.

For therapeutic applications of ES cells or iPS cells as sources of donor cells, the tumorigenic propensity of these cells has been a major barrier [49,50]. Teratoma formation ability is a native characteristic of these undifferentiated pluripotent stem cells. Moreover, these cells may acquire an even higher tumorigenic propensity as a result of mutations or chromosomal abnormalities in cultured cells. Indeed, recurrent chromosomal abnormalities in cultured human ES cells have been reported, and some of these abnormalities have been associated with oncogenic transformation [51–54]. On the other hand, proving the chromosomal normality of all donor cells is practically impossible in viable cells before transplantation. To ensure the safety of stem cell therapy, the application of the HSV-tk/GCV system has been proposed [55,56]. The introduction of the *HSV-tk* gene into donor cells in advance enables tumors originating from the donor cells to be suppressed by the administration of GCV. For this purpose, however, the transgene should be stably expressed even after the cells have transformed into any cell type. However, achieving the ubiquitous expression of the transgene constantly is rather difficult using conventional transgenesis with random integration. Our site-specific transgenesis using the endogenous *ACTB* gene promoter is especially suitable for this purpose. Thus, we established an *HSV-tk* knock-in cmES cell line using RMCE. Using drug selection, about 10 colonies were obtained. Of these, 6 clones were expanded and their genotypes were analyzed. All the clones that were analyzed exhibited correct exchanges. This high efficiency of cassette exchange is probably due to the promoter-less strategy using an IRES sequence, since most randomly integrated clones do not express the transgene except for promoter trapping. BR29-TK, an established *HSV-tk* knock-in cmES cell line, exhibited sensitivity to GCV in vitro. This finding indicates the expression of a functional *HSV-tk* gene. Next, we performed tumor formation in immunodeficient mice using BR29-TK. In the GCV administration group, the tumor growth rate was significantly suppressed. An immunohistochemical analysis revealed that the number of Ki-67-positive growing tumor cells was significantly lower than that in the control group. Therefore, for therapeutic application of ES cells or iPS cells, this HSV-tk constitutive expression from the *ACTB* locus in donor cells is a promising countermeasure to the possible donor cell-derived tumor formation upon transplantation. However, in this experiment, the Ki-67-positive cells partially persisted despite the administration of GCV (Fig. 7F). This result might have arisen because a certain population of the cells that had been in a quiescent state when GCV was administered survived and grew within our moderate GCV administration

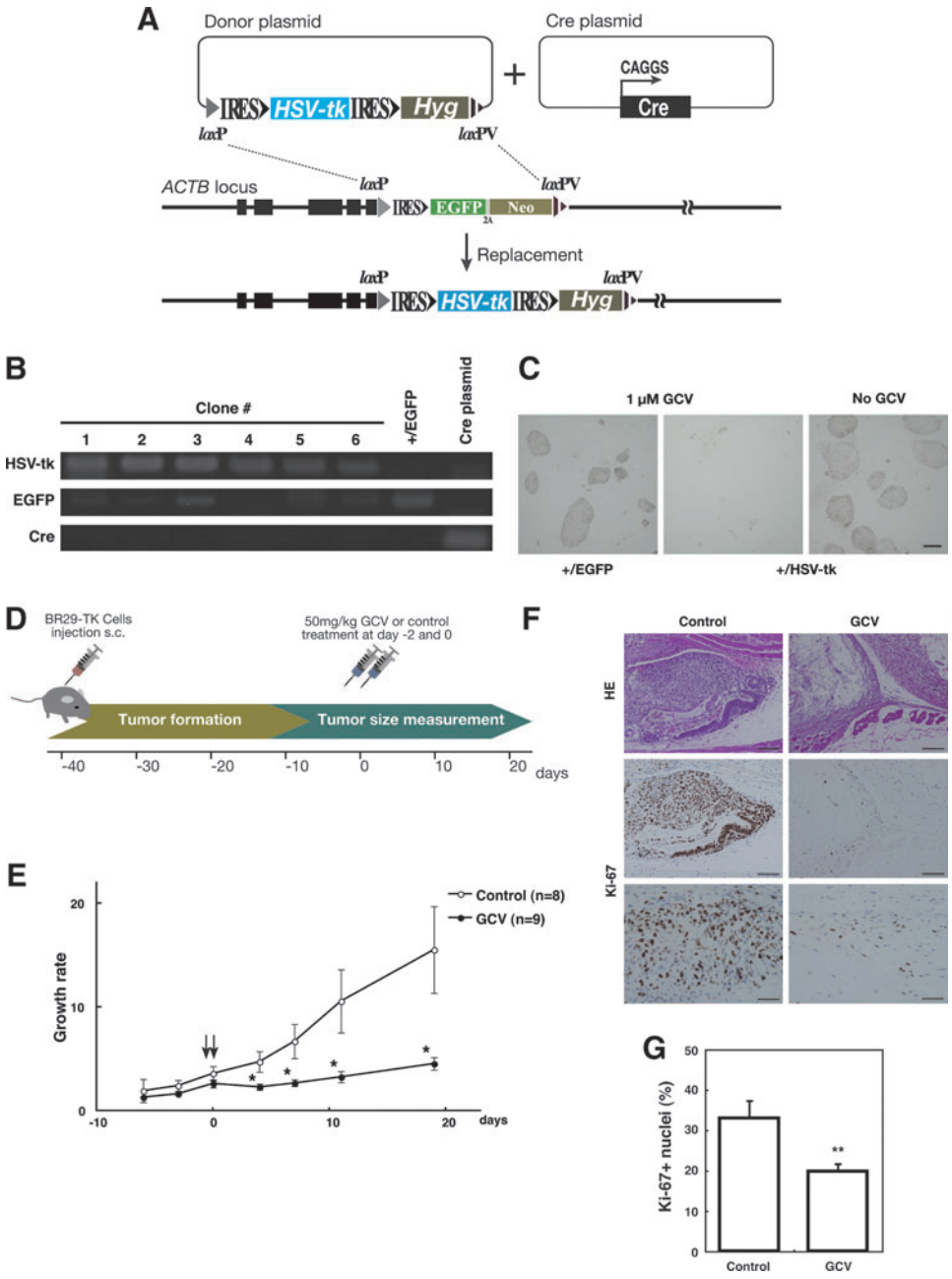

FIG. 7. Establishment of herpes simplex virus-thymidine kinase (*HSV-tk*) knock-in cmES cell line using RMCE. **(A)** Schematic representation of *HSV-tk* knock-in ES cell line using RMCE. The exchanged clone loses EGFP expression and acquires resistance to hygromycin. **(B)** Genotyping results for hygromycin-resistant clones using genomic PCR. **(C)** In vitro study of sensitivity to ganciclovir (GCV) in BR29-TK cells (+/HSV-tk). Bar, 200 μm. **(D)** Schedule of the in vivo study for GCV administration in tumors derived from BR29-TK cells. The cells were injected into immunodeficient mice and developed into tumors after about 1 month. Thereafter, mice harboring tumors were treated with GCV or water as a control on days −2 and 0. The tumor volume was measured during each time period. **(E)** Transitions of tumor growth rate. The *arrows* indicate the timing of the administration. The graph presents the mean of the relative values of the tumor volume against that of day 10. The error bars indicate the standard error of the mean. The *P* values obtained using the Student's *t*-test indicate a significant difference ($^*P < 0.05$). **(F)** The mice were sacrificed on day 20 and the tumors were examined. The *upper panels* show HE staining of the control (*left*) and GCV-treated (*right*) tumors. The *middle* and *lower panels* show Ki-67 immunostaining. Bars in the *upper* and *middle panel*, 100 μm. Bar in the *lower panel*, 50 μm. **(G)** Quantitative analysis of Ki-67-positive cells in the control and GCV-treated tumors. The results are expressed as the percentage of the Ki-67-positive nuclei. The error bars indicate the standard error of the mean (GCV, $n = 8$; Control, $n = 10$). *P* values obtained using the Student's *t*-test indicate a significant difference ($^{**}P < 0.01$).

protocol. In many reports, the HSV-tk/GCV tumor suppression system was given GCV once or twice a day for 5–14 days [57–61]. In our experiment, GCV was administered only once on both days −2 and 0 to reduce adverse effect-related damage to the animals, but this regimen was sufficient to produce a tumor suppression effect. Nonetheless, to eliminate the Ki-67-positive tumor cells completely, a longer GCV administration period is likely needed. Further studies to assess the efficacy of this system using disease model marmosets with states similar to those experienced in clinical settings are expected.

In conclusion, we created a transgene acceptor site at the *ACTB* locus of cmES cells for performing RMCE. This report is the first to demonstrate gene targeting in cmES cells. This cell line allows the efficient targeting of various transgenes using Cre recombinase activity. The transgene expression enabled by the endogenous *ACTB* promoter is ubiquitous and stable. These techniques are expected to be useful for

preclinical studies and stem cell biology using transgenes in the common marmoset.

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Author Disclosure Statement

All authors declare that they have no potential conflict of interest in connection with the submitted article.

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