

Generation of transgenic non-human primates with germline transmission

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The common marmoset (*Callithrix jacchus*) is increasingly attractive for use as a non-human primate animal model in biomedical research. It has a relatively high reproduction rate for a primate, making it potentially suitable for transgenic modification. Although several attempts have been made to produce non-human transgenic primates, transgene expression in the somatic tissues of live infants has not been demonstrated by objective analyses such as polymerase chain reaction with reverse transcription or western blots. Here we show that the injection of a self-inactivating lentiviral vector in sucrose solution into marmoset embryos results in transgenic common marmosets that expressed the transgene in several organs. Notably, we achieved germline transmission of the transgene, and the transgenic offspring developed normally. The successful creation of transgenic marmosets provides a new animal model for human disease that has the great advantage of a close genetic relationship with humans. This model will be valuable to many fields of biomedical research.

The use of transgenic mice has contributed immensely to biomedical science. However, the genetic and physiological differences between primates and mice—including their neurophysiological functions, metabolic pathways, and drug sensitivities—hamper the extrapolation of results from mouse disease models to direct clinical applications in humans. Thus, the development of non-human primate models that mimic various human systems would accelerate the advance of biomedical research. In particular, genetically modified primates would be a powerful human disease model for preclinical assessment of the safety and efficacy of stem-cell or gene therapy.

The common marmoset (*Callithrix jacchus*) is a small New World primate that, because of its size, availability, and unique biological characteristics¹, has attracted considerable attention as a potentially useful biomedical research animal in fields such as neuroscience, stem cell research, drug toxicology, immunity and autoimmune diseases, and reproductive biology. Marmosets have a relatively short gestation period (about 144 days), reach sexual maturity at 12–18 months, and females have 40–80 offspring during their life. Therefore, the application of transgenic techniques to marmosets may be feasible, and would greatly facilitate the study of human disease. In contrast, the more commonly used Old World primates, such as the rhesus monkey (*Macaca mulatta*) and cynomolgus monkey (*Macaca fascicularis*), show slow sexual maturation (about 3 years) and have fewer offspring (around 10) over the female lifespan. Thus, even though marmosets are less closely related to humans than either apes or Old World primates, their potential as transgenic primate models of human disease means they may be uniquely valuable.

Obtaining large numbers of oocytes from primates for transgenic experiments is limited by ethical and economic constraints. However, because retroviral vectors allow the efficient integration of a provirus into the host genome^{2–4}, their use requires fewer oocytes

than some other techniques. Furthermore, the injection of a lentiviral vector into the perivitelline space of a pre-implantation embryo, which is less invasive than injection into the pronucleus, is an advantageous method for generating transgenic animals. In fact, transgenic modification of rhesus monkeys using retroviral vectors and a lentiviral vector^{5–7} has been attempted. In these studies, genomic integration and expression of the transgene was observed in the placenta, but not in the infants' somatic tissues, by objective analyses such as PCR with reverse transcription (RT-PCR) or western blotting.

The recombinant adeno-associated virus has been used for the targeted knockout of the cystic fibrosis transmembrane conductance receptor gene in swine fetal fibroblasts, and targeted gene knockout pigs have been generated by somatic cell nuclear transfer (SCNT) of the fibroblast nuclei into oocytes^{8,9}. Although conceptually this method could be used to make targeted gene-knockout primates, marmoset SCNT techniques are not available at present.

Here we successfully produced transgenic marmosets, by injecting a lentiviral vector containing an enhanced green fluorescent protein (EGFP) transgene¹⁰ into marmoset embryos. Four out of five transgenic marmosets expressed the EGFP transgene in neonatal tissues; the fifth expressed it in the placenta. Two showed transgene expression in the germ cells, and one fathered a healthy transgenic neonate. Our method for producing transgenic primates promises to be a powerful tool for studying the mechanisms of human diseases and developing new therapies.

Production of transgenic marmosets

In a pilot study, we showed that pre-implantation marmoset embryos obtained through natural intercourse had much better developmental potential than embryos obtained by *in vitro* fertilization (IVF). Therefore, both natural and IVF embryos were used in this study.

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To introduce the EGFP gene into the marmoset embryo, three kinds of self-inactivating lentiviral vectors were constructed on the basis of human immunodeficiency virus type 1 (HIV-1), and each carried a different promoter, CAG, CMV or EF1- α . The self-inactivating lentiviral vectors were named CAG-EGFP, CMV-EGFP and EF1- α -EGFP, respectively.

All lentiviral vector injections were performed at the earliest embryonic stage possible using an Eppendorf FemtoJet express and a Narishige micromanipulator. Twenty-seven IVF embryos and 64 natural embryos were injected with a high titre of the lentiviral vector, from 5.6×10^9 to 5.6×10^{11} transducing units per ml (Table 1). Because the perivitelline space of the marmoset early embryo is rather small, 16 of the 27 IVF embryos, and 49 of the 64 natural embryos, at the pronuclear-to-morula stage, were first placed in 0.25 M sucrose in PB1 medium (0.25 M sucrose medium), which made the perivitelline space expand 1.2–7.5-fold (data not shown). The lentiviral vector was then injected into the perivitelline space (Supplementary Data 1). Virus was injected into the blastocoel of the remaining 11 IVF and 15 natural embryos at the blastocyst stage, without the 0.25 M sucrose treatment (Supplementary Data 1).

Immediately after injection, 4 of the IVF and 12 of the natural embryos were transferred to recipient females. The rest were examined for the expression of EGFP, starting 48 h after injection. Among the sucrose-treated IVF and natural early embryos at 48 h after injection, 68.8% and 97.7% expressed EGFP, respectively; of the non-sucrose-treated IVF and natural embryos injected with lentivirus as blastocysts, 85.7% and 87.5% expressed EGFP, respectively (Supplementary Data 1). Therefore, 61 of the natural embryos and 19 of the IVF embryos were transferred to surrogate mothers (Table 1). For the transfers, the recipients were synchronized with the donor oocyte cycle; each recipient received 1–3 embryos per cycle, and 50 surrogate mother animals were used.

Of the surrogate mothers, seven that received natural or IVF embryos became pregnant. Three recipients miscarried on days 43, 62 and 82, and the other four delivered five healthy offspring (three singletons, one pair of twins), one male (number 666) and four females, on days 144–147 after ovulation (Fig. 1). For the infants, the lentiviral vector injection had been performed at the four-cell stage (584), the pronuclear stage (587), and the morula stage (588, 594 and 666). The EGFP transgene was driven by the CAG promoter in three newborns (584, 587 and 588) and by the CMV promoter in the other two (594 and 666; Supplementary Data 1).

Table 1 | Production rates of transgenic marmosets

	Artificial reproductive technique	Natural
Number of GV oocytes	460	No data
Number of matured oocytes (only MII)	201	No data
Number of IVFs performed (including MI)	272	No data
Number of fertilized oocytes	121	No data
Fertilization rate (fertilization per GV)	26.3%	No data
Fertilization rate (fertilization per IVF)	44.5%	No data
Lentiviral injections	27	64
EGFP expression confirmed after 48 h or later	23	52
EGFP expression	17	50
EGFP expression rate	73.9%	96.2%*
ETs	19	61
Number of surrogates	13	37
Number of pregnancies	1	6
Number of deliveries	1	3
Births	1	4
Birth rate (birth per ET)	5.20%	6.55%
Number of Tgs	1	4
Production rate (Tg per injection)	3.70%	6.25%
Production rate (Tg per ET)	5.26%	6.25%
Production rate (Tg per birth)	100	100

ET, embryo transfer; GV, germinal vesicle; MI, metaphase I; MII, metaphase II; Tg, transgenes. * $P < 0.01$, chi-squared analysis.

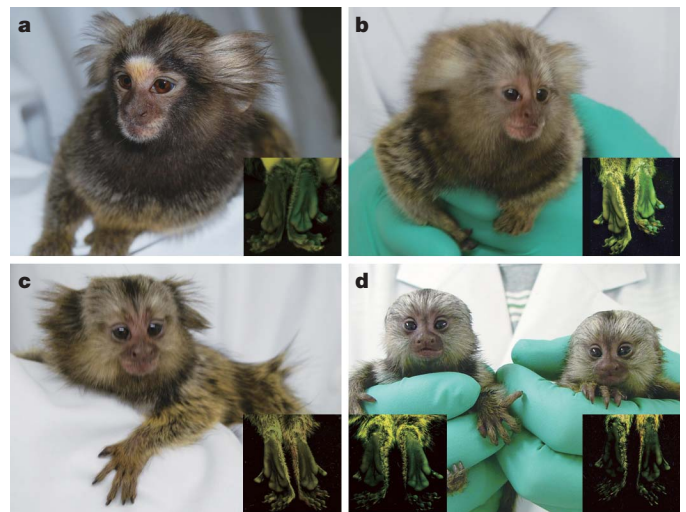


Figure 1 | Self-inactivating lentiviral vector-derived EGFP transgenic marmosets. a–d, The transgenic marmoset infants are shown. Shown are 584 (Hisui) (a), 587 (Wakaba) (b), 588 (Banko) (c), and twin infants 594 (Kei)/666 (Kou) (d). 584, 587 and 588 contained CAG-EGFP and 594/666 carried CMV-EGFP. Inset boxes in each panel show epifluorescent images of the paw of a transgenic animal (right), compared to a wild-type animal's foot pad (left). All animals except 588 expressed EGFP in their paw. 666 expressed EGFP at a slightly lower level.

EGFP transgene integration in the genome

The integration, transcription and expression of the transgene in the infant marmosets were examined using tissues that could be acquired noninvasively (placenta, hair roots, skin and peripheral blood cells). Because marmosets usually eat the placenta after delivery, only three placentae (584, 588 and that shared by twins 594/666) were collected and available for analysis¹¹.

The placental DNA from infants 584 and 588 showed high levels of the transgene content by real-time PCR, whereas that from 594/666 showed a relatively low level (Supplementary Data 2). The transgene was detected in the hair roots, skin and peripheral blood from infants 584, 587, 594 and 666.

Copy numbers of the integrated transgene were determined by Southern blotting analysis. At least four copies of the transgene were integrated into the genome of animal 584, and two copies were present in the genome of animal 587 (Fig. 2). Several integration sites in the genomic DNA of skin fibroblast cells, peripheral blood, the placenta of 594 and 666, and the placenta of 588 were found. Infant 588 showed transgene integration only in the placenta (Fig. 2).

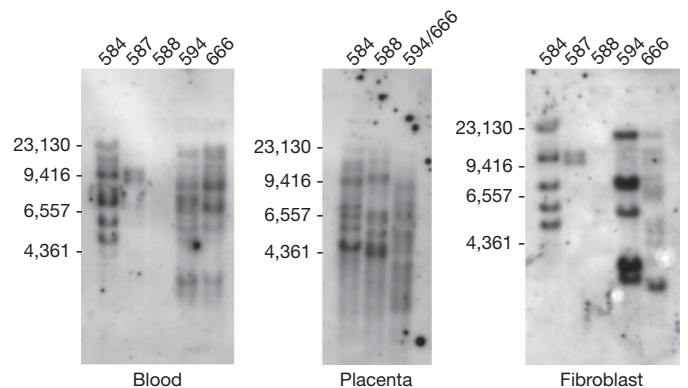


Figure 2 | Transgene insertions in several infant tissues. Southern blot analysis. All infants except 588 showed transgene integration in the skin fibroblast cells and blood, whereas 588 showed transgene integration in the placenta. The lane markers on the left of each gel represent base pairs.

To identify the chromosomal transgene integration sites, fluorescence *in situ* hybridization (FISH) was performed. Consistent with the Southern blotting analysis, the FISH results showed several integration sites in the chromosomes of peripheral blood mononuclear cells (MNCs), and further showed that each infant had different transgene integration patterns with patterns sometimes varied among different MNCs (Supplementary Fig. 1 and Supplementary Data 3). In 584, four transgene integration sites were seen, on chromosomes 2, 7 and 13; in 587, two distinct signals were recognized in the peripheral blood lymphocyte DNA, on chromosomes 3 and 12. No signal was detected in the peripheral blood lymphocyte samples from 588, and several transgene integration patterns were seen in 594 and 666. Infant 594 had at least three different transgene integration patterns, and more than six patterns may have occurred. Infant 666 showed the largest number of integration patterns, up to 13. Moreover, although this animal was male, of the 13 investigated karyograms, eight samples were of the female karyotype, owing to haematopoietic chimaerism caused by blood exchange with his twin, 594.

Expression of the EGFP transgene

EGFP messenger RNA was detected in the hair roots of all the infants except 588 and in the peripheral blood cells of 584 and 587, by RT-PCR. Transcription of the EGFP gene was indicated in all of the placental samples, 584, 588 and 594/666 (Fig. 3a–c).

To assess EGFP expression in tissues, EGFP fluorescence was examined directly by fluorescence microscopy, and immunohistochemical analysis of the hair roots, frozen sections of a small piece of ear tissue, and placenta samples was performed (Fig. 3d–g). EGFP was strongly expressed in the epidermal cells of the ear tissue and stromal cells of the placenta. In all of the animals except 588, EGFP expression was observed in the hair roots and skin. Placental samples from 584 and 588 also showed high levels of EGFP, but it was undetectable in 594/666 (Supplementary Figs 2–4).

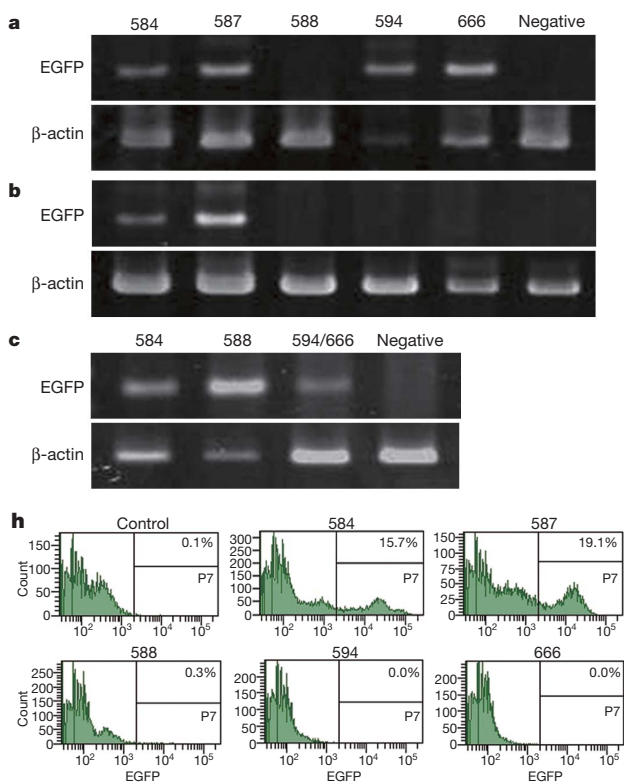


Figure 3 | Transgene transcription and expression in several infant tissues. a–c, RT-PCR results from hair roots (a), peripheral blood (b) and placenta (c). Each lane indicates the animal number. d–g, Immunohistochemical (d, f) and epifluorescence (e, g) analyses using an anti-EGFP antibody, of

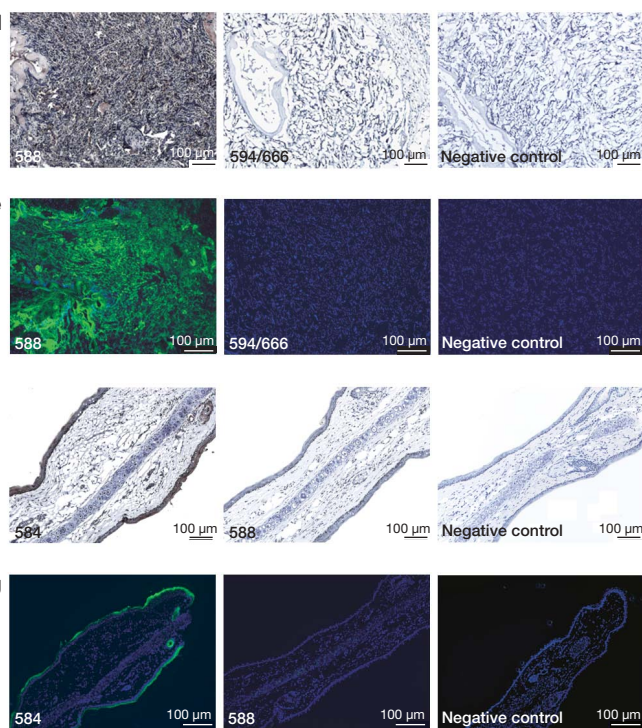
Peripheral blood samples were subjected to flow cytometric analysis using a FACScan. FACS analysis showed EGFP-positive peripheral blood MNCs in 584 and 587. The proportion of EGFP-positive cells was 15.7 and 19.1%, respectively (Fig. 3h). The flow cytometry results corresponded well with those from RT-PCR. Among the peripheral blood cells, the EGFP-positive percentage of granulocytes, lymphocytes and monocytes was 34.5, 3.3 and 18.0% in 584, and 47.7, 4.6 and 20.0% in 587, respectively (Supplementary Fig. 5).

Germline transmission of the transgene

At the moment when two of the animals (666 and 584) became sexually mature, the transgene expression in their gametes was analysed. Semen samples were collected from 666, and live spermatozoa were obtained by the swim-up method in TYH medium. RT-PCR analysis demonstrated the presence and expression of the transgene in the germ cells of 666 (Fig. 4a). IVFs were then performed using semen collected from 666 and wild-type oocytes to analyse the fertility of the germ cells carrying the transgene. Fluorescence microscopy showed that 20–25% of the IVF embryos strongly expressed EGFP, as shown in Fig. 4b. Furthermore, three pre-implantation live natural embryos were collected from female animal 584, and one of these embryos strongly expressed EGFP. The IVF embryos from 666 and two of the natural blastocyst embryos from 584 were shown to express the EGFP transgene by RT-PCR (Fig. 4a). Three EGFP-positive IVF embryos from the male animal (666) were then transferred into a surrogate mother. One neonate (687) was delivered at full term by caesarean section, and this neonate carried the EGFP gene and expressed the transgene in skin (Fig. 4c–e), but not in the placenta and hair.

Discussion

To our knowledge, this is the first report of transgenic non-human primates showing not only the transgene expression in somatic tissues, but also germline transmission of the transgene with the full, normal



frozen ear tissues (f, g) and placentae (d, e). Scale bars, 100 μ m. h, Results of FACS analysis using whole peripheral blood cells. The percentage of EGFP-positive cells is shown in the top right of each panel.

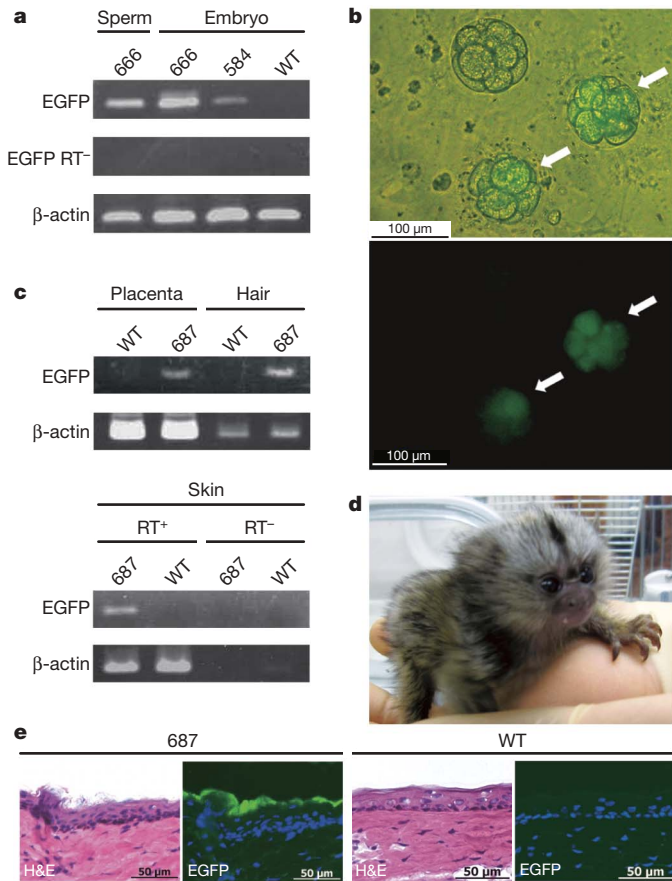


Figure 4 | Germline transmission of the transgene. **a**, RT-PCR analysis of spermatozoa and IVF embryos from 666, and natural embryos from 584. RT- denotes the absence of reverse transcriptase as a control. **b**, Bright-field and dark-field of epifluorescence images of IVF embryos. EGFP-positive IVF embryos produced with 666 spermatozoa are indicated by white arrows. **c**, PCR (top panel) and RT-PCR (bottom panel) analysis of the tissues from the F₁ neonate. **d**, Photograph of the F₁ offspring (687) from 666. **e**, Haematoxylin and eosin (H&E) staining and epifluorescence imaging of frozen skin tissue from the neonate. WT, wild-type control.

development of the embryo. We obtained five transgenic marmosets, four of which expressed the transgene in several somatic cell lineages, such as hair root, skin fibroblast and peripheral blood cells. The remaining animal expressed the transgene only in the placenta. Two of these animals reached sexual maturity and showed the transgene insertion and expression in germ cells. Epifluorescence microscopic observation and RT-PCR analysis of embryos generated by transgene-bearing gametes strongly indicated that the transgenic germ cells from animals 666 and 584 were fertile, and this was proved for the male (666) who fathered one healthy, transgenic infant (687) with the transgene expression in the somatic cells. These findings suggest that it should be possible to establish transgenic non-human primate colonies, opening the door to their use in biomedical research.

Because the manipulation of embryos for viral injection and their subsequent culture may affect embryonic development, the birth rate after embryo transfer (6.25%) was lower than that for normal embryos (30.7%, data not shown). The miscarriage rates were not significantly different between embryonic transfers performed using normal embryos (28.6%) and transgenic embryos (42.6%). Despite considerable effort, transgenic marmosets have not been produced by DNA pronuclear microinjection. The production rate that we obtained using lentivirus (5.26–6.25%) suggests that our technique is sufficiently effective for the production and use of genetically modified marmosets as human disease models.

The 100% birth rate of transgenic marmosets achieved in the present study could be due to several technical advantages. First, we used EGFP

as the transgene, enabling us to monitor the presence and expression of the transgene at each experimental step in live embryos and the transgenic animals. Accordingly, we were able to select unambiguously EGFP-expressing embryos for transfer into surrogate mothers. This selection was effective, not only for increasing the birth rate of transgenic animals, but also for reducing the number of surrogate mother animals needed.

Second, we used pre-implantation embryos obtained by natural intercourse, high-titre lentiviral vectors, and 0.25 M sucrose solution as a medium for injection. Even though the birth rates (birth per embryonic transfer) were no different between IVF and natural embryos, the fertilization rate of the germinal vesicle-stage oocytes was quite low. Because it is difficult to collect large quantities of oocytes, it was advantageous to use marmoset natural embryos. To inject as much lentiviral vector as possible into the perivitelline space, the embryos were placed in 0.25 M sucrose medium at the time of lentiviral vector injection, which expanded the volume of the perivitelline space 1.2–7.5-fold. For example, the estimated volume of the perivitelline space of one marmoset pronuclear stage embryo was approximately 31.5 pl, but when placed in 0.25 M sucrose medium, it expanded to about 231 pl. A high titre of the lentiviral vector solution was used so that many lentiviral vector particles were injected into the expanded perivitelline space; approximately 1.3×10^9 – 1.3×10^5 transducing units of lentiviral vector were injected in this study. Each of these steps probably contributed to the successful production of transgenic marmosets.

The high number of injected lentiviral vector particles resulted in several transgene integrations, as observed by Southern blot analysis and FISH. The embryos injected with the transgene before the four-cell stage (584 and 587) showed fewer than four copies of the transgene per genome by Southern blotting and FISH. The three other embryos (588, 594 and 666), which received the injection at the morula stage, exhibited several integrations of the transgene by Southern blotting and FISH. As the FISH analysis was performed using only peripheral blood MNCs, other patterns of transgene integration cells may have existed in other tissues. The FISH results for 666 were consistent with this hypothesis, as the integration sites in the chimaeric blood MNCs from his twin, 594, were different from those in the blood MNCs of 594.

The lentiviral vector used in the present study can be used to transmit only relatively small transgenes, 8.5 kilobases of DNA or less. Therefore, further study will be necessary to enable the introduction of larger transgenes into marmoset embryos. Furthermore, to study human diseases involving the malfunctions of specific genes, targeted gene-knockdown marmosets could be developed using RNA interference (RNAi) lentiviruses.

The results of the present study indicate that transgenic marmosets may be used as experimental animals for biomedical research. Recently, somatic cell nuclear-transferred embryonic stem cells from the rhesus macaque and induced pluripotent stem cells from adult human fibroblasts were reportedly established^{12–15}. Those studies indicated that the obstacle caused by immunogenetic incompatibility has at least theoretically been resolved, and that a new era of regenerative medicine using somatic cell nuclear-transferred embryonic stem cells in primates¹⁴ or human induced pluripotent stem cells^{12,13,15} has become possible. However, before such stem cells can be used in clinical applications, preclinical assessments of their safety and efficacy are essential. We previously reported that marmosets with injured spinal cords can recover motor function after the transplantation of human neural stem/progenitor cells¹⁶, highlighting the usefulness of the marmoset for assessing the safety and efficacy of, not only these cells, but also of other stem cells, such as human embryonic stem cells¹⁷ or induced pluripotent stem cells. Human disease models in non-human primates have so far been limited to mechanical injury models (for example, spinal cord injury¹⁸) and drug administration models (for example, MPTP-induced Parkinson's^{19,20}). The only transgene-induced primate disease model is of Huntington's disease⁷, in rhesus

monkeys expressing a mutant human huntingtin gene. In that report, although the transgene was inserted into the genome of founder infants and its expression was detected in post-mortem animals, the germline transmission of the transgene has not yet been confirmed⁷. Thus, at this point, it is not certain how reproducible the effects of various therapeutic interventions would be using a large number of animals.

The technique by which we achieved transgene expression in several tissues, along with germline transmission, may provide the means to obtain genetically modified non-human primate models for translational research, investigations of regenerative medicine and gene therapy, and clarification of the scientific gaps among transgenic mice, human disease models, and real human diseases.

METHODS SUMMARY

All animal experiments were approved by the institutional animal care and use committee, and were performed in accordance with Central Institution for Experimental Animal (CIEA) guidelines.

To obtain oocytes, recombinant human follicle stimulating hormone (r-hFSH; 50 international units (IU); Fertinome, Serono) was administered daily by intramuscular injection for 11 days. Human chorionic gonadotropin (hCG; 75 IU; Gonatropin, Teikoku-zouki) was administered by intramuscular injection at 17:30 on day 12. On day 13, the animals were anaesthetized and follicular aspiration was performed surgically. Oocytes were incubated for 24 h at 38 °C, 5% CO₂ in air, for *in vitro* maturation. After incubation, only matured oocytes (metaphase II) were collected and used for IVF.

Ejaculated semen was collected in TYH medium (Mitsubishi Kagaku Iatron), using a Ferti Care personal vibrator. Hyaluronidase-treated oocytes were placed in 70- μ l drops of TYH, and an aliquot of sperm (4×10^5) was added to each oocyte incubation drop. After 26–30 h of insemination, the fertilized oocytes were placed into ISM1 (Medicult) medium, and lentiviral vector injection was performed in 0.25 M sucrose.

Natural embryo collection was performed as previously described²¹. Embryos at the pronuclear-to-morula stage were placed in 0.25 M sucrose supplemented PB1 medium (Mitsubishi Chemical Medicine Corporation) and injected with lentiviral vector. Blastocysts were not treated with sucrose. Lentiviral vector injection was performed using an Eppendorf FemtoJet express and a Narishige micromanipulator. The embryos were cultured until GFP expression was confirmed.

The ovulation cycles of donor and recipient animals were synchronized, and EGFP-expressing embryos were transferred as previously described^{22,23}. After embryo transfer, the recipients were tested for pregnancy by plasma progesterone once a week. The resulting infants were analysed for transgene integration, transcription and expression, by real-time PCR, Southern blot analysis, RT-PCR, immunohistochemical analysis, FACS and FISH.

Full Methods and any associated references are available in the online version of the paper at www.nature.com/nature.

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Supplementary Information is linked to the online version of the paper at www.nature.com/nature.

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Author Contributions E.S. designed the experiments, conducted the project, and wrote the paper. A.S., Y.S., T.E., I.T. and R.H. assisted in embryological technique development. K.H., R.O. and M.K. developed surgical techniques for embryo collection and transfer. H.S., C.K. and C.Y. performed or assisted with the real-time PCR and parentage evaluation test. S.S. and T.M. assisted with the Southern blot analysis and tissue collection. M.I. raised the anti-marmoset CD45 antibody. R.I. performed the FACS analysis, and K.K. performed the immunohistochemical analysis. H.M. provided the lentiviral vectors. Y.T., H.O., S.H., N.T. and T.N. designed the project, and H.O., S.H. and N.T. also participated in writing the paper. The whole project was supervised by E.S. and H.O.

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METHODS

Animals. Adult common marmosets more than 2 years old were obtained from a marmoset breeding colony for experimental animals. Female marmosets with normal ovarian cycles were paired with intact males for natural embryo collection. Recipient females were paired with vasectomised males or intact females. This study was approved by the Institutional Animal Care and Use Committee of CIEA, and was performed in accordance with CIEA guidelines.

In vitro fertilization. Semen was collected as previously described for common marmosets²⁴. Ejaculated semen was collected in TYH medium (Mitsubishi Kagaku Iatron) and washed twice with TYH. The semen was placed in a CO₂ incubator for 10 min in a test tube inclined at a 30° angle to allow the sperm to swim up. Hyaluronidase-treated metaphase-II-arrested oocytes were inseminated with a final concentration of 5×10^6 sperm ml⁻¹ for 26–30 h. Fertilized embryos were cultured in ISM medium (Medicult, Nosan Corp.).

Embryo collection and transfer. Embryo collection and transfer were performed as previously described²⁵. After embryo transfer, the recipients were monitored for pregnancy by measuring their plasma progesterone once a week until the pregnancies could be monitored by transabdominal palpation of the uterus.

Lentiviral vector preparation and transduction. The lentiviral vectors were produced as previously described²⁶. The medium containing viral particles was spun at 4 °C, 50,000g for 4 h, and the viral pellet was then resuspended in ISM2 medium, in 1/1,000 of the volume of the original lentiviral vector supernatant. To measure the lentivirus titre, serially diluted (10^{-2} to 10^{-8}) lentiviral vector was used to infect 10^5 293T cells. The number of EGFP-positive cells was counted by FACS to quantify the titre.

Pronuclear-to-morula stage embryos were placed in 0.25 M sucrose supplemented PB1 medium (Mitsubishi Chemical Medience Corporation), and the virus was injected into the perivitelline space. For blastocyst embryos, the viral vector was injected into the blastocoel. All viral injections were performed using an Eppendorf FemtoJet Express and a Narishige micromanipulator.

Southern blot analysis. Five micrograms of genomic DNA was digested with BamHI for animals that had been injected with CAG-EGFP, and with EcoRI for those that had been injected with CMV-EGFP. The digested genomic DNA was separated on a 0.8% agarose gel and transferred to a Hybond-N+ nylon membrane (GE Healthcare Biosciences). Southern blot analysis was performed using the DIG system (Roche Diagnostics K.K.), according to the manufacturer's protocol. CMV-EGFP was digested with EcoRI and then labelled with DIG using the PCR DIG probe synthesis kit, according to the manufacturer's instructions (Roche Diagnostics K.K.).

RT-PCR. To detect EGFP gene expression, EGFP5-5 (5'-GCACAAGCTGGAGT ACAACTACAACAGC-3') and EGFP3-1 (5'-TCACGAACTCCAGCAGGACC AT-3') primers were used. To detect β -actin expression, β -actin 001 (5'-TCCTG ACCCTGAAGTACCCC-3') and β -actin 002 (5'-GTGGTGGTGAAGCTGTA GCC-3') primers were used. PCR was performed for 35 cycles of denaturation at 94 °C for 30 s, annealing for 30 s at 58 °C for EGFP primers or 62 °C for β -actin primers, and elongation at 72 °C for 30 s.

To detect EGFP gene expression in germ cells and neonatal tissues, PCR was performed using the EGFP5-4 (5'-CAAGGACGACGGCAACTACAAGACC-3')

and EGFP3-3es (5'-GCTCGTCCATGCCGAGAGTGA-3') primers. Then, 1 μ l of the PCR products was re-amplified with the EGFP5-6 (5'-TCGAGCTGA AGGGCATCGAC-3') and EGFP3-1 (5'-TCACGAACTCCAGCAGGACCAT-3') primers. To detect β -actin expression, the PCR primers β -actin 003 (5'-TGGACTTCGAGCAGGAGAT-3') and β -actin 006R (5'-CCTGCTTGCTG ATCCACATG-3') were used. Then, 0.5 μ l of the PCR products was re-amplified with the 004 (5'-TCCCTGGAGAAGAGCTATG-3') and 005R (5'-GAGC CACCAATCCACACTGA-3') primers. PCR was performed for 30 cycles of denaturation at 98 °C for 10 s, annealing at 60 °C for 10 s, and elongation at 72 °C for 30 s.

Immunohistochemical analysis. Tissues were embedded in OCT compound, frozen in liquid nitrogen, and sliced into 5- μ m sections, which were fixed in 4% paraformaldehyde for 30 min at 4 °C. Endogenous peroxidase activity was quenched using 0.03% hydrogen peroxidase for 30 min at room temperature. The slides were blocked with 10% goat serum (Nichirei) for 10 min at room temperature and then reacted with the rabbit anti-GFP polyclonal antibody (A.v. peptide antibody, BD Bioscience) overnight at 4 °C. The slides were incubated with the biotinylated secondary antibody Simple Stain Mouse MAX PO (Nichirei) for 30 min at room temperature. The bound antibodies were detected with DAB (3,3'-diaminobenzidine tetrahydrochloride) horseradish peroxidase complex. The samples were then stained with H&E and examined by microscopy.

FACS analysis. Whole blood cells were washed with PBS and suspended in 0.13 M NH₄Cl. The pellet was incubated with the mouse IgG1 anti-marmoset CD45, 6C9 antibody for 30 min on ice²⁷, then mixed with an allophycocyanin (APC)-labelled anti-mouse IgG antibody, and incubated for 30 min on ice. The sample was washed with PBS and resuspended in 200 μ l of propidium iodide solution. FACSscan analysis was then performed.

Fluorescent in situ hybridization. Peripheral blood samples were cultured in RPMI 1640 containing phytohaemagglutinin, concanavalin A, lipopolysaccharide, and 2-mercaptoethanol for 2–3 days. After 2–3 h of incubation with BrdU (final concentration 30 μ g ml⁻¹), colcemid (final concentration 0.02 μ g ml⁻¹) was added to the medium, and the samples were incubated for another 2 h. After lymphocyte fixation, the cells were spread on slides and air-dried overnight, then stained with Hoechst 33258 and treated with ultraviolet light. CAG-EGFP was labelled with digoxigenin-11-dUTP as a probe, and hybridized at 37 °C overnight. After stringent washes, the bound label was detected using anti-Dig-Cy3. For karyotyping, Leica CW4000 FISH and Leica CW4000 Karyo were used.

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