

# Assisted Reproductive Techniques and Genetic Manipulation in the Common Marmoset

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## Abstract

Genetic modification of nonhuman primate (NHP) zygotes is a useful method for the development of NHP models of human diseases. This review summarizes the recent advances in the development of assisted reproductive and genetic manipulation techniques in NHP, providing the basis for the generation of genetically modified NHP disease models. In this study, we review assisted reproductive techniques, including ovarian stimulation, in vitro maturation of oocytes, in vitro fertilization, embryo culture, embryo transfer, and intracytoplasmic sperm injection protocols in marmosets. Furthermore, we review genetic manipulation techniques, including transgenic strategies, target gene knock-out and knock-in using gene editing protocols, and newly developed gene-editing approaches that may potentially impact the production of genetically manipulated NHP models. We further discuss the progress of assisted reproductive and genetic manipulation techniques in NHP; future prospects on genetically modified NHP models for biomedical research are also highlighted.

**Key words:** assisted reproductive techniques, genetic manipulation, marmosets, nonhuman primates, oocytes and embryos, transgenic animals, gene editing

## Introduction

Genetically modified organisms, particularly mice, have provided many insights into previously unknown gene functions and molecular mechanisms behind disease onset or/and progression. However, because of the physiological differences, not all research findings using murine models can be extrapolated to humans. To overcome the issues, nonhuman primate (NHP) species, sharing many biological characteristics with humans, have been used as model animals to bridge the gap between in vitro studies and clinical medicine.<sup>1,2</sup> Among NHPs, the small body size (350–450 g) of adult marmosets is beneficial considering research purposes; they are easier to handle, and smaller quantities of highly valuable recombinant proteins or synthetically produced materials can be tested, for example, to evaluate efficacy and safety during drug development.<sup>3</sup> Moreover, the

advantage of the small body size can be extended to the development of regenerative medicine using stem cells; since obtaining large quantities of highly valuable differentiated tissues from pluripotent cells is challenging, when marmoset is used as a model animal, only small amounts of the differentiated tissues are required.

Due to the high cognitive function of NHPs, they are predicted to be excellent models for the research of neurological disorders, enabling insights into in vivo events occurring at the onset of disease or into the neuronal circuits responsible for the promotion of pathology. Furthermore, marmosets display similar social structures to those in humans, with social groups consisting of a pair of breeding animals (parents) and their offspring (children). Marmosets display cooperative breeding, whereby the youngest babies in a family are not only raised by the breeding

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female (mother) but also by the breeding male (father) and elder siblings.<sup>3</sup> Moreover, marmosets are diurnal animals with high visual acuity and frequently communicate vocally.<sup>4,5</sup> These social characteristics may make these animals suitable for modeling psychiatric disorders such as autism, schizophrenia, and depression.

To date, various types of nongenetically modified marmoset disease models have been established for the purposes of translational research. Currently, Parkinson's disease,<sup>6,7</sup> spinal cord injury,<sup>8–10</sup> stroke,<sup>1</sup> multiple sclerosis,<sup>1</sup> hepatic fibrosis,<sup>11</sup> and type 1 diabetes<sup>12</sup> models generated via drug administration or surgery are available. Of note, the average lifespan of a marmoset is a maximum of 16.5 years, and, similarly to humans, they display age-related changes in pathologies such as cancer, amyloidosis, diabetes, and chronic renal disease.<sup>13</sup> However, many neurological and psychiatric disorders are known to be associated with genetic alterations, which are difficult to mimic through the use of drugs or surgical manipulation.<sup>14–17</sup> Therefore, there is an urgent call for the development of genetically modified NHP disease models.

Recently, genetic modification techniques have been developed in the context of NHPs, allowing the generation of humanized disease models.<sup>18</sup> Among NHPs, marmosets exhibit distinct reproductive traits, particularly regarding the prolific nature of their reproductive activity. They quickly undergo sexual maturation (at approximately 1.5 years for males and 2 years for females), and adult females usually deliver twins or triplets. Under captivity, triplets can be raised in the parent's home cage with supplementary feeding. With respect to the production of genetically modified marmosets, the multiple birth aspect has pros and cons. Multiple births are beneficial in terms of obtaining high offspring numbers. Of note, in the context of these multiple pregnancies, marmoset siblings are born as blood chimeras, which come with a potential disadvantage.<sup>19,20</sup> Sato et al<sup>21</sup> produced interleukin 2 receptor common gamma (*il2rg*) knockout marmosets using gene editing and reported that hematopoietic stem/progenitor cells of the wild-type littermate, which was failed for gene manipulation, develop into leukocytes in the *il2rg* knockout animal because of the blood chimerism. Therefore, to avoid this issue, Sato et al.<sup>21</sup> performed single embryo transfers thereafter.

Moreover, short gestation intervals mean that females usually deliver twice per year.<sup>3,22</sup> These unique reproductive features make marmosets highly suited for studies related to reproductive engineering. In this review, we describe the progress in the development of assisted reproductive techniques (ART) and genetic engineering technologies in NHPs, including the use of marmosets for biomedical research.

## Assisted Reproductive Techniques

In vitro production of embryos and ART in marmosets have already been reported; the effective application of ART has become of particular interest to researchers aiming for the creation of genetically modified NHP models in biomedical research. In this section, we review ovarian stimulation, in vitro maturation (IVM) of oocytes, in vitro fertilization (IVF), in vitro embryo culture (IVC) and embryo transfer, and intracytoplasmic sperm injection (ICSI) protocols, particularly in the context of marmosets. Tables 1 and 2 summarize the research efforts to develop and employ in vitro embryo production protocols in marmosets.

## Cycle Synchronization and Ovarian Stimulation

A reliable source of oocytes is important for the effective implementation of ART in marmosets. Detailed information on the reproductive physiology and changes in the endocrine profile during the ovarian cycle provides the basis for further studies on the development of reproductive technologies in marmosets, including ovarian stimulation protocols. The measurement of circulating progesterone throughout the ovarian cycle allowed the accurate detection of the ovulation timing and onset of the luteal phase, defined as the day that progesterone levels first exceed 10 ng/mL.<sup>23,24</sup> Levels of marmoset progesterone show a mean ovarian cycle length of 28 days, with approximately 8 to 10 days comprising the follicular phase and the remaining 18 to 20 days comprising the luteal phase.<sup>23,24</sup> Importantly, these cycles can be controlled by the administration of a prostaglandin F-2 $\alpha$  analog, cloprostenol (Estrumate), which induces luteolysis within 24 hours of treatment as indicated by the rapid decline in the peripheral concentration of progesterone.<sup>25,26</sup> In fact, the administration of cloprostenol is an efficient and reliable method for the control of the ovulation timing in marmosets; of note, the posttreatment ovarian cycle is not affected by the premature regression of the corpus luteum.<sup>25,27</sup> These characteristics have enabled researchers to recover oocytes and embryos at precisely determined timepoints and to synchronize the cycles of oocyte donors and embryo recipients.

In an earlier effort to produce marmoset embryos in vitro, timed laparotomy with follicular aspiration or ovariectomy was performed after resetting the ovarian cycle via cloprostenol administration and subsequently induced ovulation in a new cycle with or without the injection of human chorionic gonadotropin (hCG) (Table 1). Lopata et al<sup>28</sup> demonstrated the control of the ovarian cycle in marmosets via the administration of cloprostenol and promoted ovulation with hCG in the late follicular phase, allowing the retrieval of fertilizable preovulatory oocytes. In fact, the administration of 75 IU hCG on day 7 or 8 postcloprostenol treatment yielded up to 2.2 oocytes per animal. Oocytes were graded as per the follicle size; oocytes derived from follicles greater than 2.5 mm in size displayed higher maturation rates than those from smaller follicles. Subsequently, Wilton et al<sup>29</sup> optimized follicular aspiration via the modification of the hCG injection timing relative to that of cloprostenol: precisely at 13:00 on day 7. This protocol induced the successful follicular maturation so that no animals had ovulated at the time of laparotomy and an average of 2.1 oocytes per animal were recovered from the follicles larger than 2 mm. Later, Marshall et al<sup>30</sup> injected 75 IU of hCG at 13:00, 7 days after cloprostenol administration, with a mean number of 2.4 oocytes successfully recovered per animal. The resulting oocytes were subjected to parthenogenetic activation, and the authors of the study found hormonal and histological evidence of implantation following embryo transfer to female recipients. Alternatively, ovaries were collected from nonstimulated adult marmosets at day 7 of the follicular phase in an effort to better understand marmoset oocyte biology.<sup>31,32</sup> An average 24.6–33.2 follicles were excised from each animal ovary and separated into groups based on size. These studies indicated that large numbers of nonovulatory antral follicles were recovered from nonstimulated ovaries, and the meiotic maturation potential of the collected oocytes was confirmed in vitro. In another study, a relatively large number of oocytes was dissected from ovaries without exogenous hormonal stimulation on days 1 to 3 of the follicular phase.<sup>33</sup> A mean of 44.7 oocytes per animal was recovered and used the optimization of IVM and in vitro embryo development. Together,

**Table 1** In Vitro Production of Marmoset Embryos

References	Ovarian Stimulation Protocol	IVM Medium	Developmental Competence
28	(1) 75 IU hCG (Chorulon, Intervet Laboratories) on d 8 at 9:00 (2) 75 IU hCG on d 7 at 9:00 (3) 75 IU hCG on d 7 at 17:00 Follicular aspiration at 24 h after hCG treatment; 1.4–2.2 oocytes/retrieval	MEM + 25 mM sodium bicarbonate + 10% heat-inactivated human cord serum + 100 mg/L penicillin G + 50 mg/L streptomycin at 37°C, 5% CO <sub>2</sub>	IVF with epididymal and electroejaculated sperm, Swim-up method 1–5 × 10 <sup>5</sup> sperm/mL for 6–18 h MEM + 10% heat-inactivated human cord serum + 10 μM dibutyryl cAMP + 10 μM caffeine at 37°C, 5% CO <sub>2</sub> Fertilization rate: 21/33 (64%) Production of live infants following IVF
29	75 IU hCG (Chorulon, Intervet Laboratories) on d 7 at 13:00 Follicular aspiration on d 8; 2.1 oocytes/retrieval	MEM + 10% heat-inactivated marmoset serum + 10 μM dibutyryl cAMP + 10 μM caffeine + 60 mg/L penicillin + 50 mg/L streptomycin at 37°C, 5% CO <sub>2</sub> , 5% O <sub>2</sub> in air	IVF with epididymal sperm 0.5–1 × 10 <sup>6</sup> sperm/mL for 6–8 h or 12–22 h MEM + 10% heat-inactivated marmoset serum + 10 μM dibutyryl cAMP + 10 μM caffeine + 60 mg/L penicillin + 50 mg/L streptomycin at 37°C, 5% CO <sub>2</sub> , 5% O <sub>2</sub> in air Fertilization rate: 68/83 (82%)
31	NA Ovary pairs were collected on d 7; 24.6 follicles/animal	Waymouth MB752/1 + 1 μg/mL hFSH + 10 μg/mL hLH + 10% FBS + 0.23 mM sodium pyruvate + 1 mM glutamine + 75 mg/L penicillin G/K + 50 mg/L streptomycin at 37°C, 5% CO <sub>2</sub> in air IVM rate 72.3%	Cytogenetic analysis Partially naked oocytes from small antral follicles exhibit a high incidence of spindle and meiotic abnormalities
34	(1) Control: normal saline (2) FSH primed: 1.5 IU hFSH (Fertinorm HP75, Serono) twice daily × 3 d Ovary pairs collected on d 4 (1) Control: 91 oocytes/animal (2) FSH primed: 88 oocytes/animal	Waymouth MB752/1 + 1 μg/mL hFSH + 10 μg/mL hLH + 1 μg/mL estradiol + 20% FBS + 0.5 mM sodium pyruvate + 1 mM glutamine + 10 mM sodium lactate + 4 mM hypotaurine + 66 mg/L penicillin G/K + 50 mg/L gentamycin, 5% CO <sub>2</sub> in air IVM rate: 421/546 (77%)	IVF with epididymal and ejaculated sperm (vaginal washing), swim-up method 4 × 10 <sup>6</sup> sperm/mL for 18–24 h TALP medium + 100 μM dibutyryl cAMP + 100 μM caffeine at 37°C, 5% CO <sub>2</sub> in air Fertilization rate Epididymal: 21/44 (93%) Ejaculated: 11/33 (33%)
30	75 IU hCG (Chorulon, Centaur) on d 7 at 13:00 Follicular aspiration on d 8; 2.4 oocytes/retrieval	MEM + 10% heat-inactivated marmoset serum at 37°C, 5% CO <sub>2</sub> in air IVM rate: 74/122 (60.7%)	IVF with epididymal sperm 10–15 × 10 <sup>6</sup> sperm/mL for 12–20 h MEM + 10 μM dibutyryl cAMP + 10 μM caffeine + 60 mg/L penicillin + 50 mg/L streptomycin at 37°C, 5% CO <sub>2</sub> PA activation rate: 68/74 (92%)
35	1, 10, 25, or 50 IU/d rhFSH (Gonal F, Laboratories Serono SA) × 5 or 6 d, 75 IU hCG on d 6 or 7 at 15:30 Follicular aspiration on d 7 or 8; 1.8–14.1 follicles/retrieval	G1.2 at 37°C, 5% CO <sub>2</sub> in air IVM rate: 109/128 (85%)	IVF with fresh ejaculate sperm (PVS), swim-up method 1–3.6 × 10 <sup>6</sup> sperm/mL for 18–20 h G1.2 medium + 100 μM dibutyryl cAMP + 10 μM caffeine at 37°C, 5% CO <sub>2</sub> in air Fertilization rate: 56/99 (57%)
32	NA Ovary pairs collected on d 7 33.2 oocytes/animal	MEM + 20% FBS + 10 IU hFSH + 10 IU hCG + 1 μg/mL beta estradiol + 1 mM Glutamax + 0.5 mM sodium pyruvate + 10 mM sodium lactate + 50 μg/mL L-ascorbic acid at 38.4°C, 5% CO <sub>2</sub> in air IVM rate: 47/117 (40%)	Cytogenetic analysis High rate of aneuploidy and spindle defects occurred in oocytes from small antral follicles
36	(1) 25 IU rhFSH (Gonal-F, Serono Australia) twice daily × 6 d Ovary pairs collected on d 7 (2) 25 IU rhFSH twice daily × 6 d 500 IU hCG (Pregnyl, Organon Pty. Ltd.) on d 7 at 14:00 Ovary pairs collected on d 8 (1) rhFSH alone: 43.7 oocytes/animal (2) rhFSH + hCG: 59.8 oocytes/animal	G2.2 + 10 ng/mL EGF + 0.1 mM cysteamine + 5 IU/mL r-hFSH + 5 IU/mL hCG + 5 mg/mL human serum albumin + 10% FBS at 37°C, 6% CO <sub>2</sub> , 5% O <sub>2</sub> in air IVM rate (>1 mm follicle) (1) FSH alone: 90.9% (2) FSH + hCG: 79.5%	IVF or ICSI using in vivo matured oocytes with fresh ejaculate sperm (PVS), Density gradient method 5 × 10 <sup>6</sup> sperm/mL for 14–16 h G2.2 medium + 5 mg/mL human serum albumin at 37°C, 6% CO <sub>2</sub> in air Fertilization rate IVF or ICSI: 66.9%
37	50 IU FSH × 11 d at 10:00 75 IU hCG on d 12 at 17:30 Follicular aspiration on d 13; 10.5 oocytes/retrieval	Waymouth MB752/1 + 1 μg/mL estradiol + 20% FBS + 0.5 mM sodium pyruvate + 1 mM glutamine + 10 mM sodium lactate + 4 mM hypotaurine at 38°C in air using transportation incubator IVM rate: 578/1092 (52.9%)	PA and SCNT PA activation rate: 57/57 (100%) SCNT activation rate: 43/45 (95.6%)
38	50 IU rhFSH (Fertinome, Serono) × 11 d 75 IU hCG (Teikoku-zouki) on d 12 at 17:30 Follicular aspiration on d 13	NA IVM rate: 201/460 (43.7%)	IVF with fresh ejaculate sperm (PVS), swim-up method 5 × 10 <sup>6</sup> sperm/mL for 26–30 h TYH medium Fertilization rate: 121/272 (44.5%) Generation of GFP transgenic marmosets

(Continued)

Table 1 Continued.

References	Ovarian Stimulation Protocol	IVM Medium	Developmental Competence
33	NA Ovary pairs collected on d 1–3; 44.7 oocytes/animal	MEM + 20% FBS + 1 or 10 IU/mL hFSH + 1 or 10 IU/mL hCG + 1 µg/mL beta estradiol + 10 ng/mL hEGF + 1 mM Glutamax + 0.5 mM sodium pyruvate + 10 mM sodium lactate + 50 µg/mL L-ascorbic acid + 5 IU/mL Penicillin + 5 µg/mL streptomycin at 38.3°C, 5% CO <sub>2</sub> in air IVM rate (1) 47/76 (62%) (2) 38/66 (58%) (3) 27/56 (48%) (4) 42/70 (60%)	IVF with fresh ejaculate sperm (PVS), density gradient method 1.6–2.4 × 10 <sup>6</sup> sperm/mL for 15–16 h Tyrodes-lactate medium without Ca <sup>2+</sup> medium + 0.5 mM sodium pyruvate + 0.5 M Glutamax + 1.7 µM CaCl <sub>2</sub> ·2H <sub>2</sub> O + 1% v/v MEM NEAA + 0.3% w/v BSA + 5 IU/mL Penicillin + 5 µg/mL streptomycin at 38.3°C, 5% CO <sub>2</sub> , 5% O <sub>2</sub> in air Fertilization rate (1) 26/47 (55%) (2) 14/38 (37%) (3) 11/27 (41%) (4) 10/41 (48%)
43	NA Ovaries obtained from euthanized female	(1) Waymouth MB 752/1 + 5% FBS + 1 µg/mL Estradiol + 0.5 mM sodium pyruvate + 10 mM sodium lactate + 4 mM hypotaurine + 100 mIU/mL FSH (2) Waymouth medium + 10% porcine follicular fluid (3) POM + 100 mIU/mL FSH At 37.5°C, 5% CO <sub>2</sub> , 5% O <sub>2</sub> in air IVM rate (1) 71/286 (24.8%) (2) 105/291 (36.1%) (3) 102/275 (37.1%)	IVF with fresh ejaculate sperm (PVS), swim-up method 5 × 10 <sup>6</sup> sperm/mL for 12–17 h TYH medium Fertilization rate (1) Waymouth: 55/70 (78.6%) (2) POM: 45/75 (60%) Generation of marmoset offspring derived from oocytes cultured in chemically defined medium
39	50 IU rhFSH (Folymon-P injection, Fuji pharma) × 9 d at 10:00 75 IU hCG (Gonotropin, Aska Pharmaceutical) on d 9 at 17:30 Follicular aspiration on d 10; 8.5–8.7 oocytes/retrieval	Porcine oocyte medium (POM) + 5% FBS + 100 IU FSH at 37.5°C, 5% CO <sub>2</sub> , 5% O <sub>2</sub> in air	IVF or ICSI with fresh ejaculate sperm (PVS), swim-up method 3.6 × 10 <sup>6</sup> sperm/mL for 18 h TYH medium at 37.5°C, 5% CO <sub>2</sub> , 5% O <sub>2</sub> in air Fertilization rate (1) IVF: 74/90 (82.2%) (2) ICSI: 82/88 (93.2%) Production of healthy marmosets following ICSI
42	NA Ovary pairs collected on d 1–3 before probable day of ovulation	MEM + 20% FBS + 1 IU hFSH + 1 IU hCG + 0, 0.1, 1, or 10 µg/mL beta estradiol + 1 mM Glutamax + 0.5 mM sodium pyruvate + 10 mM sodium lactate + 50 µg/mL L-ascorbic acid + 5 IU/mL penicillin + 5 µg/mL streptomycin at 38.3°C, 5% CO <sub>2</sub> in air IVM rate (1) 0 E <sub>2</sub> . 17/37 (46%) (2) 0.1 E <sub>2</sub> . 18/25 (72%) (3) 1 E <sub>2</sub> . 20/37 (54%) (4) 10 E <sub>2</sub> . 15/24 (63%)	IVF with fresh ejaculate sperm (PVS), density gradient method 1.6–2.4 × 10 <sup>6</sup> sperm/mL for 15–16 h Tyrodes-lactate medium + 0.5 mM sodium pyruvate + 0.5 M Glutamax + 1.7 µM CaCl <sub>2</sub> ·2H <sub>2</sub> O + 1% v/v MEM NEAA + 0.3% w/v BSA at 37°C, 5% CO <sub>2</sub> , 5% O <sub>2</sub> in air Fertilization rate (1) 0 E <sub>2</sub> . 10/17 (59%) (2) 0.1 E <sub>2</sub> . 11/18 (61%) (3) 1 E <sub>2</sub> . 12/20 (60%) (4) 10 E <sub>2</sub> . 7/15 (47%)
21	25 IU rhFSH (Folymon-P injection, Fuji Pharma) × 9 d at 10:00 75 IU hCG (Gonotropin, Aska Pharmaceutical) on d 10 Follicular aspiration on d 11	POM	IVF with fresh ejaculate sperm (PVS), swim-up method 3.6 × 10 <sup>6</sup> sperm/mL for 10–16 h TYH medium at 37°C, 5% CO <sub>2</sub> , 5% O <sub>2</sub> in air Generation of IL2RG KO marmosets
40	25 IU rhFSH (Gonal-F, EMD Serono) × 8 d on d 3–10 75 IU hCG (Novarel, Ferring Pharmaceutical) on d 11 at 16:00 Follicular aspiration on d 12; 46 oocytes/retrieval	POM + 5% FBS + 5 IU/mL FSH + 5 IU/mL hCG at 38°C, 5% CO <sub>2</sub> , 5% O <sub>2</sub> in air IVM rate: 625/787 (79.4%)	IVF with fresh ejaculate sperm (PVS), swim-up method 5 × 10 <sup>6</sup> sperm/mL for 16–20 h TALP medium at 38°C, 5% CO <sub>2</sub> , 5% O <sub>2</sub> in air Fertilization rate: 551/625 (88.2%) Generation of GCaMP transgenic marmosets
41	50 IU FSH (Fuji Pharma) × 9 days at 12:00 50 IU hCG (Aska Pharmaceutical) on day 10 at 12:00 Follicular aspiration on day 11 16.7 oocytes/retrieval	HP-POM + 10% FBS + 0.1 IU/mL FSH at 38°C, 5% CO <sub>2</sub> in air IVM rate: 53/143 (37.1%)	IVF with fresh ejaculate sperm (PVS), swim-up method 1 × 10 <sup>6</sup> sperm/mL for 4 h IVF100 medium Fertilization rate IVO oocytes: 14/22 (63.6%) IVM oocytes: 20/32 (62.5%)

ICSI = intracytoplasmic sperm injection; IVF = in vitro fertilization; IVM = in vitro maturation; NA = not applicable; PA = parthenogenic activation; PVS = penile vibratory stimulation sperm collection method; SCNT = somatic cell nuclear transfer; IU = international unit; hCG = human chorionic gonadotropin; MEM = Minimum Essential Medium; cAMP = Cyclic adenosine monophosphate; FSH = follicle stimulating hormone; TALP = Tyrode's medium with albumin, lactate and pyruvate; TYH = Toyoda, Yokoyama and Hosi; NEAA = Non-essential Amino Acids; POM = porcine oocyte maturation; IVO = in vivo matured oocytes.

Table 2 In Vitro Culture and Embryo Transfer of Marmoset Embryos

References	IVC	Embryo Transfer
28	MEM + 10% human cord serum + insulin-transferrin-sodium selenite at 37°C, 5% CO <sub>2</sub> 20 CL/21 IVF (95.2%) 3 BL/15 CL (20%, 1 hatched BL)	75 IU hCG on d 8 ET on d 3 after ovulation date Surgical ET ET at 4- to 6-cell stage (on d 3 IVC) 2 pregnant/3 IVF-ET (66.7%) 2 pregnant/2 in vivo fertilized embryos (100%) NA
29	MEM + 10% marmoset serum at 37°C, 5% CO <sub>2</sub> 60 CL/66 IVF (91%)	NA
34	Two-step culture at 37°C, 5% CO <sub>2</sub> 10% O <sub>2</sub> in air (1) First 48 h: TL medium +3 mg/mL BSA + 10% FBS + 0.5 mM sodium pyruvate +1 mM hypotaurine +1 mM glutamine +50 µg/mL gentamycin (2) CMRL-1066 + 20% FBS + 0.5 mM sodium pyruvate +10 mM sodium lactate +1 mM glutamine +1 mM hypotaurine +66 µg/mL penicillin G-K + 50 µg/mL gentamycin 93 CL/97 IVF (95.9%) 5 BL/93 CL (5.4%)	NA
30	MEM + 10% female marmoset serum +60 mg/L penicillin +50 mg/L streptomycin at 37°C, 5% CO <sub>2</sub>	75 IU hCG on d 8 ET on d 3 after hCG injection Surgical ET ET at 4-cell stage NA
35	Two-step culture at 37°C, 5% CO <sub>2</sub> 10% O <sub>2</sub> in air (1) First 48 h: G1.2 (2) G2.2 medium 47 CL/53 IVF (88.7%) 25 BL/47 CL (53.2%, 18 hatched BL)	NA
36	Three-step culture at 37°C, 6% CO <sub>2</sub> , 5% O <sub>2</sub> in air (1) First 72 h: G1.2 medium +5 mg/mL human serum albumin (2) Next 24 h: G2.2 medium +5 mg/mL human serum albumin (3) G2.2 medium +5 mg/mL human serum albumin +10% FBS CL: 44.7% BL: 46.7%	NA
37	Two-step culture at 38°C, 5% CO <sub>2</sub> in air (1) First 48 h: ISM1 (2) ISM2 + 10% FBS + inactivated marmoset embryonic fibroblast coculture	NA
38	ISM medium	Surgical ET 1 pregnant/19 IVF-ET (5.3%) 6 pregnant/61 in vivo fertilized embryos (9.8%) NA
33	CMRL-1066 + 26 mM NaHCO <sub>4</sub> + 10% FBS + 0.5 mM sodium pyruvate + 10 mM sodium lactate + 1 mM GlutaMax + 1 IU/mL Penicillin + 1 µg/mL streptomycin in 5% CO <sub>2</sub> , 5% O <sub>2</sub> in air (1) 19 CL/26 IVF (73.1%) (2) 7 CL/14 IVF (50%) (3) 2 CL/11 IVF (18.2%) (4) 12 CL/29 IVF (41.4%)	NA
43	Two-step culture (1) First 48 h: ISM1 (2) ISM2 (1) Waymouth 51 CL/55 IVF (92.7%) 5 BL/51 CL (9.8%) (2) POM 43 CL/45 IVF (95.6%) 6 BL/43 CL (14%)	Surgical ET ET at 8-cell to morula stage 2 pregnant/11 IVF-ET (18.2%)
39	Two-step culture at 37.5°C, 5% CO <sub>2</sub> , 5% O <sub>2</sub> in air (1) First 48 h: ISM1 (2) ISM2 (1) IVF 69 CL/74 IVF (93.2%) 29 BL/69 CL (42%) (2) ICSI 80 CL/82 ICSI (97.6%) 29 BL/80 CL (36.3%)	Nonsurgical ET ET at 6-cell to blastocyst stage (1) 6- to 8-cell stage ET: 3 pregnant/8 ICSI-ET (37.5%) (2) Blastocyst stage ET: 4 pregnant/20 ICSI-ET (20%)
42	CMRL-1066 + 26 mM NaHCO <sub>4</sub> + 10% FBS + 0.5 mM sodium pyruvate + 10 mM sodium lactate + 1 mM GlutaMax + 1 mM hypotaurine + 1 IU/mL penicillin + 1 µg/mL streptomycin in 5% CO <sub>2</sub> , 5% O <sub>2</sub> in air (1) 0 E <sub>2</sub> : 6 CL/10 IVF (60%) (2) 0.1 E <sub>2</sub> : 9 CL/11 IVF (82%) (3) 1 E <sub>2</sub> : 9 CL/12 IVF (75%) (4) 10 E <sub>2</sub> : 4 CL/7 IVF (57%)	NA
21	ISM1 at 37°C, 5% CO <sub>2</sub> , 5% O <sub>2</sub> in air	Nonsurgical ET 19 pregnant/113 IVF-ET (16.8%)
40	ISM1 at 38°C, 5% CO <sub>2</sub> , 5% O <sub>2</sub> in air 385 CL/551 IVF (80.9%)	Nonsurgical ET 13 pregnant/51 IVF-ET (25.5%) 9 pregnant/31 in vivo fertilized embryos (29%) NA
41	Two-step culture at 38°C, 5% CO <sub>2</sub> , 5% O <sub>2</sub> in air (1) First 48 h: Cleav medium (2) BlastAssist medium +10% FBS + cocultured with inactivated mouse embryonic fibroblasts (1) IVO oocytes 12 CL/14 IVF (85.7%) 0 BL/12 CL (0%) (2) IVM oocytes 16 CL/20 IVF (80%) 4 BL/16 CL (25%)	NA

BL = blastocysts; CL = cleaved embryos; ET = embryo transfer; IVC = in vitro culture; MEM = Minimum essential medium; IVO = in vivo matured; NA = not applicable.

these studies provide a protocol for the collection of reliable numbers of viable oocytes from marmosets within the follicular phase. Results suggest that the maturation rate of oocytes from larger follicles is higher than that from smaller follicles.

The establishment of a consistent ovarian stimulation protocol for marmosets will be useful to further increase the population of preovulatory follicles as well as to enhance the developmental competence of oocytes. An early attempt of *in vivo* priming with follicle-stimulating hormone (FSH) in marmosets demonstrated that the subsequent IVM potential of oocytes was marginally improved following 2 daily injections of 1.5 IU human FSH from days 0 to 3 of the follicular phase.<sup>34</sup> The FSH dosage used in this study was comparable with the dosage used in rhesus monkey stimulation, and twice that generally used in humans. However, the proportion of oocytes enclosed in cumulus cells was not changed by FSH priming compared with that in nontreated animals. Later, Marshall et al<sup>35</sup> compared the ovarian response to different concentrations of recombinant human FSH (r-hFSH) injections at the doses of 1, 10, 25, or 50 IU/d for 5 or 6 days, followed by the injection of 75 IU hCG. There were no differences in the number of preovulatory follicles obtained from the untreated control group and from the animals receiving 1–25 IU/d r-hFSH. However, animals that received 50 IU r-hFSH displayed major increases in the number of follicles. The levels of r-hFSH required for ovarian hyperstimulation in marmosets were extremely high at around 12.5 times those used in rhesus monkeys. To further optimize the ovarian stimulation protocol for marmosets, the ovarian response to an FSH priming regimen combined with the administration of a single high dose of hCG was compared with that caused by FSH priming alone.<sup>36</sup> On days 1 to 6 of the follicular phase, 25 IU r-hFSH was administered twice per day in the FSH only group, and animals in the FSH + hCG group were additionally treated with 500 IU hCG on day 7. As a result, the numbers of oocytes recovered from small antral follicles were similar regardless of the stimulation protocol; however, the group treated with FSH + hCG displayed more expanded cumulus-oocyte complexes compared with those from the group primed with FSH alone. More recently, the stimulation protocol was further modified by extension of FSH daily injection to days 9–11 of the follicular phase, with a dose of 25 to 50 IU followed by 75 IU hCG treatment.<sup>21,37–41</sup> Altogether, these studies demonstrated that marmoset oocytes can be effectively collected via ovarian follicular aspiration with extended duration of FSH treatment in an ovarian stimulation protocol. Furthermore, they demonstrated that a reliably large number of oocytes is retrieved, with a mean of 8.5–46 oocytes per animal (Table 1).

When oocytes are collected from FSH-primed marmosets, samples include both premature oocytes that have not yet reached metaphase II (MII) and mature oocytes that have already reached MII. Kanda et al.<sup>41</sup> described postmature oocytes (*in vivo* matured [IVO]) at the time of follicular aspiration and evaluated their developmental capacity following IVF, as well as the cytoskeletal status of embryos originating from IVO and IVM oocytes. Although both IVM and IVO oocytes were similar in appearance at the MII stage and showed a comparable fertilization rate following IVF, the developmental competence of IVO oocytes beyond the 4-cells stage was significantly impaired compared with that of IVM oocytes. IVO oocytes also displayed abnormal cytoskeletal formation, indicating that further optimization of the ovarian stimulation protocol is needed to reduce the number of IVO oocytes and generate a high number of developmentally competent MII stage oocytes.

In general, FSH priming combined with a timed hCG treatment successfully increases the number of preovulatory follicles in marmosets and the ability to collect large numbers of viable

oocytes, making the marmoset an important primate model in the context of reproductive and biomedical research.

### In Vitro Maturation

It was reported that marmoset ovaries possess abundant small and medium-sized antral follicles and that the oocytes contained within are capable of undergoing meiotic maturation *in vitro* under proper conditions.<sup>31</sup> In recent years, substantial progress has been made in IVM procedures; however, further improvements are necessary to maximize the production of developmentally competent marmoset embryos. One critical point of the maturation culture is the grading and assortment of oocytes. Results obtained by Gilchrist et al<sup>31</sup> indicate that oocytes from large follicles (>1 mm in diameter) have a greater ability to undergo maturation with low incidence of meiotic and spindle abnormalities. They further demonstrated that oocytes from large follicles have enhanced developmental potential compared with oocytes from small follicles.<sup>31,34</sup> Collected follicles were graded according to size, with class 1 being the smallest and class 4 the largest. Overall, 77% of the oocytes completed meiotic maturation, but class 1 follicles exhibited significantly lower germinal vesicle breakdown and MII competency regardless of the enclosed cumulus cell status.<sup>34</sup> Moreover, when ovaries were collected from FSH-primed animals, oocytes from class 1 follicles demonstrated a more advanced rate of meiosis. In contrast, oocytes from other follicle classes displayed no changes in the rate of meiosis, indicating a marginal effect of FSH priming on oocyte meiotic competence.<sup>34</sup>

Various culture conditions have been implemented to promote the maturation of marmoset oocytes. Several basal media such as alpha-modified minimum essential medium (MEM),<sup>28–30,32,33,42</sup> Waymouth's Medium MB752/1,<sup>31,34,37,43</sup> G1.2,<sup>35</sup> G2.2,<sup>36</sup> and Porcine oocyte medium<sup>21,39–41,43</sup> are available for the successful culture of marmoset oocytes. In addition to basal medium, diverse forms of protein were added, such as fetal bovine serum (FBS),<sup>31–34,36,37,39–43</sup> porcine follicular fluid,<sup>43</sup> human cord serum,<sup>28</sup> human serum albumin,<sup>36</sup> or marmoset serum.<sup>29,30</sup> These provided substrates essential for energy production while maintaining the osmotic and pH balance.<sup>44</sup> To further optimize the culture conditions of marmoset oocytes, Tkechenko et al<sup>33</sup> examined the effect of combinations and concentrations of gonadotrophins and local growth factors, such as epidermal growth factor (EGF). In the presence of gonadotropins, supplementation with EGF did not influence nuclear mutations but suppressed oocyte radial expansion patterns and increased their degeneration rates during culture, demonstrating the negative effect of EGF on IVM. In another study by the same group, the dose-dependent effect of estradiol during marmoset oocytes IVM was investigated.<sup>42</sup> As a result, the group that was supplemented with 0.1  $\mu\text{g}/\text{mL}$  estradiol appeared to have the highest rate of MII progression and improved first cleavage rates compared with the control group. However, the highest concentration of estradiol tested (10  $\mu\text{g}/\text{mL}$ ) resulted in a high proportion of metaphase spindle abnormalities, with low embryo progression, suggesting a negative effect of overdose estradiol supplementation. In current IVM protocols, reliable rates of oocyte maturation (up to 90.9%) are achieved after 26–30 hours incubation in terms of oocytes at MII stage with extrusion of the first polar body.<sup>36,40</sup> Thereafter, mature oocytes can be directly cultured with spermatozoa or manipulated for ICSI to obtain viable embryos *in vitro*.

### In Vitro Fertilization

Successful IVF has been performed via the co-incubation of matured oocytes and spermatozoa for up to 30 hours, providing

access to marmoset preimplantation embryos for the study of development *in vitro*. Initially, it was challenging to collect high-quality marmoset semen samples in a timely manner as mammalian MII oocytes have a limited window of time for normal fertilization.<sup>45,46</sup> The early methods of semen collection in the marmoset were via rectal probe electro-ejaculation or vaginal washing after natural copulation.<sup>28,47–49</sup> The electro-ejaculation method requires sedation or anesthesia, and therefore it is less suitable for studies needing repeated sample collection.<sup>28,47,49</sup> The vaginal washing method is labor- and time-intensive; there is the need for timed mating between selected pairs of animals, and the ejaculates are usually contaminated with cells from the female genital tract.<sup>34,47,48</sup> Other methods of semen collection, such as hemicastration and cauda epididymis rinsing, have been reported to yield semen samples for use in IVF research; however, their practice is not appropriate since the collection cannot be replicated on the same male.<sup>28,29,34</sup> The penile vibratory stimulation (PVS) method was later suggested as a noninvasive and practical alternative to the electro-ejaculation method to yield semen of higher quality without contamination.<sup>50,51</sup> In a study by Kuederling et al,<sup>50</sup> the success rate of PVS was 35.2%, but it was increased to 89.2% in a later study that further modified the PVS collection technique.<sup>51</sup> Moreover, PVS yielded 3 to 4 times more motile spermatozoa than electro-ejaculation.<sup>51</sup> Enhanced success rates with higher rates of motile sperm made the PVS sperm collection strategy the method of choice in marmosets (Table 1).

Typically, the spermatozoa are washed and selected using swim-up or density gradient centrifugation procedures followed by semen collection to eliminate seminal plasma, debris, and dead spermatozoa and to select the more motile and morphologically normal sperm.<sup>36,52,53</sup> Then, spermatozoa require treatment with capacitation factors to achieve functional maturation and to acquire the ability to penetrate the oocyte zona pellucida. *In vitro* capacitation of ejaculated rhesus sperm was accomplished via incubation with caffeine and dibutyryl cyclic AMP (dbcAMP).<sup>54,55</sup> This sperm preparation protocol was adapted in the earlier marmoset IVF studies; however, ejaculated spermatozoa exhibited notable hyperactivity followed by rapid loss of motility in response to dbcAMP and caffeine treatment, indicating further work would be necessary to optimize marmoset sperm capacitation conditions.<sup>34</sup> Additionally, *in vitro* capacitation of spermatozoa can be achieved by the simple removal of seminal plasma and incubation with media containing protein sources such as bovine serum albumin, as well as different ions, including bicarbonate and calcium, which are present in the majority of culture media used for sperm selection or IVF.<sup>56,57</sup> Presently, fertilization rates, measured as per the extrusion of the 2 polar bodies with pronuclear formation at 12 to 18 hours postinsemination, were achieved in up to 88.2% of trials without dbcAMP and caffeine supplementation in fertilization medium for capacitation.<sup>39,40,42</sup> Regarding the culture media used for marmoset IVF, MEM,<sup>28,29</sup> modified Tyrode's albumin lactate pyruvate,<sup>33,34,40,42</sup> G1.2 medium,<sup>35</sup> G2.2 medium,<sup>36</sup> Toyoda, Yokoyama, Hoshi (TYH) medium,<sup>21,38,39,43</sup> and IVF100 medium<sup>41</sup> have been successfully used for the production of developmentally competent zygotes. The final sperm concentration used in the IVF drop can vary from 0.1 to  $15 \times 10^6$  spermatozoa/mL depending on the study and IVF system used (Table 1). Sperm and oocytes are usually co-incubated for 4–30 hours at 37–38.4°C. At the end of the co-culture period, the presumable zygotes are usually cleaned using a pipette to remove adhering sperm and cumulus cells. They are then placed in fresh culture medium to inspect

successful fertilization before performing the periodic monitoring of embryo development.

### In Vitro Culture

After fertilization, presumptive zygotes are cultured *in vitro* until the blastocyst stage. Therefore, the development of appropriate culture conditions is essential for enhancing the developmental competence of *in vitro* produced embryos. Various media have been used in the context of marmoset embryo cultures across different laboratories and experiments (Table 2). In earlier studies, more than 90% of embryos successfully underwent at least 1 cleavage division, and 20% of cleaved embryos developed to the blastocyst stage in MEM medium supplemented with 10% serum.<sup>28,29</sup> Changes in energy substrate requirements for the development of early- and late-stage embryos in cattle and sheep led to the design of a “sequential” media system<sup>58</sup> later applied to IVC of marmoset embryos. Embryos were cultured in the first medium for about 48 hours and then transferred to the second medium and cultured for up to 2 weeks until the development of the blastocyst stage. These sequential culture medium systems included the TL medium to CMRL-1066,<sup>34</sup> G1.2 to G2.2,<sup>35,36</sup> ISM1 to ISM2,<sup>37,39,43</sup> and Cleave to BlastAssist.<sup>41</sup> Of the fertilized embryos, 44.7%–97.6% was cleaved to 2 cells, with 5.4%–53.2% of cleaved embryos reaching to the blastocyst stage (including hatching from zona pellucida). Alternatively, co-culture with inactivated mouse or marmoset embryonic fibroblasts has been used in the context of marmoset embryo IVC systems.<sup>37,41</sup> Although co-culture systems have been shown to support the development to the blastocyst stage, further research is needed to determine the factors influencing embryo development, which will enable the satisfactory usage of the system. Overall, a wide variety of approaches to culture marmoset embryos are available, including modulation of media composition, gas conditions, and temperature, with varying degrees of success (Table 2). Of note, it is important to optimize the culture conditions and establish standard culture protocols for marmoset embryos, which will ultimately result in normal embryo development and allow comparisons of research outcomes across studies.

### Embryo Transfer

The establishment of appropriate conditions for oocyte collection and embryo generation via IVF followed by IVC resulted in the consequent development of embryo transfer techniques. Once IVF embryos reach the 8- to 16-cell stage, there is a choice of whether to transfer them to a synchronized recipient female or to continue culture *in vitro* and allow their development into blastocysts. The majority of our research has taken the embryo transfer approach as a way to obtain as many of the desired marmoset models as possible. In most cases, between 1 and 3 embryos were transferred to each recipient. Practical marmoset reproductive technologies for generating genetically modified marmosets have been summarized by Kurotaki and Sasaki et al.<sup>59</sup> Since it is possible to synchronize the ovarian cycle of the oocyte donor and recipient marmosets via the timed injection of cloprostenol, the ovulation timing was monitored as per the serum progesterone levels to permit an embryo transfer operation between days 2 and 10 postovulation.<sup>60–62</sup> Marmoset embryo transfer techniques can be based on both surgical and non-surgical methods. Primarily, successful surgical embryo transfer techniques have been developed via the delivery of fresh or frozen-thawed embryos to the uterine lumen after laparotomy, resulting in a pregnancy rate of 66% with successful full-term

gestation.<sup>28,60</sup> However, since the excessive repetition of invasive surgical procedures on the same animal is not an appropriate practice, nonsurgical uterine approaches for embryo transfer were developed. Although it is difficult to use this approach due to the location of the fornix near the external os of the cervix,<sup>63,64</sup> a skilled operator is able to gently guide a blunt cannula and stylet through the cervical canal into the uterine lumen, and deposit medium-containing embryos at the uterine fundus.<sup>60–62</sup> In a study by Marshall et al,<sup>61</sup> 4 animals became pregnant (44.4%) among 9 recipients to which 17 embryos were transferred via this nonsurgical approach, and 3 recipients delivered 6 babies. There are, however, a number of problems associated with this approach, including the possibility of impacting the transferred embryos into viscous cervical mucus secretions, the structural or physiological disruption of the uterine endometrium due to the manipulation of the cannula in the lumen, the inadequate synchronization of the embryo and the recipient, or the transfer of insufficiently viable embryos. The proportionally wider and shorter marmoset uterus is one of the inconvenient factors for nonsurgical embryo transfer.<sup>64</sup> However, the successful placement of embryos in the uterine lumen can be ensured via the careful monitoring of echogenic signals in the uterine cavity using abdominal ultrasonography during the transfer procedure. Furthermore, the successfulness of the placement can be improved via flushing the cannula with collection medium following its withdrawal such that no embryos remain in the cannula.<sup>39,61,62</sup>

A variety of factors can affect the pregnancy rate, including the transfer volume and the relationship between the embryonic stage and uterine synchrony. Marshall et al<sup>61</sup> compared the pregnancy rate in synchronous condition (the embryo donor and the embryo recipient ovulated on the same day) or asynchronous condition (the embryo donor ovulated at least 2 days before the embryo recipient). As a result, the asynchronous transfer led to a pregnancy rate of 44% with 6 live births, whereas the synchronous transfer resulted in a pregnancy rate of 9% with no full-term pregnancies.<sup>61</sup> Moreover, to find out the effect of the embryo stage on the pregnancy rate, the transfer of early-stage or late-stage embryos was compared.<sup>60</sup> Higher pregnancy rates were achieved following the transfer of early cleavage stage embryos into the uterus compared with the transfer of morula to blastocyst stage embryos.<sup>39,60,61</sup> Another factor considered to influence the outcomes of nonsurgical embryo transfer is the volume of the embryo-containing medium used for the transfer. When the transfer volume was 1  $\mu$ L or less, a higher pregnancy rate (of about 80%) was achieved; transfer volumes of 2–3  $\mu$ L resulted in a lower pregnancy rate of around 50%. This may be associated with the embryo expulsion from the uterus due to an excessive volume of media.<sup>62</sup>

Overall, successful pregnancies and live births have been achieved following the transfer of in vitro–produced marmoset embryos to recipient animals. Of note, the gestation length has been described as being within the normal range, and the newborns were viable without any abnormalities. These techniques provide a valuable toolbox for increasing the number of genetically valuable founder animals.

### Intracytoplasmic Sperm Injection

ICSI is one of the ART techniques described for marmosets and involves microinjection of a single spermatozoon into the cytoplasm of a matured oocyte, bypassing the natural process of sperm-oocyte interactions.<sup>36,39</sup> The ICSI procedure provides an opportunity to investigate the fundamental components of

the fertilization process, such as the sperm triggering oocyte activation and following early embryo development.<sup>39</sup> Takahashi et al<sup>39</sup> reported the successful generation of healthy offspring following ICSI, demonstrating that this technique is an effective fertilization method for marmosets. To determine the optimal timing of oocyte fertilization after IVF, ICSI was performed at various time points after the extrusion of the first polar body. Although there were no significant differences between groups, the group with 2- to 4-hour intervals showed the highest blastocyst rate, suggesting that the optimal timing of fertilization is more than 2 hours after oocyte maturation. Successful in vivo developmental competency of ICSI embryos was confirmed, with a pregnancy rate of 20% from ICSI blastocyst transfers and 37.5% from 6- to 8-cell-stage ICSI embryo transfers. One of the major applications of ICSI is the reproduction of high-value founders and the production of transgenic animals via ICSI-mediated transgenesis, which is discussed later in this article.

## Genetic Manipulation Techniques in Mammals

There are several methods for the genetic manipulation of mammalian genomes (Table 3). Genetic modifications are classified into 2 categories: transgenic or gene targeting technologies. The transgenic technology comprises the insertion of an exogenous gene into a host genome, whereas gene targeting technology is the introduction of genetic modifications into a specific region of a target gene via homologous recombination or gene-editing techniques. In general, there are various transgenic approaches available, including pronuclei DNA microinjection,<sup>65</sup> retroviral transduction,<sup>66</sup> and gene transfer into embryonic stem cells (ESCs) or induced pluripotent stem cells (iPSCs). The injection of these modified ESCs or iPSCs into 8-cell-stage to blastocyst-stage embryos results in the genes transfer to germ cells.<sup>67,68</sup> Similarly, genes can be transfected into germline stem cells (GSCs). Other approaches include microinjection into the seminiferous tubules of infertile recipient mice,<sup>69–71</sup> ICSI-mediated transgenesis by co-injecting unfertilized oocytes with sperm and exogenous DNA,<sup>72,73</sup> or somatic cell nuclear transfer (SCNT).<sup>74–76</sup> Combined with the genetic manipulation of ESCs, iPSCs, GSCs, and SCNTs, these methods can be used to produce genetically manipulated animals, such as transgenic and target gene knock-out/knock-in models. Furthermore, the recently developed “gene-editing techniques” are also useful for generating target gene knock-out/knock-in animals.<sup>77–80</sup> In this section, several approaches for transgenesis, both established and currently not successfully achieved methods, in NHPs will be introduced; the successful approaches in NHPs are summarized in Table 3.

### Pronuclei DNA Microinjection

Pronuclei DNA microinjection is widely used in several vertebrate species, including mice, rabbits, sheep, and pigs.<sup>81,82</sup> Injected DNA is randomly integrated into the host genome during DNA replication and maintained during embryo development, resulting in neonates that harbor the foreign DNA. An advantage of pronuclei DNA microinjection is that it can introduce long sequences of DNA (5–10 kb). Furthermore, when artificial chromosomes are used, 300 kb (bacterial artificial chromosome vectors) to 2 Mb (yeast artificial chromosome vectors) can be introduced.<sup>83,84</sup> On the other hand, the production efficiency of transgenic animals is around 1% of injected



**Table 3** Genetic Manipulation Methods and Production of Genetically Modified NHPs

		Gene Manipulation			Report of Successful Production of Offspring in NHPs		
		Gene Translation	Target Gene Knock-out	Target Gene Knock-in	Insert Point Mutation	Marmoset	Other NHPs
Pronuclei DNA microinjection		Random integration	NA	NA	NA	NR	NR
Retroviral/lentiviral vectors		Random integration	NA	NA	NA	38,40,215	100,101,102,106
ICSI-mediated transgenesis		Random integration	NA	NA	NA	NR	NR
Pluripotent stem cells (ESCs/iPSCs)		Random integration	Precise target gene knock-out by cell selection	Precise target gene knock-in by cell selection	Available by HDR	NR	NR
Germline stem cells		Random integration	Precise target gene knock-out by cell selection	Precise target gene knock-in by cell selection	Available by HDR	NR	NR
Somatic cell nuclear transfer		Random integration	Precise target gene knock-out by cell selection	Precise target gene knock-in by cell selection	Available by HDR	NR	184,185
Gene editing	ZFN	NA	Target gene knock-out by insertion-deletion			21	NR
	TALEN	NA	Target gene knock-out by insertion-deletion			21	187
	CRISPR/Cas9	NA	Target gene knock-out by insertion-deletion	Target gene knock-in by ssODN, dsDNA, or AAV			186,194
	Base Editor/Target AID	NA	NA	NA	Transitions cytosine-to-thymine (C > T)		207
	ABE	NA	NA	NA	Transitions adenine-to-guanine (A > G)		

NA = not applicable; NHP = non human primate; ICSI = intracytoplasmic sperm injection; HDR = homology directed repair; ssODN = single-stranded donor oligonucleotides; dsDNA = double strand DNA; AAV = Adeno-associated virus; NR = not reported.

embryos in mice.<sup>84</sup> Due to this low success rate, the DNA pronuclear injection requires large numbers of oocytes to obtain transgenic animals. However, the NHP oocyte supply is limited. For example, marmoset ovum pickup retrieves approximately 15–50 GV stage ova from 1 animal after ovarian stimulation.<sup>85</sup> This makes it difficult to apply this technique to NHPs, and therefore the generation of transgenic NHPs by DNA microinjection is not reported.

### Retroviral Vectors

Retroviruses synthesize DNA from viral RNA via reverse transcription and integrate it into the host genome as a DNA provirus. Due to this property, retroviral vectors can be leveraged to introduce genes into the animal genome. Retroviral vector-mediated transgenic mice have been produced by the co-culture of zona pellucida-free, 8-cell-stage embryos with cells producing replication-competent<sup>86,87</sup> or defective retroviruses.<sup>88–91</sup> The world's first transgenic mice with germline transmission of transgenes were produced in 1976 via Moloney leukemia virus (M-MuLV) infection of early mouse embryos. Exogenous M-MuLV was shown to be integrated into the mouse genome and transmitted through the germline.<sup>87</sup> A replication-defective vector based on M-MuLV, pseudotyped with the envelope glycoprotein of vesicular stomatitis virus (VSV-G) interacts with phospholipid components of the host cell plasma membrane and expands the host range of infectivity. Furthermore, pseudotyped retroviruses can be concentrated to titers 100–1000-fold higher than those of authentic retroviral

vectors.<sup>92</sup> Since this VSV-G pseudotyped retroviral vector allows high efficiency of transduction, the production of transgenic domestic animals has been reported.<sup>93–99</sup>

For the production of transgenic NHPs, several kinds of VSV-G pseudotyped retroviral vectors have been used. The first transgenic monkeys were produced via the injection of the VSV-G pseudotyped M-MuLV vector carrying the green fluorescent protein (GFP) gene into the perivitelline space of mature oocytes from rhesus monkeys.<sup>100</sup> However, the transgene expression was reduced (or absent), suggesting the transgene was silenced, a frequently observed phenomenon in retroviral vector-mediated transgenesis. In the same year, Wolfgang et al<sup>101</sup> also reported the generation of another transgenic rhesus monkey using VSV-G pseudotyped self-inactivating lentiviral particles; they were injected into blastocysts, and their integration was confirmed via the observation of GFP expression in placental tissues. In 2010, 2 live rhesus monkey infants, stably expressing enhanced GFP in somatic tissues, were obtained via the transduction of a simian immunodeficiency virus-based lentiviral vector into 4- to 8-cell-stage embryos.<sup>102</sup>

Transgenic marmosets were produced via the injection of a lentivirus vector encoding GFP into the perivitelline space of preimplantation stage embryos (2 pronuclear stages to blastocyst stage), and the first germline transmission of a transgene was reported.<sup>38</sup> Out of 5 animals, 1 expressed GFP in the placenta only, similar to the results of Wolfgang et al.<sup>101</sup> Both animals were obtained through the injection of viral vectors into blastocyst-stage embryos,<sup>38,101</sup> which may result in placenta-specific gene manipulation.<sup>103–105</sup>

Following these reports, GFP-expressing transgenic cynomolgus monkey infants were obtained after the injection of lentiviral vectors into the perivitelline space of MII stage oocytes, followed by ICSI 4 hours after injection.<sup>106</sup> The use of lentiviral vectors with a human cytomegalovirus immediate-early enhancer and chicken beta-actin promoter (CAG) to genetically manipulate NHP zygotes or oocytes resulted in the ubiquitous expression of the transgene in several somatic tissues and a lower transgene silencing rate.<sup>38,102,106</sup> After these reports, several transgenic NHPs were produced; these studies were well-reviewed by Park and Silva.<sup>18</sup>

As described above, retroviral vectors, including lentivirus, have proven to be excellent tools for highly efficient introduction of exogenous genes into the NHP genome; therefore, the retroviral vector transgenesis methods are well established in NHPs, including in marmosets. However, the size of the sequence that can be inserted into retroviral vectors is limited due to packaging limitations. Although the maximum packaging size of the transgene in the lentiviral vector has been reported as 13.5 kb,<sup>107</sup> in reality, the most efficient packaging occurs with sequences up to 5 kb in length.

### ICSI-Mediated Transgenesis

It has been reported that mixing DNA and sperm prior to IVF can produce transgenic mice with a success rate of approximately 30%, with the transgene transmitted to the offspring.<sup>108</sup> This method can be performed easily; the DNA is mixed with sperm cells during *in vitro* fertilization, this can be performed via the intrinsic ability of sperm to bind and transport exogenous DNA into an oocyte. These techniques are considered to be simple and inexpensive methods, because highly skilled personnel and specialist equipment such as micromanipulators are unnecessary. However, lack of reproducibility and the effectiveness of integrating transgenes into the host genome have led to controversy.<sup>109,110</sup> Nevertheless, numerous reports have been published confirming that sperm-mediated gene transfer is effective for the delivery of transgenes into the animal genome in many species. To improve transgenesis efficiency, augmentation techniques have been combined, such as lipofection or electroporation, which increase exogenous DNA uptake in spermatozoa and improve the integration of the transgene.<sup>111</sup>

An alternative approach is ICSI-mediated transgenesis, also called MII-mediated transgenesis.<sup>112</sup> In mice, ICSI-mediated transgenesis can either be used with intact or membrane-disrupted spermatozoa incubated with foreign DNA followed by injection into MII oocytes.<sup>73</sup> The advantage of ICSI-mediated transgenesis is the ability to introduce large transgenes using bacterial artificial chromosomes<sup>113,114</sup> or yeast artificial chromosomes,<sup>112,115</sup> allowing the insertion of 100–1000 kbp of DNA with relatively high efficiency. Only 1 trial has been reported to produce transgenic rhesus monkeys using ICSI-mediated transgenesis via GFP-expressing embryo transfers. However, although GFP epifluorescence was detected in the stillborn animals, no transgenic live offspring was obtained.<sup>116</sup> These results may suggest the transgene DNA have not been integrated into the host embryo chromosome while selecting the GFP expressing preimplanted embryos. Although transgenic offspring have not been obtained by ICSI-mediated transgenesis in NHPs to date, ICSI is a conventional method to generate zygotes in NHPs.<sup>39,106,116–118</sup> Therefore, it is considered to be a potential method to overcome the transgene size limitations associated with retroviral and lentiviral vectors.

### Pluripotent Stem Cells

Mouse pluripotent stem cells, ESCs, and iPSCs can contribute to germline manipulation via direct injection into host embryos.<sup>119–122</sup> These chimeric competent pluripotent stem cells are termed “naïve” or “ground” state ESCs.<sup>123</sup> Although naïve ESCs have been routinely derived from mice since 1981,<sup>124,125</sup> ESCs with chimeric competency have not been established in other species except rats. Similarly, in NHPs, although many lines of ESCs and iPSCs have been established, exhibiting pluripotency and enabling the differentiation into the 3 germ layers, chimeric competency has not been proven.<sup>126–130</sup> In rodents, a defined culture system combining 2 inhibitors of the Erk pathway and glycogen synthase kinase-3 with LIF allowed the efficient derivation and clonal expansion of ESCs from dissociated cells in the mouse and rat strains, supporting the establishment of chimeric competent ESCs.<sup>131–134</sup> However, in other mammals, including NHPs, these 2 inhibitors were not sufficient to establish chimeric competent ESCs/iPSCs. Many studies have reported the successful resetting of human pluripotent stem cells into a naïve-like phenotype.<sup>123,135–137</sup> However, these reports did not provide evidence for chimeric competency due to ethical reasons. Although putative naïve-like NHP pluripotent cells also have been established, meaning that these cells can be analyzed in terms of contribution to embryos or fetal development, neither chimeric neonates nor germline chimeras have been reported in NHPs.<sup>138,139</sup> In fact, Tachibana et al.<sup>140</sup> demonstrated that chimeric NHP production is difficult since the primitive endodermal cells of blastocysts prevent the incorporation of injected cells into the host embryos. It is still unclear whether this inability to produce chimeric NHPs via pluripotent stem cells is due to the failed uptake of the injected cells into embryos or if there are other reasons. Moreover, due to the long lifespan of NHPs, the generation of chimeric monkeys is not thought to be a practical approach to establish genetically modified NHP model colonies, as obtaining the next generation requires 2.5–5 years. Tetraploid (4N) complementation would be one solution to overcome this issue. When diploid ESCs/iPSCs were injected into tetraploid host embryos, only injected diploid ESCs/iPSCs contributed to viable live-born animals, while the tetraploid host embryos contributed to the placenta.<sup>141–143</sup> Since all somatic and germ cells of live animals originate from injected genetically manipulated ESCs/iPSCs, first-generation animals can be used as models for research. In marmosets, tetraploid blastocysts are easily developed via SCNT to intact MII oocytes.<sup>37</sup> Currently, producing genetically modified animals using pluripotent stem cells has not been achieved in NHPs, including in marmosets. However, if chimeric competent pluripotent stem cells were available, we believe that it would be feasible to use precise gene-manipulated stem cells for the production of genetically modified NHPs.

### Germline Stem Cells

Besides chimeric competent pluripotent stem cells, GSCs that are established from spermatogonial stem cells (SSCs) are another option for producing precise gene-manipulated marmosets. SSCs are germ stem cells that have two characteristics: self-renewal and the ability to give rise to spermatozoa, spermatids, and spermatozoa. Although the SSCs exist at extremely low levels (0.02%–0.03% in mouse and 0.06%–18% in NHPs) in the testes,<sup>144,145</sup> they can be isolated from both neonate and adult testes and expanded in long-term cultures. Furthermore, they maintain their biological ability to produce offspring by spermatogonial transplantation into host male

animal testis.<sup>146,147</sup> Using GSCs, transgenic and knock-out mice have been produced through genetic transduction and drug selection.<sup>148</sup> Originally, SSCs/GSCs are unipotent cells that are involved only in spermatogenesis. However, when SSCs/GSCs are cultured under ESCs culture conditions, they can be converted into pluripotent cells and used to produce chimeric mice.<sup>149–151</sup> To date, while the establishment of GSCs has been attempted in various animal species, the generation of offspring has been reported in rats, sheep, and goats<sup>152–155</sup> but not in NHPs, including marmosets.

### Somatic Cell Nuclear Transfer

In most cases, reproductive biological studies or development of artificial reproductive technologies have been performed in mice first, and only then have they been applied to other animal species. In contrast, the first offspring obtained by SCNT was reported in sheep.<sup>156</sup> Following this report, SCNT was used to produce offspring in many species, including mice, pigs, cattle, goats, mules, horses, dogs, and cats.<sup>76,157–168</sup> SCNT is an advanced technique for the generation of genetically identical animals and involves the culture of donor somatic cells and oocytes, transfer of donor cells into enucleated oocytes, artificial activation of reconstructed embryos, and transfer of cloned embryos into a recipient.<sup>5</sup> This said, SCNT can produce not only cloned animals (genetically identical offspring) but also transgenic animals or target gene knock-out/-in animals when genetically manipulated cells are used as donor cells. Importantly, SCNT can also be used to restore useful genetically modified animal models that have died. The advantage of this method is that it allows the generation of animals with arbitrary genetic modification via selection of genetically modified donor cells prior to the nuclear transfer. The first research on nuclear transfer in NHPs was performed in 1997; rhesus monkey embryonic blastomeres were transferred into oocytes, giving rise to genetically identical newborn offspring.<sup>169</sup> Since donor cells were not somatic, the resultant offspring were not SCNT monkeys. However, this research was important for the establishment of basic SCNT techniques in NHPs. After this report, many attempts to produce SCNT embryos or NHPs have been performed, mostly in rhesus monkeys; one study has been performed in marmosets. Although the results showed that SCNT embryos developed later than the 8-cell stage, indicating successful zygotic genome activation, live offspring were not produced.<sup>5,37,170–175</sup> Even in mice, the success rate of the generation of offspring by SCNT is roughly 2%–3%, and many studies have been performed to overcome this low efficiency. In particular, stimulation of oocyte activation by treatment with ionomycin and histone deacetylase inhibitors such as dimethylaminopurine or trichostatin A has shown to improve the efficiency of SCNT.<sup>5,170,176–179</sup>

Matoba et al<sup>180</sup> reported that reprogramming resistant regions are enriched by the repressive histone modification, histone 3 lysine 9 trimethylation (H3K9me3). These regions resist zygotic genome activation in SCNT embryos; importantly, the expression of an H3K9me3-specific demethylase reduces the levels of H3K9me3 in cloned embryos, improving the reprogramming efficiency of SCNT embryos and increasing the development rates in mice and NHPs.<sup>180–183</sup> Applying trichostatin A and histone demethylase Kdm4d mRNA treatments to the cloning protocols for cynomolgus monkeys successfully produced live offspring. Moreover, 5 cloned cynomolgus monkeys were obtained by SCNT using gene-edited cynomolgus monkey's fibroblast cells as a donor cells and the same protocol.<sup>184,185</sup> This is especially pertinent for the generation of a large number of

cloned NHPs models of human disease with a uniform genetic background. As described above, in marmosets, SCNT has been attempted; however, no cloned animals were obtained so far.

### Gene Editing

As described above, germline-competent pluripotent stem cells are not available in animals other than mice and rats. Gene editing techniques are those that allow the modification of the endogenous target gene in the genome of cells including embryos without the need to use pluripotent stem cells. Gene editing tools make double-stranded breaks in the targeted region in the genome and subsequently repair them via the error-prone nonhomologous end-joining (NHEJ) or homology-directed repair (HDR) pathways. This can result in frameshift mutations in the DNA sequence; of note, DNA can also be repaired using exogenous DNA templates containing a sequence of interest flanked by the relevant homology arms. Consequently, the modified gene is translated into truncated amino acid sequences, disrupting or altering gene function. DNA repair via NHEJ is the predominant cellular repair pathway with a high frequency of mutagenesis. Therefore, target gene knock-outs have been produced in many animal species, including NHPs.<sup>21,186–191</sup> Currently, the most popular method of gene editing is clustered regularly interspaced short palindromic repeat (CRISPR)/CRISPR associated (Cas) 9 system. This system uses a guide RNA that can be a single guide (sg) RNA or crisp (cr) RNA and transactivating crisp (tracr) RNA, alongside a Cas9 nuclease. Briefly, in the CRISPR/Cas9 system, the guide RNA recruits Cas9 to the target DNA sequence, and the Cas9 digests the double-stranded target DNA.<sup>79,80,192</sup> This system usually results in mosaic embryos with mutant and wild-type blastomeres.<sup>193</sup> Therefore, in some cases, the mutated gene function, which causes the disease or objective phenotype, is compensated by the expression of the intact gene, and these mosaic embryos develop into animals that exhibit a wild-type phenotype. However, when a knocked-out gene leads to embryonic lethality, this mosaicism is beneficial, enabling the embryos to develop to full term. In NHPs, obtaining nonmosaic second-generation offspring with objective phenotypes requires more than 2 to 5 years. Further, due to the long lifespan of NHPs, the onset of the objective disease may take a longer time.

Zinc finger nucleases (ZFNs) are recombinant proteins with zinc finger motifs that bind specifically to target DNA sequences.<sup>78</sup> Transcription activator-like effector nucleases (TALENs) are fusion proteins with a transcription activator-like effector (TALE) DNA binding domain.<sup>77</sup> Both ZFNs and TALENs use the FokI nuclease to digest target DNA sequences. In contrast to the CRISPR/Cas9 system, mosaic alterations were not observed when ZFNs or platinum TALENs were used to knock-out the *il2rg*; all of the male animals displayed immune deficiency.<sup>21</sup> For these reasons, ZFNs and TALENs are suitable tools when complete gene targeting is required in NHPs. To increase the success rates of production of phenotypic marmosets, Sato et al<sup>21</sup> screened high-efficiency ZFNs and TALENs using marmoset fibroblasts. Embryos mosaicism was estimated from 8-cell-stage embryos following injection with the gene-editing tool, and gene alteration of each blastomere was analyzed.

Gene editing techniques were also applied to generate target gene knock-in organisms using the HDR mechanism to insert artificial DNA sequences or to induce single-nucleotide substitutions in target loci. Since the efficiency of the HDR pathway in embryos is extremely low, many conditions have been investigated to increase knock-in efficiency, including the length of homology arms and types of donor DNA, such as

single-stranded DNA or double-stranded DNA.<sup>192–198</sup> In cynomolgus monkeys, two studies reported the production of target gene knock-in. One study reported the insertion of humanized recombinant GFP into the last codon of Oct4 using Cas9/sgRNA-mediated HDR,<sup>199</sup> and another study achieved the expression of mCherry under the beta-actin gene promoter.<sup>200</sup> However, although both studies successfully generated target gene knock-in monkeys, insertions/deletions in the target gene<sup>199</sup> and mosaicism<sup>200</sup> were observed. Thus, further investigations to overcome such limitations are needed. Recently, CRISPR/Cas9-mediated target gene knock-in using an adeno-associated viral vector in mice and rats has been reported by two research groups.<sup>201,202</sup> Without the need for the micromanipulation of embryos, these studies reported relatively high efficiency of target gene knock-in (6.3%–100%) using electroporation and incubation with adeno-associated viral vector in a petri dish. While one report described the precise sequence modification in the target locus without indel mutations, the other reported insertions/deletions in the target locus.<sup>201,202</sup>

To knock-out a target gene, recent CRISPR/Cas9-based technologies have been developed to insert point mutations within a target gene without the need for double-stranded breaks. Recently, 2 groups simultaneously reported the development of a technology called Base Editors (BEs) or activation-induced cytidine deaminase, based on a fusion protein comprising the catalytically dead Cas9 and cytidine deaminase APOBEC or activation-induced cytidine deaminase.<sup>203,204</sup> In addition, some BEs fuse a uracil DNA glycosylase inhibitor at the C-terminus of dead Cas9 to protect G:U from the endogenous DNA mismatch repair mechanism and improve mutation efficiency.<sup>203</sup> The BEs induce cytosine-to-thymine (C > T) transitions by the cytidine deaminase within a 4- to 5-nucleotide window at the 5' end of the sgRNA target sequence. Further, adenine BEs have been developed via the evolution of wild-type tRNA adenosine deaminase and an evolved TadA\* heterodimer fused to Cas9 nickase, which converts adenine-to-guanine (A > G).<sup>205</sup> These base editing techniques may be suitable for inducing amino acid substitutions to mimic human diseases. In NHPs, base editing techniques were successfully applied to cynomolgus monkey embryos to insert mutation into specific objective genes, and in another study, the Hutchinson-Gilford progeria syndrome monkey model was produced.<sup>206,207</sup> However, several concerns remain; for example, the active windows of nucleotide transition may cause imprecise mutations, and off-target mutations can be generated. Currently, in marmosets, only production of target gene knock-out via gene editing techniques have been reported.

## Future Perspectives of Genetically Modified NHP Research

Recent developments of ART in NHP and genetic manipulation techniques have enabled the production of genetically modified NHPs, which are expected to be used as human disease models as well as models for understanding primate-specific biological features. Further, they may enable the study of early embryonic development and higher cognitive functions in the primate brain that are impossible to study using rodent models. There are several important aspects of the use of genetically modified NHPs as human surrogate models.

In NHPs, it is important to reduce the numbers of animals used based on the principles of the 3Rs (replacement, reduction, and refinement) because of ethical concerns. To comply with the 3Rs, techniques for the production of genetically modified

marmosets have been developed to be as noninvasive as possible. The noninvasive embryo collection and transfer methods are useful not only for the generation of genetically modified marmosets but also for the maintenance of a wide genetic background of global marmoset colonies. For instance, we can use these techniques and exchange frozen embryos globally without the need to cause transport-induced stress to the animals. Cryopreserved sperm and vitrified embryos have been successfully used for producing marmoset offspring.<sup>60,62,208</sup> These techniques have important implications in conserving and enabling the exchange of valuable marmoset resources worldwide.

Generally, genetically modified mice are selected after neonates are obtained. However in NHPs, to avoid killing animals in the event that genetic manipulation has failed, only those embryos with confirmed genetic modifications should be used for embryo transfer. However, although lentiviral vector-mediated transgenesis enables the selection of transgenic embryos via marker gene expression, it is challenging to select objective target gene knock-out embryos prior to embryo transfer. Therefore, development of embryo biopsy techniques for preimplantation genetic screening warrants additional exploration to overcome this limitation and enable more precise and sophisticated genome modification in NHPs.

Moreover, due to the long lifespan of NHPs, a prolonged period is required to establish a genetically modified NHP line; importantly, it is crucial to produce models with objective phenotypes from the founder generation. Another concern is that since target gene knock-out via gene editing is dependent on NHEJ-induced random DNA repair, the types of mutation at target sites and the rate of target gene mutations in animals cannot be controlled. Moreover, when the target gene is haploinsufficient, founder animals with mosaic mutations do not show an obvious phenotype, and an additional 3–5 years is required to obtain animals with objective phenotypes in the next generation. Furthermore, difficulties in producing target gene knock-in NHPs are due to the low editing efficiency of HDR.

To overcome these concerns, novel key technologies, such as SCNT or ES/iPS nuclear transfer, must be developed in marmosets. As described above, SCNT NHPs have been successfully produced in cynomolgus monkeys; however, further studies are required to accomplish the production of SCNT marmosets. The SCNT marmoset production techniques will solve the disadvantages of NHPs as laboratory animals, such as the long generation period to expand numbers of useful animal models and the inflexibility of the genetic modification using early embryos. Another possibility is to produce gametes from ES/iPS cells or establish naïve ES/iPS cells and generate tetraploid chimeras. These techniques will also enable the next generation of genetically modified marmosets to be obtained. However, further studies are required to achieve this. Taken together, developing these techniques will not only promote the advancement of neurological research but also provide insights into the molecular mechanisms of the primate-specific germ cell and early embryonic development.

## Conclusions

Genetically modified marmosets are expected to be used for modeling neuronal or psychiatric disorders such as Alzheimer's disease, Parkinson's disease, and autism spectrum disorder. Since rodent models of these diseases do not successfully recapitulate the pathophysiology of human patients, the neuronal circuits that are responsible for the disease phenotypes are mostly unidentified, as are the events occurring *in vivo* at the

onset of the disease. Therefore, NHP models are expected to offer a unique opportunity to explore disease dynamics due to their close similarities to humans and to provide a crucial platform for the development of new treatments. Currently, these NHP models are technically available to produce. However, the evaluation of their use as models as well as the establishment of new analysis methods are crucial. Particularly, as the onset of Parkinson's or Alzheimer's disease occurs after middle age in humans, the investigation of the pathogenesis of primate models will also require a long time. Therefore, once the founder of genetically modified marmoset models is obtained, these animals should be used as both disease models for analysis and as breeding animals. Consequently, the development of noninvasive analysis methods and ARTs is important. As described above, noninvasive embryo collection and transfer methods enable breeding with less stress to the animals. For noninvasive analyses of brains, various magnetic resonance imaging-based marmoset brain atlases have been published, which enable noninvasive analyses of the developmental and structural changes in the brain.<sup>209–212</sup> Although several behavioral analysis methods have also been published,<sup>213</sup> further evaluation tasks will be needed. In addition, unlike mice, statistical evaluation methods using a small number of animals, such as repeated analysis of the same animal or comparison with an average brain template,<sup>214</sup> should also be considered; still, further development of analysis methods for fewer animals is desirable. Together with these techniques, genetically modified NHP disease models will be valuable for studies throughout the fields in life sciences.

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