A non-human primate model of familial Alzheimer’s disease for translational medicine

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Summary
Aging is a primary risk factor of Alzheimer’s disease (AD), with the world-wide number of patients anticipated shortly to exceed 50 million. Despite extensive research efforts, no effective measures are available for its prevention or treatment due in part to a lack of human-like animal models. Here, we describe the generation of three mutant marmoset individuals in which exon 9 of the PSEN1 gene product is deleted (PSEN1-ΔE9); the ΔE9 mutations have been reported to cause early onset familial AD1-5. We used Transcription Activator-Like Effector Nuclease (TALEN) to delete the 3’ splice site of exon 9 in the marmoset PSEN1 gene; to this end, TALEN exhibits high genome-editing efficacy, generates few off-target effects, and produces minimal mosaicism. Indeed, whole genome sequencing and other analyses illustrated an inclusive absence of off-target effects and apparent absence of mosaicism. Fibroblasts obtained from newborns indicated occurrence of full-length presenilin 1 protein (PS1) caused by perturbation of PS1 endoproteolysis as well as an increased ratio of Aβ42/Aβ40 production, a signature of familial AD pathogenesis. To our knowledge, this is the first non-human primate model of familial AD. The model lines will be made available to the research community to facilitate the global fight against AD.
**Introduction**

AD is the most common neurodegenerative disease that deprives patients and their families of human dignity. The number of individuals with dementia in the world was approximately 50 million in 2019\(^6\), and AD accounted for 50–70% of all these cases\(^7\). Clinically, AD is characterized by early memory deficits followed by a decline in other cognitive functions\(^7,8\). The pathological changes associated with AD development precede the clinical manifestation by approximately 20 years\(^9\) and comprise the following: deposition of amyloid β peptide (Aβ) as extracellular plaques, accumulation of hyper-phosphorylated tau as intracellular neurofibrillary tangles (NFTs) and chronic neuroinflammation followed by neurodegeneration mainly in the cerebral cortex and hippocampus\(^10,11\). The genetic and pathological observations collectively support the A\(\beta\) hypothesis depicting that A\(\beta\) plays a central role in the AD pathogenesis\(^12\). Mouse AD models appear to have reached a highly advanced state of development by overcoming overexpression artefacts in recent times\(^13-17\). The amyloid precursor protein (App) knock-in mice that harbor familial AD mutations with the A\(\beta\) sequence humanized\(^13\) reproduced A\(\beta\) pathology and neuroinflammation without overexpression of APP. These mice exhibited cognitive dysfunctions as analyzed by Intellicage\(^18\) presumably through vasoconstriction by pericytes\(^19\), impairment of grid cells\(^16\) and plaque-induced gene network\(^17\), providing the mechanistic insights into the action of A\(\beta\) pathology.

These model mice however never exhibited tau pathology or neurodegeneration even when crossbred with human MAPT knock-in mice, in which the entire Mapt gene had been humanized\(^15,20\). The reason for the absence of tauopathy and neurodegeneration in these models remains elusive, but this may simply be because mice live only as long as approximately two years whereas the entire pathological changes in human brain proceed over decades\(^9\). The discrepancy between mice and humans may also be accounted for by the species differences in genetics, neuroanatomy, immunity and metabolism. In addition, various higher cognitive functions specific to primates, represented by the presence of highly developed prefrontal cortex\(^21\), are affected in AD. In this respect, outcomes from mouse models are more suitable for application to preclinical studies than directly to clinical studies. We thus came to the conclusion that a non-human primate model is needed for “near”-clinical studies\(^22-24\). The developmental engineering of primates is, however, much more challenging than that of rodents; thus far we have created three mutant primates using 810 oocytes as described below.

Common marmosets (marmosets, *Callithrix jacchus*) are small non-human primates that belong to the New World Primates. They have been increasingly utilized in neuroscience because of advantages that arose over other research primates\(^22\) (Supplementary Table 1). Marmosets possess genetic backgrounds, physiological functions, brain structures and complex cognitive/social behaviors similar to those of humans; they communicate mainly via visual and auditory measures. In association with AD research, the amino acid sequence of A\(\beta\) is identical to that of humans, and the aged wild-type marmosets start accumulating A\(\beta\) from 7 years old or even earlier\(^25,26\). In addition, adolescent
Marmosets exhibit tau hyperphosphorylation, but not NFT formation, in brain that increases with aging\textsuperscript{26}. Their life spans in captivity are as long as 10 to 15 years, making them suitable for aging research\textsuperscript{27}. Their immune systems and metabolic functions resemble those of humans\textsuperscript{27,28} and thus may affect the pathogenic processes related to AD\textsuperscript{29-31}. Because sleep disorder is an early clinical incident in AD\textsuperscript{32}, it is noteworthy that marmosets share with humans the sleep phases composed of rapid eye movement (REM) and non-REM cycles\textsuperscript{33}. Among various non-human primate species, the marmoset seems most applicable to genetic manipulation, i.e. generation of designed mutants, for which the high reproductive efficacy is advantageous\textsuperscript{22,34}. Furthermore, fecundity characteristics of marmosets, such as the short period of sexual maturity, multiple birth, short gestation interval, are suitable to produce genetically modified disease models. We decided to introduce a pathogenic mutation in the marmoset \textit{PSEN1} gene because the majority of familial AD-causing mutations reside in the \textit{PSEN1} gene\textsuperscript{35}. Typically, deletion mutations in exon 9\textsuperscript{1-5} or point mutations at the 3’ splice site (acceptor site) of exon 9 in the \textit{PSEN1} gene cause dominantly inherited familial AD. The point mutations instigate exon 9 elimination and S290C modification in the corresponding mRNA at the junction sites of exons 8 and 10 via conversion of alternative splicing\textsuperscript{36-40}. We thus set out to generate a marmoset model of AD in which the exon 9 of \textit{PSEN1} gene product is deleted using TALEN to produce AD marmoset models because TALEN exhibits high genome-editing efficacy and because TALEN generates few off-target effects and produces little mosaicism\textsuperscript{34,41}. To our knowledge, this is the first case, in which TALEN has successfully been applied to artificial exon skipping in non-human primates.

\textbf{Results}

\textbf{Evaluation of TALEN activity}

We used the Platinum TALENs designed to target 3’ splice site of exon 9 of the marmoset \textit{PSEN1} gene (Figure 1a)\textsuperscript{32,43}. After introduction of the TALEN mRNAs into the nucleus of marmoset pronuclear stage embryos, we performed surveyor assays and sequencing with the genomic DNA extracted from the developed embryos. We found that 2 out of 3 embryos exhibited deletions at the target sequences including the acceptor site as expected (Figure 1b). To confirm exclusion of exon 9 in the \textit{PSEN1} mRNA, we performed RT-PCR and sequenced the corresponding cDNA sequencing using RNAs extracted from 4-cell-stage or single blastomeres of the TALEN-injected marmoset embryos (Figure 1c)\textsuperscript{34}. We consequently verified complete exclusion of exon 9 in two out of two 4-cell-stage embryos and 2 out of 5 single blastomere, and 3 were failed to amplify the cDNA (Figure 1d). This indicated that the 3’ splice site-deletion resulted in exclusion of exon 9 of the mRNA transcribed from the \textit{PSEN1} gene. There was no wild-type sequence in the embryos or single blastomeres in which 3’ splice site was destroyed, suggesting that the mutations took place in a biallelic manner. We later noted that homozygous deletion of \textit{PSEN1} exon 9 appear to cause embryonic lethality \textit{in vivo} (see the following).
Generation of marmosets lacking \textit{PSEN1} exon 9 in the corresponding mRNA (\textit{PSEN1-ΔE9} and apparent absence of mosaicism

The embryos treated with TALEN were transferred into uterus of surrogate mother marmosets to generate \textit{PSEN1} exon9 deleted AD model marmosets. However, the developmental rate of the embryos (35%) was significantly lower than that in our previous study\textsuperscript{34}, and no pregnant animals were obtained even after 40 zygotic injections. These results implied that the deletion mutations of the \textit{PSEN1-ΔE9} in the biallelic status are likely to cause the low embryonic developmental rate and embryonic lethality presumably due to the disruption of Notch signaling\textsuperscript{44}. This was somewhat expected because mice deficient in PS1\textsuperscript{45,46} or lacking the active site aspartate \textsuperscript{47} exhibited embryonic lethality and because the protein domain corresponding to \textit{PSEN1} exon 9 is a site for endoproteolysis\textsuperscript{48}, which is perturbed by the active site destruction\textsuperscript{47}. We thus came to realize that we need to introduce the ΔE9 mutations in a monoallelic manner.

To avoid the biallelism of the \textit{PSEN1} gene mutations, we injected TALEN mRNAs into 810 ova and then performed \textit{in vitro} fertilization on the 578 (71.4%) ova that survived, using wild-type marmoset sperms. After the \textit{in vitro} fertilization, 218 (37.7%) zygotes were obtained then transferred 154 (70.6%) embryos that developed to above 6-cell stage into uterus of 77 surrogate mothers. Around the 145th day from the ovulation of the surrogate mother, we obtained 6 marmoset neonates by normal delivery or by Caesarean section (\textbf{Figure 2a, Table 1}).

Surveyor assays of the genomic DNA isolated from hair roots of the newborns suggested that 3 out of 6 neonates carried \textit{PSEN1} gene mutations. Sequence analyses of the subcloned PCR products from the genome DNA indicated that 3 different patterns of the \textit{PSEN1} gene mutations in these animals. 2 animals carried 6-bp deletions and another newborn harbored 9-bp deletions at the 3’ splicing site, envisaging deletion of the region in mRNA that corresponded to the genomic \textit{PSEN1} exon 9 (\textbf{Figure 2b}). The numbers of wild-type and truncated genes in the subclones collected from the mutants’ hair roots were 53 and 47 out of 100 in I774gmF, 51 and 49 in I943gmM, and 59 and 41 in I949gmM, respectively (\textbf{Figure 2c}): approximately 50% of the clones carried the identical mutations in each case. Consistently, RT-PCR followed by sequencing of cDNA verified the excision of exon 9 in the mRNAs extracted from hair root samples of the mutants. These observations suggest two possibilities: [1] a presence of heterozygous mutations essentially in all the cells; [2] a presence of wild-type and homozygous mutant cells in a 1:1 ratio, i.e., mosaicism. The second possibility can be ruled out because homozygous \textit{PSEN1-ΔE9} mutations result in embryonic lethality presumably via a Notch phenotype\textsuperscript{5,46,49} that would preclude half of the neural stem cells from differentiating to neurons during development. It is noteworthy that \textit{γ}-secretase cleaves the truncated Notch in a cell-autonomous manner\textsuperscript{60}. These results and speculations indicate a probable absence of mosaicism in the mutant marmosets\textsuperscript{34}. Consistently, single cell PCR analyses indicated that two of the mutants,
I774gmF and I949gmM, were mosaicism-free: ten out of ten single cells showed heterozygous mutations. However, the other mutant, I943gmM, exhibited an extensive mosaicism (>80%) and was thus excluded from further analyses (Supplementary Figure 1). The sequence analyses of the cDNAs also showed that there were no insertions or deletions in the exons 8 and 10 in the PSEN1 gene (Figure 2d, Supplementary Figure 2). We thus concluded that we successfully acquired PSEN1-ΔE9 marmoset neonates.

Off-target analyses of the mutant PSEN1-ΔE9 marmosets
A number of genome editing-based studies suffer from off-target effects. We analyzed the off-target mutations in the mutant PSEN1-ΔE9 marmosets in two ways. The online Paired Target Finder tool was used to search for candidate off-target sites for calculation an average score at each potential off-target site obtained from the search and identified the top 10 candidate sites (Supplementary Table 2). Genomic DNA extracted from hair roots of PSEN1-ΔE9 marmosets was subjected to PCR then determined the sequence by direct sequencing. None of the PSEN1-ΔE9 marmosets exhibited any mutations in the off-target candidate sites. Second, we performed whole genome sequencing (WGS) of the genomic DNA obtained from the first PSEN1-ΔE9 marmoset (I774gmF) and from her parents at the average coverage over genome of 42.1, 57.4, and 62.2x for the neonate, father, and mother, respectively (Supplementary Table 3). In order to process the WGS data sets, we performed Illumina Dynamic Read Analysis for GENomics (DRAGEN) pipeline (version 3.5.7) for mapping and variant calling process at a default parameter with “CJA1912RKC” marmoset genome reference sequence (GCA_013373975.1). We used this highly permissive parameter in variant calling and did not filter out variants to keep as many variants in off-target candidate sites as possible. By comparing all the variants from the PSEN1-ΔE9 marmoset (I774gmF) with those from her parents, we found 306,492 variants only in the PSEN1-ΔE9 marmoset (I774gmF) (Supplementary Figure 3). None of the 306,492 variants were present within the 10 off-target candidate sites. These analyses further confirmed the absence of the off-target mutations. We thus declare that application of the TALEN technology to generation of the PSEN1-ΔE9 marmosets has fully succeeded.

Endoproteolysis of the mutant marmoset-derived PS1 and secretion of Aβ42 versus Aβ40
PS1 serves as a major catalytic subunit for the γ-secretase complex that generates Aβ from the C-terminal fragment of APP after the limited proteolysis by β-secretase. Most, if not all, of the pathogenic mutations found in the PSEN1 gene cause familial AD by elevating the ratio of Aβ42/Aβ40 production. The ratio matters because Kim et al. demonstrated that Aβ42 is causal whereas Aβ40 is protective in the pathological processes. To biochemically analyze the mutant marmosets in the least invasive manner, we established primary fibroblasts from the wild-type and mutant marmosets edge of the ear lobe. We first examined the effect of exon 9 deletion on the endoproteolytic status of PS1
protein because such deletion impacts on the global PS1 endopeptidase activity. We performed Western blot analysis of fibroblasts derived from wild-type and PSEN1-ΔE9 marmosets using a set of antibodies that recognize the N- and C-termini of PS1. The exon 9 deficiency in the two independent mutants, i.e. 1774gmF and 11943gmM, gave rise to a full-length PS1 that was absent in the wild-type fibroblasts (Fig. 3a). Consistently, the quantities of the N-terminal and C-terminal fragments in the mutants were reduced to approximately half the levels compared to those in wild-type cells. These observations indicate that the monoallelic exon 9 deletion caused perturbation of the PS1 endoproteolysis in a heterozygous manner and that the nature of endoproteolysis was autolytic. The deletion also induced an amino acid sequence conversion, S290C, as shown in Supplementary Figure 2.

We then quantified Aβ42 and Aβ40 secreted from the wild-type and mutant fibroblasts by enzyme-linked immunosorbent assay (ELISA) for the reasons stated above. The quantity of Aβ42 was significantly increased in the mutant-derived media while that of Aβ40 also showed a significant reduction. Consequently, the ratio of Aβ42/Aβ40 production was statistically significantly increased approximately 3-fold (Fig. 3b), a hallmark of familial AD pathogenesis. Similar results had been reported using cultured human fibroblasts and lymphocytes obtained from PSEN1 mutation carriers. Aβ43 was undetectable. These observations indicate that the PSEN1-ΔE9 mutations introduced into the marmosets are indeed pathogenic.

**Discussion**

Steiner et al. previously demonstrated that a PSEN1-ΔE9 mutation exerted its pathogenic effect via an amino acid conversion, S290C, rather than perturbed PS1 endoproteolysis. This effect of the amino acid conversion on the Aβ42/Aβ40 ratio is also likely to apply to our observation in the fibroblasts obtained from the mutant marmosets because they also carry the S290C conversion (Supplementary Figure 2). There is however a distinct difference between the studies by Steiner et al. and by us. Steiner et al. used a cDNA overexpression paradigm in cultured cells and in Caenorhabditis elegans. This approach does not necessarily warrant the stoichiometric formation of γ-secretase complex and is comparable to homozygous deletion of exon 9 in vivo, which resulted in embryonic lethality (see Generation of marmosets lacking PSEN1 exon 9 in the corresponding mRNA (PSEN1-ΔE9) and apparent absence of mosaicism). In addition, the results shown by Steiner et al. do not exclude the possible need for simultaneous presence of exon 9 deletion and S290C mutation together to evoke the pathogenic activity in a coordinated manner, and the S290C mutation by itself is unlikely to account for the symptom of spastic paraparesis often observed in PSEN1-ΔE9 patients. The embryonic lethality caused by the biallelic presence of PSEN1-ΔE9 mutations also interrogates the pathogenic role of the S290C mutation by itself because most pathogenic point mutations in Psen1 gene generally resulted in non-lethal phenotypes in a homozygous status at least in mice except for a few cases.
We thus cannot rule out the possible role of ΔE9 mutations alone in the pathogenicity *in vivo*. These issues would be better addressed if we could create various mutant knock-in mice, but deletion of *Psen1* exon 9 in mice generated a termination codon within the *Psen1*-coding sequences (Kaori Sato and Nagata, unpublished observations), rendering such attempts difficult. These queries thus remain as open questions, but they are beyond the scope of our present study the aim of which is to establish a non-human primate model of familial AD.

Currently, the CRISPR/Cas9 technology including Base Editor (BE) appears the prevalent method for genome editing in general. Indeed, Wang *et al.* succeeded in generating a Hutchinson–Gilford progeria cyonmoligos monkey model using BE. In the present study, we used a slightly different technique, TALEN, because it is highly efficient, and produces less mosaicism. Further, application of BE to genome editing of the marmoset *PSEN1* gene has proven more challenging than expected (Kumita *et al.*, unpublished observations). Consistently, the genome editing efficacy in the present study by TALEN was as high as 50%. This study showed that targeting 3′ splice site of the *PSEN1* gene induced whole exon 9 skipping by TALEN and cDNA sequence analyses showed that neither insert nor deletion occurred at exon 8 or exon10 in the *PSEN1* gene. The strategy used in this study, induce mutation at the splice site of the target gene, is a new method for precisely deletion whole target exon at the target gene, without any mutation at neighbor exon. Therefore, destruction of the splice site in the *PSEN1* gene to induce the exon 9 skipping by this specific genome-editing tool appears the most realistic approach to generate a non-human primate model of familial AD. The oldest mutant marmoset among the three mutants, I774gmF, was born in April of 2019 thus needs some more time to become reproductive at present, making it difficult to provide direct evidence for the germ line transmission of the pathogenic mutations. However, we presume that the apparent lack of mosaicism (see Results) implies the presence of the mutations in the reproductive cells because a similar approach to generate IL2RG knock-out marmosets using TALEN resulted in 100% germ line transmission.

To our knowledge, this is the first non-human primate model of familial AD ever produced. Because the wild-type marmosets start accumulating Aβ in brain around the age of 7 or even earlier, because the age-of-onset of *PSEN1*-ΔE9 humans varies in their 40’s, and because Aβ deposition arises approximately two decades prior to the disease on-set in humans, we anticipate the mutant marmosets to start exhibiting Aβ pathology from 2-3 years old. Relevance of the mechanistic findings obtained using the model mice shall be confirmed if the mutant marmosets are utilized. Because the marmosets live much longer than mice, they may develop tau pathology and neurodegeneration, which model mice failed to recapitulate. If so, they would become convenient tools to establish the cause-and-effect relationship and to elucidate the mechanisms by which Aβ amyloidosis evokes tau pathology and neurodegeneration. They would first become applicable to primary basic studies being used for humans, including non-invasive imaging analyses by MRI to determine morphometric
changes and by positron emission tomography (PET) to search for Aβ and tau pathology. The body fluids such as blood and cerebrospinal fluid may be collected to identify biomarkers. In a more clinical sense, we can subject them to highly sensitive cognitive analyses such as touch-sensitive technology\textsuperscript{61} or can analyze the sleep disturbances\textsuperscript{33}. We can also examine the default mode network, the changes of which represent an early sign of preclinical AD, by functional MRI\textsuperscript{62,63}. Model mice are incompetent for these studies. In addition, the effect of Aβ pathology on the function of prefrontal cortex\textsuperscript{21} can be understood only by analyzing the mutant marmosets. For pharmaceutical studies, the mouse model can first be used for initial screening, and the marmoset model can then be used for the second screening before clinical trials. They would also become tools to examine the anti-tau pathology and anti-neurodegeneration medication candidates if these pathological features arise. We are making maximum efforts to expand our colonies so that the marmoset model can be shared by the global research community in the near future.
Methods

Animals
All animal experiments were carried out in accordance with guidelines for animal experimentation from the RIKEN Center for Brain Science and the Central Institute for Experimental Animals (CIEA: 18031A, 19033A). The CIEA standard guidelines are in accordance with the guidelines for the proper conduct of animal experiments determined by the Science Council of Japan. The marmosets used in this study were purchased from CLEA Japan, Inc., Tokyo, Japan. The animals ranged from 2-4.5 years-old with body weights between 300 and 450 g.

In vitro transcription of TALEN mRNA
We employed a two-step Golden Gate assembly method using the Platinum Gate TALEN Kit (Addgene; cat#1000000043) to construct Platinum TALEN plasmids containing the homodimer-type FokI nuclease domain. The assembled repeat arrays were subsequently inserted into the final destination vector, ptCMV-153/47-VR. TALEN mRNA was synthesized using the mMESSAGE mMACHINE T7 Ultra Transcription Kit (Thermo Fisher Scientific, AM1345). Transcribed mRNA was purified using the MEGAclear Transcription Clean-Up Kit (Thermo Fisher Scientific, AM1908), and Nuclease-Free water (Thermo Fisher Scientific, AM9937) was used for the elution step and subsequent dilution. For microinjection, an aqueous solution was used in which left TALEN and right TALEN mRNAs were mixed in equal amounts so that the final concentration was 8 ng/μl.

Procedures for oocytes collection and in vitro fertilization
Oocyte collection and in vitro fertilization (IVF) was performed as previously described22,64. Briefly, 62 female marmosets whose plasma progesterone levels under 10 ng/ml were used for the follicular stimulation. To collect the oocytes, the selected marmosets were intramuscularly treated with Purified Human Menopausal Gonadotrophin, follicle-stimulating hormone (hFSH, 25IU; FOLYRMON-P injection, Fuji Pharma) for five times every other day. The day after the last hFSH administration, human chorionic gonadotropin (hCG, 75IU; Gonatropin, ASKA Pharmaceutical) was subsequently administered by an intramuscular injection. The hormone-treated female marmosets were pre-anesthetized with 0.04 mg/kg medetomidine (Domitor; Nippon Zenyaku Kogyo), 0.40 mg/kg midazolam (Dormicam; Astellas Pharma) and 0.40 mg/kg butorphanol (Vetorphale; Meiji Seika Pharma). The ovum pickup was performed at 17-20 hours after the hCG injection. During the operation, the marmosets were anesthetized by isoflurane (Isoflurane Inhalation Solution; Pfizer) inhalation. The collected ova were incubated in Porcine Oocyte Medium (Research Institute for the Functional Peptides, IFP1010P) for 28-32 hours for in vitro maturation65. For IVF, semen was collected from healthy male marmosets and diluted to 3.6×10^6 sperm/ml, after washing with TYH medium (LSI Medience, DR01031). Insemination was performed via the co-incubation of 3.6×10^4 sperm per oocyte for 10-16 hours at 37°C with 5% CO2, 5% O2, and 90% N2.

Blastomere splitting
TALEN-mRNA-injected embryos that had reached the 4-cell stage and above were directed to blastomere splitting as previously described\textsuperscript{34}. Briefly, the embryo specimens were treated with acidified Tyrode’s solution (Origio, 10605000) to lyse the zona pellucida. Naked embryos were transferred into Embryo Biopsy Medium (Origio, 10620010). After several minutes, embryos that exhibited weak adhesion between blastomeres were split into single blastomeres using glass capillaries and washed in phosphate-buffered saline (PBS) (-) drops immediately. The blastomere was transferred to a test tube with a small amount of PBS and used for subsequent analyses.

**Embryo transfer**

The embryos that grew to 6-cell-stage or higher were transferred to the uteri of surrogate mothers as previously described\textsuperscript{22,64}. The method for obtaining newborns was based on natural delivery, but if delivery was judged to be difficult, a Caesarean section was performed on day 145 after embryo transfer\textsuperscript{34}.

**PCR for genotyping**

Genomic DNA was extracted from marmoset cord blood and hair roots using QIAamp DNA Micro Kit (Qiagen, 56304) according to the manufacturer’s instructions. Embryos and blastomeres were used as templates without any genome extraction processing. PCR was performed using specific primers followed by subsequent sequencing or surveyor assay (Supplementary Table 4). Each PCR mixture contained 1X PCR buffer, 0.2 mM of dNTPs, 0.5 mM of each primer, 20 ng of template DNA and 2.5 U of KOD-Plus-Neo (Toyobo, KOD-401) in a total volume of 20 μl. PCR was performed using the following PCR cycling conditions: 1st PCR (for embryo and blastomere), one cycle of 94°C for 2 min, 45 cycles of 98°C for 10 sec, 60°C for 10 sec and 68°C for 40 sec, one cycle of 68°C for 7 min. 2nd PCR (for all samples), one cycle of 94°C for 2 min, 35 cycles of 98°C for 10 sec, 60°C for 10 sec and 68°C for 30 sec, one cycle of 68°C for 7 min.

**Sequencing analyses**

To confirm the sequences of the TALEN target sites, PCR products were subcloned into the Zero Blunt PCR Cloning Kit (Thermo fisher scientific, 44-0302) according to manufacturer’s instructions. The plasmids obtained were transformed with ECOS Competent E. coli DH5α (Nippon gene, 316-06233) and cultured on LB agar containing Kanamycin. After amplifying the plasmid using the emerged single colony, it was reacted using M13 primer and Big Dye terminator v3.1 cycle sequence kit (Thermo fisher scientific, 4337455), and gene sequences were analyzed by 3130 Genetic Analyzer (Thermo fisher scientific).

**Surveyor assay**

The SURVEYOR mutation detection kit (IDT, 706025), one of the mutation analysis methods that identifies mismatches in double-strand DNA, was used to detect genetic modifications at the TALEN target site. Briefly, 8 μl of the PCR product described above. For the analysis of biallelic mutations in the \textit{PSEN1} gene, a sample in which 4 μl of the PCR product was mixed with an equal amount of the
PCR product prepared using wild-type PSEN1 as a template was also prepared. The sample digested with Nuclease was electrophoresed on Novex TBE Gels, 10%, 12 well (Thermo fisher scientific, EC62752BOX) and then subjected to nucleic acid staining with GelRed (Fujifilm Wako Chemicals, 518-24031).

**Single-cell PCR**

To establish the marmoset primary fibroblast cells, approximately 3 mm³ was excised from the edge of the earlobe of marmosets. Tissue from each animal was cultured at 37 °C and 5% CO₂ with Dulbecco’s modified Eagle’s medium + GlutaMAX (Thermo Fisher Scientific, 10566-024) containing 10% FBS (Biowest) and a penicillin-streptomycin cocktail (Thermo Fisher Scientific, 15240-062). Cultured fibroblast cells were harvested and washed in PBS (-) following which single cells were isolated one by one with a glass capillary tube and placed into individual sampling tubes. Direct PCR and sequencing were then performed, with the PCR conditions used being the same as those used for the genotyping PCR. The electrophoresis of PCR products was performed with 10%TBE Gels (Thermo Fisher, EC62752BOX), followed by nucleic acid staining with GelRed.

**RT-PCR**

Total RNA was extracted from marmoset embryos, blastomeres, and hair roots using Nucleospin RNA plus XS (Takara, U0990B) according to the manufacturer’s instructions. cDNA was synthesized using ReverTra Ace - α - (Toyobo, FSK-101F). In the reaction, the sample was divided into two: one received Reverse Transcriptase, and the other received the same amount of water as a control to evaluate genomic DNA contamination. PCR was performed the primers designed on exons (Supplementary Table 4). Each PCR mixture contained 1X PCR buffer, 0.2 mM of dNTPs, 0.5 mM of each primer, 20 ng of template DNA and 2.5 U of KOD-Plus-Neo (Toyobo, KOD-401) in a total volume of 20 μl. PCR was performed using the following PCR cycling conditions: 1st PCR (for embryo and blastomere): one cycle of 94°C for 2 min, 45cycles of 98°C for 10 sec, 60°C for 10 sec and 68°C for 40 sec, and one cycle of 68°C for 7 min. 2nd PCR (for all samples): one cycle of 94°C for 2 min, 35cycles of 98°C for 10 sec, 60°C for 10 sec and 68°C for 30 sec, one cycle of 68°C for 7 min.

**Whole-genome sequencing (WGS)**

To prepare the genomic DNA for WGS, the edge of the ear lobe (approximately 3 mm square) was cut out from the marmosets as a lowly invasive body tissue collection. Genomic DNA extraction from the ear tissue was performed using the QIAamp Micro DNA kit (Qiagen, 56304), according to the manufacturer's instructions. Briefly, the tissue was incubated in lysis buffer and Proteinase K overnight at 56°C, then genomic DNA was collected by column purification with low-TE (Tris-Hcl 10 mM, EDTA 0.1 mM, pH8.0) elution. Whole genome sequencing was performed using Illumina NovaSeq. FASTQ files for all WGS runs are deposited under accession number DRR239155, DRR239156, DRR239157 in DNA Data Bank of Japan (DDBJ) Sequence Read Archive (DRA). For processing the
WGS data sets, Illumina DRAGEN pipeline (version 3.5.7) was performed for mapping and variant calling process at a default parameter with “CJA1912RKC” marmoset genome reference sequence (GCA_013373975.1) (Supplementary Table 3). We used this highly permissive parameter in variant calling and did not filter out variants to keep as many variants in off-target regions as possible. By comparing all the variants from the mutant PSEN1 (I774gmF) marmoset with those from her parents, 306,492 variants were found only in the mutant PSEN1 marmoset without any filtering.

Off-target analysis by Paired Target Finder Tool
Paired Target Finder (https://tale-nt.cac.cornell.edu/node/add/talef-off-paired) was used to search for potential off-target sites. As the reference, CJA1912RKC (GenBank assembly accession: GCA_013373975.1), which is the whole genome sequence of marmoset, was set in the search database, and the RVD sequence of TALEN was provided as follows; RVD Sequence 1, NI HD HD NG NN NN NI NI NG NG NG NN KG HD NG; RVD Sequence 2, NI NG KG HD NI HD HD NI NI KG HD HD NI. The minimum and maximum lengths of the spacers were set to 10 and 30, respectively. Other parameters were set as recommended. Genomic DNA extracted from marmoset hair roots using QIAamp DNA Micro Kit (Qiagen, 56304) was subjected to PCR with specific primers followed by direct sequencing (Supplementary Table 5). Each PCR mixture contained 1X KOD One PCR Master Mix (Toyobo, KMM-101), 0.5 mM of each primer and 10 ng of template DNA in a total volume of 20 μl. PCR was performed using the following PCR cycling conditions: 30 cycles of 98°C for 10 sec, 60°C for 5 sec and 68°C for 1 sec. The amplicon was purified using NucleoSpin Gel and PCR Clean-up (Takara Bio, 740609), and reacted with one of the PCR primer and Big Dye terminator v3.1 cycle sequence kit (Thermo fisher scientific, 4337455), and the gene sequence was identified by 3130 Genetic Analyzer (Thermo fisher scientific).

Western blot analysis
We established primary fibroblast lines from ear skin tissues of the wild-type and PSEN1-ΔE9 marmosets, i.e. I774gmF and I943gmM. We performed Western blotting as previously described. Briefly, cells were lysed and subjected to subcellular fractionation using ProteoExtract Subcellular Proteome Extraction Kit (Merck, #539790) according to the manufacturer’s instructions. We loaded the membrane fractions onto polyacrylamide gels, electrophoresed them, blotted them onto PVDF membranes (Merck Millipore), which were then treated with the ECL Prime blocking agent (GE Healthcare) and incubated with the primary antibodies raised against the PS1 N-terminal (G1Nr5) and C-terminal (G1L3) domains. We used an antibody to sodium-potassium ATPase (EP1845Y, Abcam, ab76020) as a loading control of the membrane fraction.

Enzyme-linked immunosorbent assay (ELISA)
Aβ40 and Aβ42 was quantified using ELISA as previously described. Briefly, culture media, collected from the primary skin fibroblasts at 72 hours after incubation, were mixed with 11 times the volume of 6M guanidine-HCl. We then quantified Aβ40 and Aβ42 using Aβ ELISA kit (Wako, 294-62501 and
Quantification of Aβ43 was performed as previously described58.

**Statistic analysis**

All data are shown as the mean ± s.e.m. For comparison between two groups, data were analyzed by Student’s t-test. All the data were collected and processed in a randomized and blinded manner.

**Author Contributions**

H.S., K.N., and T.C.S. made a primary design of the AD model. T.S. and T.Y. created TALENs. K.S., W.K. and E.S. planned developmental engineering procedures. K.S., W.K., T.I., Y.K. and E.S. generated mutant marmosets and fibroblasts thereof, and performed molecular biological analyses. H.S. and N.M. performed biochemical experiments. K.S. (Kaori Sato), and K.N. performed mouse experiments. M.T., R.M., K.O., and Y.O. analyzed the WGS data. K.S., H.S., W.K., K.N., T.S., T.Y., T.C.S. and E.S. jointly analyzed and interpreted the other data. K.S., H.S., W.K., K.N., T.S., T.Y., M.T., R.M., K.O., Y.O., T.C.S. and E.S. wrote and edited the manuscript.

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**Conflicts of interest**

The authors declare no conflicts of interest in the present study.
Figure 1.

(a) Structure of TALEN-AS elements with sequences for Exon 9 and Intron 8.

(b) Genetic analysis of embryos with TALEN-AS treatment.

(c) Process of injecting TALEN-AS into embryos for whole embryo analysis.

(d) PCR analysis results showing unedited and edited DNA fragments for Exon9.
Figure 1. TALEN-mediated genome editing in marmoset embryos.

a) Left (TALEN_AS_L) and right (TALEN_AS_R) were constructed using Platinum Gate TALEN Kit (Addgene; cat#1000000439) as previously described\(^4\). Pink boxes indicate TALEN targeting sites, green boxes indicate exons of the marmoset PSEN1 gene, and red letters (AG) are the acceptors site of exon 9. b) Surveyor assay of marmoset embryos after TALEN injection. PCR that covered the acceptor site of exon 9 in genomic DNA extracted from TALEN-injected embryos (left three columns) was performed with or without Nuclease (N) treatment. The right two columns denoted experimental controls from wild-type embryos. c) Schematic representation of the TALEN mRNA injection and subsequent analysis of PSEN1 mRNA. After injection of TALEN mRNA into marmoset zygotes, RNA was extracted from four 3- to 4-cell stage whole embryos or single blastomeres from one 5-cell stage embryo after blastomere splitting. The RNA was reverse transcribed, and the cDNA was subjected to PCR (RT-PCR). d) RT-PCR of mRNA derived from TALEN-injected embryos. The lane 1-5 indicates RT-PCR products of each blastomere after splitting; the lanes 3c, 4c-1, 4c-2, and 4c-3 indicate RT-PCR of four embryos without splitting. PCR products with exclusion of exon 9 appeared with 315 bp bands while wildtype bands were 402 bp. Note that all the analyzed embryos showed only single band, suggesting that no embryos carried the mutations in a heterozygous manner.
Figure 2.

a)  

![Images of three monkeys labeled I774gmF, 943gmM, I949gmM]

b)  

|    | CB   | HR   | M    | CB   | HR   | M    | CB   | HR   | M    | CB   | HR   | M    |
|----|------|------|------|------|------|------|------|------|------|------|------|------|
| I774gmF |      |      |      |      |      |      |      |      |      |      |      |      |
| I774gmF |      |      |      |      |      |      |      |      |      |      |      |      |
| 943gmM |      |      |      |      |      |      |      |      |      |      |      |      |
| 943gmM |      |      |      |      |      |      |      |      |      |      |      |      |
| I949gmM |      |      |      |      |      |      |      |      |      |      |      |      |
| I949gmM |      |      |      |      |      |      |      |      |      |      |      |      |

CB: Cord blood genome  
HR: Hair root genome  
M: 50bp ladder  
+N: Added PCR product of Normal PSEN1  
+: Surveyor ctrl. / Nuclease added  
-: Surveyor ctrl. / Non-treatment

c)  

| Wildtype |  (I774gmF, I940gmM, I942gmM) |  TGGTTACCTGGAAATTGTGTCTTTCTCAACAGGCACAATGTTGTTGTTGGTGATATGC | Intact (50/50) |
|----------|-----------------------------|-------------------------------------------------------------|----------------|
| PSEN1-ΔE9 |  (I774gmF) | TGGTTACCTGGAAATTGTGTCTTTCTCAACAGGCACAATGTTGTTGTTGGTGATATGC |  -6bp (47/100) |
|          |                | TGGTTACCTGGAAATTGTGTCTTTCTCAACAGGCACAATGTTGTTGTTGGTGATATGC | Intact (53/100) |
| PSEN1-ΔE9 |  (I943gmM) | TGGTTACCTGGAAATTGTGTCTTTCTCAACAGGCACAATGTTGTTGTTGGTGATATGC |  -6bp (41/100) |
|          |                | TGGTTACCTGGAAATTGTGTCTTTCTCAACAGGCACAATGTTGTTGTTGGTGATATGC | Intact (59/100) |
| PSEN1-ΔE9 |  (I949gmM) | TGGTTACCTGGAAATTGTGTCTTTCTCAACAGGCACAATGTTGTTGTTGGTGATATGC |  -9bp (47/100) |
|          |                | TGGTTACCTGGAAATTGTGTCTTTCTCAACAGGCACAATGTTGTTGTTGGTGATATGC | Intact (53/100) |
d) Figure 2. The *PSEN1-ΔE9* marmosets generated by TALEN.

a) Photograph of the founder neonates. B) Surveyor assay of neonatal somatic tissues. Genomic DNAs extracted from umbilical cord blood and hair roots were subjected to surveyor assays. Samples, in which 4 µl of the PCR product were mixed in equal amounts with the PCR product from wild-type *PSEN1* DNA as a template to detect biallelic mutations in the *PSEN1* gene, are designated as +N. c) Sequences of the TALEN-targeted site in *PSEN1* gene. The underlined part corresponds to the TALEN recognition sequence: the red letters to the acceptor site, and the dashes to the missing bases. d) Result of sequence analyses of the neonates. RT-PCR products from hair roots obtained from 6 obtained neonates were subcloned and sequenced. The first column shows the marmoset identities, the second the genotypes, the 3rd the percentage of wild-type cDNA, and the last percentage of mutated cDNA.

| ID   | Genotype       | Wild type | ΔExon9          |
|------|----------------|-----------|-----------------|
| I774gmF | *PSEN1-ΔE9*    | 46% (46/100) | 54% (54/100)   |
| I780gmF | Wild type       | 100% (50/50)   | 0% (0/50)      |
| I940gmM | Wild type       | 100% (50/50)   | 0% (0/50)      |
| I942gmM | Wild type       | 100% (50/50)   | 0% (0/50)      |
| I943gmM | *PSEN1-ΔE9*    | 43% (43/100)   | 57% (57/100)   |
| I949gmM | *PSEN1-ΔE9*    | 42% (10/24)    | 58% (14/24)    |

CB: Cord blood genome
HR: Hair root genome
M: 100bp ladder
RT(+) = Added reverse transcriptase
RT(-) = Without reverse transcriptase
Figure 3.

a) Western blot analysis of the membrane fractions obtained from wild-type (WT) and mutant (ΔE9) marmoset fibroblasts using antibodies to the N-terminal fragments (NTF) (left panel) and to C-terminal fragments (CTF) (right panel) of PS1. Arrowheads indicate NTF and CTF produced by endoproteolysis of PS1; arrows indicate uncleaved full-length PS1 protein. Sodium-potassium ATPase was used as a loading control of the membrane fraction.

b) $A\beta_{40}$ and $A\beta_{42}$ in the culture media from

WT n=15
ΔE9 n=6

*p < 0.05
****p < 0.0001

Figure 3. Biochemical analysis in the $PSEN1$-ΔE9 marmoset fibroblasts.

a) Western blot analysis of the membrane fractions obtained from wild-type (WT) and mutant (ΔE9) marmoset fibroblasts using antibodies to the N-terminal fragments (NTF) (left panel) and to C-terminal fragments (CTF) (right panel) of PS1. Arrowheads indicate NTF and CTF produced by endoproteolysis of PS1; arrows indicate uncleaved full-length PS1 protein. Sodium-potassium ATPase was used as a loading control of the membrane fraction. b) $A\beta_{40}$ and $A\beta_{42}$ in the culture media from
wild-type and mutant marmoset fibroblasts were quantified by sandwich ELISA. The Aβ_{43} levels were below the detection limit. Data represent mean ± sem (n=15 (WT); 6 (ΔE9)). *P < 0.05; ****P<0.0001 (Student’s two-tailed t-test).
Table 1. Summary of animal generation

| TALEN injected ova | Matured ova (%) | Fertilized ova (%) | Transferred embryos (%) | Recipients | Pregnant animals (%) | Aborted case (%) | Delivered animals | Intact | Mutant |
|--------------------|-----------------|-------------------|-------------------------|------------|---------------------|-----------------|-------------------|--------|--------|
| 810                | 578 (71.4)      | 218 (37.7)        | 154 (70.6)              | 77         | 7 (9.1)             | 2 (28.6)        | 6                 | 3      | 3      |
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