




REVIEW ARTICLE

Local transgene expression and whole-body transgenesis to model brain diseases in nonhuman primate

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Abstract

Animal model is an essential tool in the life sciences research, notably in understanding the pathogenesis of the diseases and for further therapeutic intervention success. Rodents have been the most frequently used animals to model human disease since the establishment of gene manipulation technique. However, they remain inadequate to fully mimic the pathophysiology of human brain disease, partially due to huge differences between rodents and humans in terms of anatomy, brain function, and social behaviors. Nonhuman primates are more suitable in translational perspective. Thus, genetically modified animals have been generated to investigate neurologic and psychiatric disorders. The classical transgenesis technique is not efficient in that model; so, viral vector-mediated transgene delivery and the new genome-editing technologies have been promoted. In this review, we summarize some of the technical progress in the generation of an ad hoc animal model of brain diseases by gene delivery and real transgenic nonhuman primate.

KEYWORDS

animal models, neuroscience, nonhuman primates

1 | INTRODUCTION

Worldwide, brain disorders are among the principal cause of disease burden. However, the development of new and better clinical treatment progresses slowly, in part, due to the lack of good animal models.¹ Animal models of brain diseases are essential for understanding the pathophysiology and investigating the brain-behavior relationship that cannot be studied in humans.²

Studies on nonhuman primates (NHP) are still limited. The number of monkeys used for research was anecdotal compared to others species such as the rodents (less than 0.1% vs 80%, respectively).³ Of all animal models used in neurosciences, the brain of monkey is most similar to the human brain.⁴ Hence, the NHP brings a physiological, genetic, and morphological environment close to human, which makes it more suitable than rodent models.

Various methodologies have been explored to obtain the most similar models to human disease. Surgical and drug-inducible methods have been used for a long time but nowadays transgenic models are under development. Viral vector delivery has been largely used to obtain an ad hoc animal model. For that purpose, the method of vector delivery is as important as the vector itself to achieve transgenesis. In rodent, transgenic animals have also been obtained by introducing genetic modified embryonic stem cells (ESC) into host blastocysts. Until recently, ESCs of nonhuman primate do not have the competency to generate chimeric animals. That is why the new genome-editing tools, such as zinc finger nuclease (ZFNs), transcription activator-like effector nucleases (TALENs), and clustered regularly interspaced short palindromic repeat/CRISPR-associated protein 9 (CRISPR/Cas9), were used to disrupt a target gene (knock-out [KO]) or to replace (knockin [KI]) a gene of interest.⁵

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To date, there are few validated primate models of central nervous system (CNS) disorders. However, advances in the knowledge of genetic risk factors for brain disorders, including autism, schizophrenia, bipolar disorder, Alzheimer's disease, and Parkinson's disease (PD), open new perspectives. These progressive acquaintances in human disease genetics associated with the recent genetic targeting promise to revolutionize the generation of transgenic animal models in a translational perspective.

2 | WHY USING NONHUMAN PRIMATE MODELS?

Within neuroscience, the use of NHPs has led to a greater understanding of the brain physiology and pathology. In rodents, some important aspects of human brain function are difficult to address. Some higher cognitive functions are too complex and evolutionarily recent to be meaningfully studied in rodents.³ Notably, psychiatric diseases are difficult or impossible to model in rodents since they are diagnosed purely through behavioral symptoms.¹

Primates are the closest species to humans phylogenetically, sharing a common ancestor with macaques (Old World monkeys) and marmoset (New World monkeys).⁶ NHPs are the closest to humans in aspects of anatomy, immunology, endocrinology, genome constitution, social behavior, cognitive, metabolic, reproductive, and brain functions.⁷⁻¹¹ They share a similar brain network organization.^{4,12-14} In contrast to most mammalian models, the primate brain has kept characteristics of human brain such as a stable neuronal density with the increased number of neurons,¹⁵ complex motor skills, and a highly developed prefrontal cortex. The prefrontal cortex has no clear structural or functional homolog in rodents but is responsible for higher cognitive function in primates and it contains vulnerable domains involved in some psychiatric disorders.^{8,16,17} In the rodent, the striatum is a single entity, whereas cortical fibers in humans and NHPs are grouped into the internal capsule, separating the striatum dorsomedially into the caudate nucleus and ventrolaterally into the putamen, an anatomical characteristic with particular relevance to PD.¹⁸ Another interesting feature is the presence of neuromelanin pigment in dopaminergic neurons in the substantia nigra (SN) which can contribute to the vulnerability of this brain region in PD.¹⁹

Nonhuman primates are more sophisticated behaviorally.^{10-12,20} The motor, cognitive abilities, and social behavior of NHPs closely mimic humans.^{1,21} In humans and NHP, the brain is extremely modified via experience-dependent plasticity and learning during the juvenile phase.¹ Many biological processes implicated or disrupted in brain diseases differ between primates and rodents, such as the detailed organization of the primate oxytocine system, the neurotransmitter and neuromodulatory system, the specific receptor system, and the neuronal connectivity.¹

Several genes that are expressed only in primate neuronal system have been described.²² Even if the precise function of these genes is still unknown, it has been hypothesized that they play important roles in higher cognitive function.²³ It enables a more accurate assessment of the impact of the pathology on motor outcomes,

neuroimaging procedures, and non-motor symptoms. There are also differences in cell types and noncoding sequences of the genome between primate and rodent brains.¹ The mapping of the rhesus (*Macaca mulatta*) and common marmosets (*Callithrix jacchus*) genomes accomplished, respectively, in 2007 and 2014 improved NHP models of disease.²⁴

The high similarity in brain anatomy allows noninvasive, high-resolution imaging of the brain structures and functions by functional magnetic resonance imaging (fMRI) and positron emission topography (PET). Furthermore, for studying neurodegenerative diseases and the physiologic decline of aging in monkeys, various cognitive and behavioral tests are available.⁷ Moreover, in progressive pathology such as PD where age is the main risk factor, the use of long-lived animals is crucial. It will allow studying developmental phenotypes and prodromal disease stage, which is difficult to assess in human patients.¹

All that characteristics allow researchers to study not only physiological processing (such as visual information processing, working memory, decision-making, reward processing, social relationship, facial expression and recognition, etc.) but also pathological behaviors (anxiety and depressive-like phenotypes, addiction, autism, or schizophrenia).^{2,3} Primates are already used in pharmaceutical research for pharmacokinetic and toxicology studies that precede human clinical trials but the few number of validated primate models prevent their use for studies on drug efficacy.¹ The new precise gene-editing techniques promise to change this research field, notably after the demonstration of genetic changes in embryos from many species, including NHPs and nonviable human embryos.^{23,25-31}

3 | THE DIFFERENT TYPES OF NHP TRANSGENIC MODELS

Genetically manipulated NHPs were used to investigate disease pathophysiology. However, researchers have to face to political and technical challenge, such as the long monofetal gestation, the low chances of success due to the possible random insertion of the foreign DNA. The low efficiency of assisted reproductive technologies in producing oocytes and embryos suitable for genetic engineering and the technical challenges of embryonic stem cells derivation and cloning prevent the large-scale production of transgenic monkeys. Moreover, the high cost of production and maintenance associated with ethical concerns explain the low use of transgenic NHP models.³² For instance, the first transgenic NHP was created more than 25 years after the first transgenic mouse.³³ To date, few studies have been carried out using viral-mediated delivery of gene or gene-editing methods, notably TALEN or CRISPR-Cas9.

3.1 | The viral vector-mediated gene transfer models

Before the development of gene-based methodologies to model diseases, a number of experimental models had been developed,

including in NHPs.^{34,35} They come essentially in two flavors: pharmacological (eg, reserpine) and toxic (eg, 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine). These models can also be recast by some authors as pathogenic and symptomatic/pathophysiological, as each may contribute to our understanding of the cause, the mechanisms, and the treatment of PD.³⁴ The MPTP NHP models come into many different regimens of intoxication.³⁶ Although the construct validity could be put into question regarding the mechanisms of cell death, the symptomatic face validity is without equivalent as these models are perfect phenocopies of PD, featuring motor and non-motor symptoms, as well as the subserving anatomopathology.³⁶⁻⁴¹ Further understanding the etiology and the mechanisms of cell death required the development of gene-based models. Among the initial proposals was the local transgenesis that brings the advantages to induce a prolonged effect and to mimic the ongoing disease pathophysiology by promoting the production of the pathological protein.

3.1.1 | Postnatal stereotaxic delivery

Among the various viral vectors available for transgenesis, adeno-associated virus (AAV) and lentivirus are the main virus used. As a prelude to translational studies, as well as clinical trials in patients with PD for example, the transduction efficiency of AAV2/1-GFP, AAV2/5-GFP, and AAV2/8-GFP was examined in NHPs. Viral particles were injected into the either striatum or SN via stereotaxic surgery. AAV2/5-GFP and AAV2/1-GFP transduce significantly higher number of cells than AAV2/8-GFP. The transduction was predominantly neuronal with a GFP expression around the injection site: in the cell bodies and fibers in the striatal region or throughout the SN.⁴² This study confirms that transgene expression in some brain areas can be achieved by postnatal stereotaxic viral delivery.

In PD, new models have emerged in the last few years based on native or mutated α -synuclein (α -syn) overexpression. Viral vector-mediated gene transfer models appear to be promising by mimicking the time course of neuropathology. For the first time, Kirik and colleagues created a NHP α -syn overexpression model⁴³ based on the protocol tested initially in rats.⁴⁴ They injected high-titer recombinant AAV2 unilaterally in the SN in adult marmosets to express the wild-type (WT) or A53T human α -syn. 90%-95% of all nigral dopamine neurons expressed the α -syn protein which was distributed by anterograde transport throughout axonal and dendritic projections. The overexpression of either WT or mutant α -syn is sufficient to induce a PD-like neuropathology in the primate including dopaminergic neuron loss (30%-60%) and motor impairments 16 weeks post-transduction. Another study was performed by the same group with 24 adult marmosets injected unilaterally into the SN with rAAV2/5-WT α -syn or rAAV2/5-A53T α -syn. The follow-up was performed during 1 year. A decline in motor coordination, associated with a decrease in TH+ fibers density with neurodegeneration (especially in the A53T- α -syn injected animals), the presence of pathological Ser129-phosphorylated α -syn, PK-resistant α -syn aggregates, and ubiquitin containing aggregates were described.⁴⁵ In 2015, Yang et al used lentivirus to deliver A53T α -syn unilaterally

in the SN of Old World Rhesus monkeys. Aging increased the accumulation of A53T in neurites and its associated neuropathology.⁴⁶ In the same year, our team reported the lack of an additive role of aging in nigrostriatal neurodegeneration after the injection of rAAV2/9-A53T α -syn unilaterally in the SN pars compacta in two age groups marmosets (1-2 years old: n = 8, >6 years old: n = 5).⁴⁷ Another team described different experiments of injection in order to obtain the best NHP model to test developmental therapeutics. They designed a rAAV1/2-A53T α -syn injected bilaterally in six cynomolgus macaques. α -Synuclein was expressed in >85% of nigral neurons, the number of TH+ neurons decreased by 50%, and dopamine neurochemistry showed a 21%-72% reduction according to the groups.⁴⁸ Based on α -syn delivery, these studies demonstrated the efficacy of this strategy to model PD. Recently, recombinant AAV1/2 vector was also used to deliver human α -synuclein under the myelin basic protein promoter to obtain oligodendroglial α -syn expression following striatal delivery. The aim was to obtain a model for multiple system atrophy with >60% expression in oligodendrocytes.⁴⁹

In the Huntington's disease (HD), NHP models were first generated by stereotaxic delivery of lentivirus expressed mutant *HTT*. The lentiviral vector encoding for the first 171 amino acids of the Huntingtin protein with 19 (wild-type) or 82 (mutated: Htt171-82Q) polyglutamine repeats was injected into the dorsolateral sensorimotor putamen of macaques. The monkeys injected with the Htt171-82Q presented the classic symptoms of HD in humans, associating to neuritic and nuclear huntingtin aggregates, astrocytosis and neuronal loss.⁵⁰

These studies show that stereotaxic delivery allows the generation of different brain disease models but that the transgene expression is relatively concentrated around the injection site. To induce a wider and global central nervous transduction, postnatal systemic delivery was performed.

3.1.2 | Postnatal systemic delivery

In a systemic injection approach, using viral vector-mediated transgene delivery in mice, the cell types targeted shifted from neurons to astrocytes when the viral vector was injected in adults compared to neonate injection.^{51,52} In monkeys, injections have also been performed either in newborn or adults. A male cynomolgus macaque was injected intravenously with $1-3 \times 10^{14}$ particles of scAAV9-GFP on postnatal day 1 (P1). Robust GFP expression was described within the dorsal root ganglia and motor neurons along the entire neuraxis.⁵³ Dehay et al also reported intravenously injection with four different AAV9 viral titers (from 9.5×10^9 to 1.33×10^{12} particles/g of body weight) in newborn rhesus macaques (P1). All rAAV2/9-GFP injections resulted in efficient neuronal transduction of brain areas such as the cortex, midbrain, hippocampus and cerebellum.⁵⁴ Different time points for the intravenous injection were tested by another group: P1, P30, P90 animals injected with $1-3 \times 10^{14}$ vg/kg of sc-AAV2/9-GFP and 3-year-old cynomolgus macaques injected with 2.7×10^{13} vg/kg of sc-AAV2/9-GFP. An efficient motor neurons targeting was

described but injection in adults was less powerful compared to injection in young animals (partially explained by the lower dose injected). However, all GFP-injected animals had extensive transgene expression throughout the entire brain. The cortical region, lateral geniculate, midbrain, pons, and medulla had the higher GFP+ cell density compared to subcortical structures. Interestingly, the glial transduction with microglia and astrocytes were the most prominent cell types targeted whatever the age of the animals.⁵⁵ In 3- to 4-year-old rhesus macaques, a reduction in brain transduction was observed compared to mice, along with a clear shift toward mostly glial transduction after intravenous or intra-arterial injection of sc-AAV9-GFP.⁵⁶ The results obtained with AAV-GFP in primates confirmed the preliminary results observed in mice with a global neuronal and glial brain transduction but with a preferentially glial transduction, and that particularly in older animals. In another study, different routes of viral vector administration were compared: infusion of AAV-9 GFP into the internal carotid artery (ICA) or into the cisterna magna (CM). A mainly astrocytic transduction was described in both routes of administration but CM infusion led to a stronger expression throughout the cortex and cerebellum.⁵⁷ This last study confirms that the transgene expression was mostly observed in superficial postnatally developed structures.

Since the neonatal injection allows a better neuronal transduction compared to adult injection, earlier injections have been performed via in utero delivery. The goal was to obtain an exclusive and efficient neuronal expression.

3.1.3 | In utero delivery

In utero viral vector delivery has already shown widespread brain transduction in rodents using retrovirus,^{58,59} adenovirus⁶⁰ or AAV.⁶¹⁻⁶³ In 2003, Garrett et al performed gene transfer with rAAV2-GFP via an in utero approach in a rhesus macaque. The recombinant virus was delivered by transabdominal infection into the amniotic fluid under ultrasound control. The tissues analyzed at 15 months of age revealed GFP expression in lung cells and intestines villi.⁶⁴ Another report focused on the transduction of the central and peripheral nervous systems. scAAV2/9-CMV-eGFP vectors genomes (1.10^{13}) were infused slowly into the intrahepatic vein at E140 of a 155-day gestation. The authors showed that GFP expression throughout the CNS was variable following in utero gene transfer for up to 14 weeks. The transduction was clearly observed in the cerebral cortex. Moreover, both the injected offspring showed transduction of the majority of Purkinje cells in the cerebellum and neurons, including motor neurons in the spinal cord, compared with the greater variation in neuronal transduction found within the cerebrum.⁶⁵ Further efforts have been done to obtain a homogeneous transgene expression in the cortex and deep brain structures. A widespread neuronal transduction was obtained after in utero delivery of scAAV2/2-GFP to monkey embryos via intracerebroventricular injection.⁶⁶

All these promising studies are probably the prelude to the production of animal models after in utero transgene injection.

Moreover, researchers have focused on the development of the classical transgenesis in NHP.

3.2 | The classical transgenesis

The criteria for a successful transgenic animal are (a) stable integration of the transgene into the host cell genome, (b) expression of the transgene and bioactivity, and (c) germline transmission.⁶⁷ The advancement in transgenic technology in the end of the 1990 has led to the generation of the first transgenic monkeys in 2001.^{68,69} The first transgenic monkey model of human disease, associating genetic modification and disease phenotype, was generated in 2008.⁷⁰ The germline transmission of the transgene was described in 2009 in common marmoset.⁷¹ The efficiency of transgenesis was improved in this study by injecting a higher volume of lentiviral solution into dehydrated embryos with a large perivitelline space and by selecting the transgenic embryos before transfer. Transgenic F1 and F2 animals have been obtained.⁵ A new era in modeling human genetic disorders has been opened by this success.

3.2.1 | What are the available methodologies?

Pluripotent stem cells

Different methodologies were published with the goal to generate chimeric offspring. In mice, genetic modified pluripotent stem cells, such as embryonic stem cells (ESCs) or induced pluripotent cells (iPS), are powerful tool to obtain KO mice.²³ The first attempts to translate the methodology used for transgenesis in mouse, such as linear DNA injection into the fertilized eggs and blastocyst injection of marmoset pluripotent stem cells carrying transgene, failed to produce transgenic NHP.^{72,73} Established ESCs and freshly isolated inner cell mass (ICMs) failed to produce chimeras when injected into host blastocysts. However, ICMs developed into separate fetuses with placental support from the host embryos. Aggregating of several four cells embryos efficiently produced live chimeric offspring.⁷⁴ Few years later, Chen et al published the production of chimeric monkey fetuses by injection of ESCs grown in adjusted culture conditions into host morulae.⁷⁵ Nuclear transfer embryo derived from embryonic blastomeres or fetal fibroblasts was also achieved for the cloning of rhesus monkeys. The transfer of embryonic cell nuclear transfer embryos resulted in a term pregnancy.⁷⁶ However, for a transgenic model, a foreign gene needs to be introduced into the somatic cell donor of nucleus. This method fails to produce any live monkey.⁷⁷⁻⁷⁹ Until now, these techniques are not optimal in NHPs. Others strategies have also been explored.

Viral vector delivery

Viral vector delivery, such as lentiviral gene transfer, has shown to be a powerful tool and is one of the most efficient methods in creating transgenic animals.⁷ A simian immunodeficiency virus (SIV)-based vector that encodes EGFP was used to infect one- to two-cell stage embryos or four- to eight-cell stage rhesus monkey embryos. Two offspring exhibited whole-body expression of the EGFP reporter.⁸⁰

Then, lentivirus was used to create transgenic marmoset and cynomolgus monkey models.⁸¹⁻⁸⁴

This methodology was then used to model diseases. To study HD, transgenic rhesus monkeys were generated by microinjection of lentivirus carrying exon-1 of the *htt* gene containing 147 CAG repeats into MII oocytes before in vitro fertilization. Animals died following a premature birth at 4 months of gestation and showed abundant neuropils aggregates in swelling neuronal processes.⁸⁵ The same methodology was performed by Yang et al. where high-titer lentivirus expressing exon 1 of the human *htt* gene with 84 CAG repeats was injected. Five monkeys were born, all carrying the transgenic mutant HTT. No clear sign of neurodegeneration was detected but animals develop chorea, dystonia, and other involuntary motor deficiencies similar to HD.⁷⁰ The germline transmission of the pathogenic mutant HTT in HD monkey was demonstrated by the production of embryos and subsequent derivation of HD monkey embryonic stem cells.⁸⁶ Longitudinal studies until the 2 or 5 years old of transgenic animals described progressive motor dysfunction and a lack of behavioral inhibitory control, suggesting a functional decline of the frontostriatal pathway in HD monkeys. It is associated to a decreased brain volume and neuronal loss. Thus, these transgenic models closely display the progressive clinical features of HD.^{87,88} The emerging symptoms of HD were explored in two male transgenic HD rhesus monkeys. They expressed anxiety and irritability/aggression to an acute stressor as compared to control. Moreover, HD monkey exhibited increased proinflammatory cytokines and higher induction of immune pathway genes compared to controls.⁸⁹ Another seven transgenic monkeys were produced for longitudinal studies to study the transcriptomic pattern ($n = 4$)⁹⁰ or the whole brain white matter integrity ($n = 3$).⁹¹ Interestingly, germline transmission was confirmed by the detection of the transgene directly not only in the male germ cells, but also in embryonic stem cells derived from blastocyst obtained after microinjection of spermatozoa from transgenic male. Moreover, transgenic F1 offspring was produced after artificial insemination.⁹² These different transgenic animals recapitulate the characteristics of the disease and offer the opportunities to create a transgenic line.

For a Rett syndrome monkey model, Liu et al reported that the transgenic monkeys obtained after the microinjection of lentivirus expressing human MECP2 into the perivitelline space of oocytes exhibited autism-like behaviors. Male germline transmission of the transgene was also obtained. Five F1 monkeys with defective social behaviors carrying the human MECP2 transgene were born.⁹³ In another report, transgenic animals were generated after ICSI with spermatozoa collected in immunodeficient mice after xenografting of testicular tissue from one transgenic monkey.⁹⁴ This technique shortens the time necessary to obtain mature spermatozoa and enables the researchers to accelerate the reproduction of transgenic monkeys.¹⁶

For PD, Niu et al injected a lentivirus expressing A53T- α -syn into the perivitelline space of 133 MII oocytes before fertilization. Six of the seven live newborns and five of the eight aborted fetuses were positive for transgenic A53T.⁹⁵ They described in the stillborn

monkey brain a number of neurons expressing A53T in the SN and some punctate staining that may reflect the enrichment A53T in synaptic terminals but without S129-phosphorylated synuclein labeling. Moreover, no neurodegeneration was obtained in the SN, cortex, and striatum. The oldest A53T transgenic animal began to display cognitive defects and an anxiety phenotype at the age of 2.5 years old which can be consistent with the non-motor symptoms of PD patients at the early disease stage. However, MRI analysis revealed no obvious degeneration in the transgenic monkey brain.⁹⁵

More recently, a transgenic marmoset model of the polyglutamine disease, a neurodegenerative disease, was reported which recapitulates progressive neurological symptoms 3-4 months after birth, accumulation of misfolded protein, and neurodegeneration.⁸³ In this study, a self-inactivating lentiviral vector carrying full-length human ataxin 3 cDNA with 120 CAGs was introduced in four-cell to morula-stage embryos. Six of the seven offspring expressed the transgene and three of them showed age-related neurological symptoms. The same group successfully generated transgenic marmosets using a tetracyclin-inducible transgene expression (tet-on) system. The mutant human ataxin 3 gene controlled by the tet-on system was injected into marmoset's embryos via lentiviral transduction. Four of the seven live offspring were transgenic and carried the transgene controlled by the tet-on system.⁸⁴ With the objective to monitor in vivo neural activity, Park et al reported the generation of a transgenic marmoset expressing GCaMP, a genetically encoded calcium indicator, under ubiquitous and neuronal promoters.⁸¹ Probably, others transgenic monkey models will be published in few years since the generation of transgenic models of various neurodegenerative diseases (ie, amyotrophic lateral sclerosis, Alzheimer's disease, and PD) are being planned.^{16,96}

Viral vector-mediated gene transfer has shown its efficacy but has limitations, such as size limitation of the transgene, random insertion in the genome, and uncontrollable expression levels.¹⁶ The recent breakthroughs in techniques for site-specific gene editing and transfection is paving a new way forward for studying disease progression in NHPs.

Site-specific gene editing

The most popular techniques are the ZFN, the TALENs, and the CRISPRs (Figure 1). Once the breakpoint done, the cell's endogenous homologous recombination machinery allows the insertion of exogenous DNA sequence. The ZFNs combine the N-terminal zinc finger DNA-binding domain, a variable peptide linker, and a C-terminal Fn domain with a nuclease activity for digesting target genes.⁹⁷ The TALENs are chimeric proteins comprising the transcription activator-like effector for the DNA binding and FokI nuclease to induce a specific DNA double-strand break.⁹⁸ The CRISPR-Cas9 system associates a specific RNA guide to the Cas9 nuclease to digest double-strand target DNA.⁹⁹

Using these recent technologies, the first models were generated in 2014. The CRISPR/Cas 9 system was used in one-cell embryos to modify the monkey genome and obtain founder animals harboring two gene modifications (disruption of *Ppar- γ* and *Rag1*

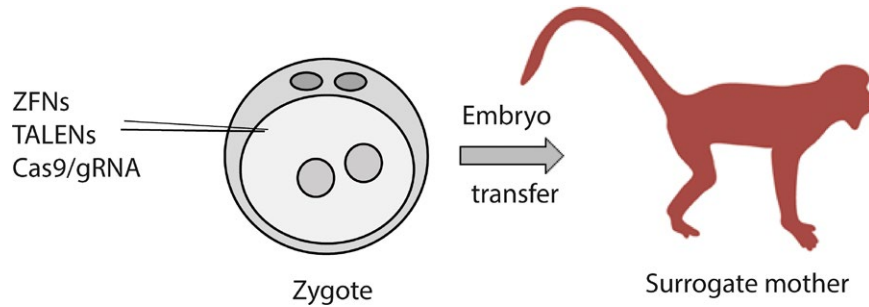


FIGURE 1 Illustration of the method for transgenesis in nonhuman primates (NHP) with the new genome-editing technologies. Metaphase II oocytes were subjected to intracytoplasmic sperm injection (ICSI). At 8–10 h after ICSI, the fertilization was checked and two pronuclei zygotes were injected with ZFN, TALEN or CRISPR/Cas9. The zygotes were then transferred into surrogate females and pregnancy was confirmed by ultrasonography

in founder animals).²⁹ In another studies, CRISPR/Cas9 was used via zygote injection to KO the p53 gene or to realize the dystrophin gene KO to generate Duchenne muscular dystrophy monkey models.^{26,100} This technology was also used in order to model the X-linked adrenal hypoplasia congenital and hypogonadotropic hypogonadism obtained from *DAX1* KO. The *DAX1*-deficient monkey displayed defect in adrenal gland development with abnormal architecture of the adrenal cortex and of the testis.¹⁰¹ A recent paper also demonstrated the generation of a *SIRT6*-null cynomolgus monkey. *SIRT6*-specific sgRNAs and Cas9 mRNA were injected into 98 monkey zygotes. Three female infants were born with efficient KO of the *SIRT6* gene and no detectable off-target. All three animals showed a global development delay in utero compared to wild-type newborns with prematurity and developmental retardation at the tissue level. Notably, a smaller brain and thinner cortical layers in the cerebral and cerebellar hemispheres were described in the transgenic newborn monkeys, evoking neuronal maturation delay. It was consistent to the loss-of-function mutation of *SIRT6* described in humans that causes late fetal loss with intrauterine growth restriction.¹⁰² So, the use of CRISPR/Cas9 appears to be a promising way to obtain new transgenic animal model. However, technological limitations induce difficulties to obtain homozygous mutations at a high rate in NHPs, compared to mice. Nowadays, the challenge consists in developing more precise gene-editing technologies, by increasing the efficiency, producing more homozygotes, and reducing the mosaicism and the off-targets,³³ even if cases of off-targeting were still limited in previous studies.^{29,101}

Direct injection of transcription activator-like effector nuclease (TALEN) plasmids in rhesus and cynomolgus monkey zygotes was also performed for genetic modeling of human disease. This technology has been employed for effective gene editing of *MECP2* gene in modeling Rett syndrome but without any behavioral deficits.²⁸ Further detailed analyses using eye tracking and magnetic resonance imaging showed homologies between Rett syndrome in humans and the phenotype observed in monkeys.¹⁰³ Another five fetuses were developed, all but one were stillborn. The remaining one failed to survive after birth but was the only one who showed a specific deletion in the *MECP2* gene.¹⁰⁴ Others report detailed the feasibility to generate KO monkey using ZFN and TALEN.^{9,105,106} Notably, TALEN

was used to produce microcephaly-associated MCPH1 KO cynomolgus monkeys. One monkey recapitulated most of the important clinical features observed in microcephalic patients, including reduction in head circumference, premature chromosome condensation, hypoplasia of the corpus callosum, and upper limb spasticity.¹⁰⁵

4 | CONCLUSIONS

The new genome-editing tools associated to the development of NHP-assisted reproductive technologies have allowed for the development of NHP disease models, notably by transgene overexpression and mutagenesis of endogenous genome DNA. Many studies have been conducted, especially in the neuroscience field and in particular in neurodegenerative diseases, psychiatric disorders, and cognitive functions. Further experiments are needed for evaluating the phenotype of the transgenic animals and for demonstrating the reproducibility while keeping in mind the three “R” principles (replacement, reduction, and refinement) for NHP experimental technique.

The research community expects that new findings will be obtained, since these new animal models have been developed in order to better understand the pathophysiology and to identify novel therapies. It will therefore be desirable to improve the emerging reproductive technologies which appear to be a promising and efficient way to establish animal models. Various strategies used in mice and rats could be translated to NHP, for example, genome editing of spermatogonial stem cells, gene modification of androgenetic (male) and parthenogenetic (female) haploid ES cells used to fertilized an oocyte, testis xenografting, nuclear transfer from cultured cell lines, differentiation of ES cells into primordial germ cells followed by ovary or testis transplantation.^{8,94,107–111}

All these strategies developed to model human disease can also be diverted for gene therapy. In humans, the use of viral vector, notably the AAVs, offers a promising way in clinical trials such as muscular dystrophy, hemophilia, PD, Leber's congenital amaurosis, and macular degeneration.¹¹² In human preimplantation embryos, CRISPR-Cas9 was used to correct the heterozygous *MYPBC3* mutation which causes hypertrophic cardiomyopathy. To minimize the

mosaicism, the coinjection of sperm and CRISPR-Cas9 components was performed into metaphase II oocytes. The yield of MYBCP3^{WT/WT} embryo (72.4%) in the injected group was significantly higher than in untreated controls (47.4%).¹¹³ Another team demonstrated efficient correction of point mutation in *HBB* and *G6PD* gene.¹¹⁴ The clinical application is not for today but these studies open the field of efficient gene therapy in human embryos.

To conclude, the development of all these novel models is paving a new way forward for investigating disease pathogenesis and for preclinical drug studies, notably for intractable neurodegenerative diseases such as PD and amyotrophic lateral sclerosis, as well as mental disorder such as schizophrenia and autism spectrum disorders.

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CONFLICT OF INTEREST

None.

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