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Investigation of brain science and neurological/psychiatric disorders using genetically modified non-human primates

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Although mice have been the most frequently used experimental animals in many research fields due to well-established gene manipulation techniques, recent evidence has revealed that rodent models do not always recapitulate pathophysiology of human neurological and psychiatric diseases due to the differences between humans and rodents. The recent developments in gene manipulation of non-human primate have been attracting much attention in the biomedical research field, because non-human primates have more applicable brain structure and function than rodents. In this review, we summarize recent progress on genetically-modified non-human primates including transgenic and knockout animals using genome editing technology.

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Introduction

Our human brains are composed of structures conserved through evolution and those unique to primates. Recently-evolved brain structures involve the enlargement of the cerebral neocortex and provide essential substrates for acquisition of novel brain functions unique to primates, and eventually humans. Because of the unique structure and function of the primate brain, it is impossible to gain a full, accurate understanding of either normal human brain function or mental illness (neurological and psychiatric disorders) through rodent-based studies. Traditionally-used rats and mice only possess fundamental neuronal circuits conserved across mammalian species [1•,2••]. Particularly, the primate prefrontal cortex is responsible for higher cognitive processes, and it contains vulnerable domains involved in some psychiatric disorders. The prefrontal cortex has no clear structural or functional homolog in rodents [1•,3], which suggests advantages of using non-human primates to model human neurological and psychiatric diseases. The pathophysiology of human neurological and psychiatric diseases is not always recapitulated in genetically modified (GM) rodent models, possibly due to the differences in genome information, life span, and brain structure and functions between humans and rodents. For example, mice that lack *Parkin* (a causative gene for a form of familial Parkinson Disease) do not show the spontaneous degeneration of the dopamine neurons in nigrostriatal pathways that is typical in humans suffering from Parkinson Disease, although the mice do display some changes relevant to this disease [4].

Given the limitations of rodent models, it is essential to explore genetic manipulation of non-human primates to investigate brain structures and functions and to develop *in vivo* models for human neurological and psychiatric diseases. In 2009, GM non-human primates with germline transmission were successfully developed through lentivirus mediated gene transfer to fertilized and unfertilized eggs of common marmosets (*Callithrix jacchus*) [5•]. Marmosets are small New World primates that have attracted brain researchers' attention as potential models [6,7] because of their human-like neurological traits [8]. Furthermore, the marmoset brain is small (about 8 g in adults), but provides a complete primate brain structure, including a prefrontal cortex, which makes it a suitable model for comprehensive brain mapping. Another advantage of using marmosets is that their smooth brains are easier to analyze through electrophysiology and optical imaging than highly gyrencephalic brains. It is notable that basic research tools are available for marmosets including whole genome sequencing data [9,10] and histological and MRI brain atlases [11–13]. Furthermore, various neurological diseases models are available in marmosets even without GM technologies, including stroke [14], spinal cord injury [15,16], multiple sclerosis (experimental allergic encephalitis model), age-related spontaneous and induced β amyloid deposition [17] and drug-induced Parkinsonism [18,19]. Because of marmosets' characteristics, Japan started a national brain project, Brain/MINDS (Brain Mapping by Integrated

Neurotechnologies for Disease Studies) to focus on marmoset brain mapping and disease modelling using wild-type and GM marmosets [2••,20–22].

Development of the first generation of GM marmosets using lentiviral mediated gene transfer

To develop transgenic technology in marmosets, our group has explored several methods based on mouse transgenic technologies such as linear DNA injection into the fertilized eggs, blastocyst injection of marmoset pluripotent stem cells (e.g. embryonic stem cells (ESCs) [23] carrying transgene [24] and nuclear transfer of somatic cells with transgene). However, none of these has been successful. Germline-competent marmoset ESCs are not currently available [2••]. Finally, the first achievement was obtained by injecting a non-replicating lentiviral vector carrying a foreign gene encoding *EGFP*; this technique could ultimately create transgenic marmosets [5•]. Furthermore, this study represents the first successful generation of transgenic non-human primates with germline transmission (reviewed in [2••]). Using this technology, several groups have developed marmoset models of neurodegenerative disorders by overexpressing the transgene with toxic mutations [25] and marmosets expressing the functional reporter gene [26]. Tomioka *et al.* generated transgenic marmoset models of the polyglutamine (polyQ) diseases by lentiviral transduction of the human ataxin 3 gene with 120 CAG repeats encoding an expanded polyQ stretch, showing progressive neurological symptoms including motor impairment [25]. Park *et al.* generated transgenic marmosets expressing GCaMP, a genetically encoded calcium indicator (GECI), under ubiquitous and neuronal promoters, allowing chronic *in vivo* monitoring of neural activity [26]. In Brain/MINDS, generations of several transgenic marmoset lines which express Cre under neuronal subtype-specific promoter/enhancer elements are ongoing (e.g. Orexin promoter-Cre). Transgenic models of other neurodegenerative diseases (e.g. Parkinsons disease, Alzheimers disease, and amyotrophic lateral sclerosis) are being planned [2••]. Collectively, it is expected that these transgenic marmosets will contribute to structural and functional brain mapping and the diagnosis, prevention and treatment of human brain disorders.

On the other hand, lentiviral vector-mediated gene transfer has clear limitations, such as random insertion on the genome, size limitation of insert, and uncontrollable expression levels. To overcome these issues, the 2nd generation GM marmosets have been developed using genome editing technologies [27,28•].

Development of the second generation of GM marmosets using genome editing

Most human genetic diseases are caused by a loss-of-function, such as a point mutation, a deletion, or insertion of endogenous genes, rather than multiplication of gene copies. Therefore, new technologies that manipulate endogenous genes of marmosets are required for broader disease studies. For a long time, it had been impossible to create knock-out and knock-in marmosets. This was due to a lack of naïve ES cells that can contribute to development of chimeric animals [1•,24]. However, emergence of engineered nucleases has overcome this obstacle [29–31], and enabled generation of GM marmosets. An engineered nuclease is an artificially engineered restriction enzyme that facilitates targeted editing of the genome by inducing either homology-directed repair (HDR) or non-homologous end-joining (NHEJ) via double stranded breaks at specific locations. Zinc finger nucleases (ZFNs) [29], transcription activator-like effector nucleases (TALENs) [32], and clustered regularly interspaced short palindromic repeat (CRISPR)/Cas system [33,34] are currently used in various research fields.

ZFN is composed of a DNA-binding domain that is made of tandem zinc finger-binding motifs and a cleavage domain of the restriction endonuclease *Fok*I. This chimeric protein specifically binds to a genomic sequence based on a combination of zinc finger motifs, and the endonuclease cleaves the genome sequence near the binding site. TALEN has a similar structure to ZFN, and this enzyme uses a transcription activator-like effector (TALE) DNA binding domain that is derived from *Xanthomonas* bacteria as a DNA-binding domain, instead of a zinc finger motif. Most recently, the CRISPR/Cas system was reported as a new genome editing tool [33,34], and this system works very rapidly. While ZFN and TALEN use a protein for specific DNA-binding, CRISPR/Cas system uses a base-pair complementarity between DNA and RNA. The system is originated from the RNA-based adaptive immune system in bacteria and archaea [35,36], and guide RNA identifies binding sites of Cas9 nuclease. While ZNF and TALEN require a complex DNA-binding protein that specifically fits a target sequence, the CRISPR/Cas system only needs a guide RNA that complimentarily binds to a target genome sequence. Although this system has more off-target effects than the other engineered nucleases, easy design of guide RNA has made CRISPR/Cas system a leading role in genome editing tools.

GM animals have been successfully generated in many species using those engineered nucleases [37–42]. A recent paper reveals that this genome editing technology can apply to marmosets [28•]. The authors generated a model of severe combined immunodeficiency (SCID) by targeting the *interleukin-2 receptor subunit gamma* (*IL2RG*) gene on the X chromosome. They synthesized mRNAs from two vectors encoding ZFN and TALEN against *IL2RG*, and introduced them into pronuclear stage embryos by microinjection (Figure 1). The injected embryos were transferred to surrogate mothers, and