

Neuroscience Research Using Non-human Primate Models and Genome Editing

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Abstract The common marmoset (*Callithrix jacchus*) is a small New World non-human primate indigenous to northeastern Brazil. This species has been attracting the attention of biomedical researchers and neuroscientists for its ease of handling and colony maintenance, unique behavioral characteristics, and several human-like traits, such as enriched social vocal communication and strong relationships between parents and offspring. Its high reproductive efficiency makes it particularly amenable for use in the development of transgenic and genome editing technologies in a non-human primate model. Our group has recently generated transgenic marmosets with germ line transmission, opening new avenues in primate research.

In this chapter, we describe recent advances in neuroscience and disease research using common marmosets, and we outline potential uses of genome editing in non-human primates toward the development of knock-in/knock-out marmosets.

Introduction

Rodent models have long played important roles in neuroscience and medical research, made possible in part by the advent of robust genetic technologies. Knock-out/knock-in mouse models have shown particular utility in the neurosciences. There are nonetheless substantial anatomical, physiological, and cognitive differences between rodents and humans. The human brain consists of two major functional domains, one that is evolutionarily conserved and a second that is primate-specific and the locus of many higher cognitive functions. For many human neurological and psychiatric diseases involving higher cognitive dysfunctions, studies using rodent models may thus not be informative with respect to the relevant pathophysiological mechanisms. To gain a better understanding of the

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pathogenesis of such diseases, we need animal models that exhibit brain functions more closely similar to those in humans.

This need has led to increased interest in the development of genetically engineered non-human primates for use in the study of both functional domains. Our group has recently generated a transgenic common marmoset, a New World monkey (Sasaki et al. 2009). Emerging genome editing techniques are also opening new possibilities for the creation of better non-human primate models for use in the study of neurodegenerative and mental disorders (Izpisua Belmonte et al. 2015).

This chapter is an updated and modified version of previously published review articles on marmosets (Okano et al. 2016; Kishi et al. 2014) and work presented by Hideyuki Okano at the “Genome Editing in Neurosciences” symposium.

Characteristics of the Common Marmoset

Common marmosets (*Callithrix jacchus*) are New World primates native to the Atlantic coastal forests of northeastern Brazil (Abbott et al. 2003; Carrion and Patterson 2012; Mansfield 2003; Okano et al. 2016; Tokuno et al. 2012; Kishi et al. 2014; Izpisua Belmonte et al. 2015). These small monkeys (adult height: 20–30 cm; weight: 350–400 g) have ear tufts and relatively long banded tails, and they are omnivorous, eating plant exudates, lizards, and infant mammals. Common marmosets are monogamous and, unlike many other non-human primates, live in stable families of approximately ten members (Tardif et al. 2003). Females commonly give birth to two babies per litter and are ready to breed again about 10 days after giving birth; they typically have two litters per year. Since mothers need to nurse infants during gestation and the perinatal period, the male partner and other members of the group also provide infant care. This remarkably human-like trait is a focus of attention among neuroscientists and behavioral scientists.

Although common marmosets have been used for biomedical research since the 1960s, macaque monkeys are more widely used in research, due to their closer similarity to humans. The recent rapid advances in genome editing are now calling new attention to the advantages offered by the marmoset because of its size, availability, and high reproductivity.

Macaques are evolutionarily closer to humans than common marmosets, but some marmoset traits are more similar to those of humans, perhaps due either to geographical segregation or convergent evolution. New World primates are estimated to have diverged from Old World primates ~35 mya, and these monkeys have adapted to neotropical environments. Despite this phylogenetic distance, common marmosets, like humans, exhibit strong intergenerational kin relationships and social vocal communications (Dell’Mour et al. 2009; Eliades and Wang 2008; Gordon and Rogers 2010), which may indicate a convergent trajectory in their evolution. The genomic basis of the origins of such traits may be addressable through genome editing studies in the future.

Advantages of Using Common Marmosets for Biomedical Research

Rodents play a crucial role in biomedical investigations in many research fields. Powerful genetic tools, such as knock-out/knock-in mice, have informed the study of gene functions, but the significant anatomical and physiological differences between rodents and humans mean that a more closely similar animal model is needed to advance our understanding of human biology in areas such as the neurosciences.

For biomedical use, the common marmoset offers many advantages. Marmoset endocrinology and metabolism are more similar to those of humans than of rodents, which is important in pharmacological and toxicological studies of new drug candidates. The marmoset is also more closely phylogenetically related to humans (Kitamura et al. 2011; t'Hart et al. 2003, 2012). In Europe, the marmoset is now being used as a non-rodent second species in drug safety tests (Smith et al. 2001).

The common marmoset can be handled with greater ease than many other non-human primates. Along with the appropriateness of the model to the research question, animal welfare and availability are important factors in selecting a model species. Marmosets are readily obtained for laboratory use and, as distinct from macaques, have not been reported to carry herpes b virus (*Macacine herpesvirus 1*), providing a safety benefit to researchers and animal facility staff (Mansfield 2003). The small size of marmosets is also beneficial as it reduces costs and floor space requirements (Smith et al. 2001).

Common marmosets are among the most highly reproductive of all primates. The ovarian cycle is approximately 28 days, similar to that in human (Summers et al. 1985). The gestation period is approximately 145–148 days. Female animals are ready to breed again 10 days after delivery. Usually, female marmosets have two litters per year, which is strongly advantageous when compared to macaques, which require 5 years to sexual maturation and breed only once per year (Austad and Fischer 2011). The remarkable reproductive efficiency of marmosets is extremely well-suited to the development of transgenic and genome editing techniques.

Lastly, a number of basic research tools have been developed for use in marmosets, which is important for encouraging broader adoption by the scientific community. Although the annotated sequencing of its genome has not been completed, a draft sequence with 6× coverage using whole-genome shotgun sequencing is available on GenBank (The Marmoset Genome Sequencing and Analysis Consortium 2014; URL: <https://www.hgsc.bcm.edu/content/marmoset-genome-project>).

Our group has also sequenced the marmoset genome using animals from the colony maintained by the Central Institute for Experimental Animals (CIEA) in Kawasaki, Japan (Sato et al. 2015). Resequencing and assembly of the genome were performed by deep sequencing with high-throughput sequencing technology using a next-generation sequencer, giving approximately 60× coverage. This

enabled us to generate genome assemblies and gene-coding sequence analysis more efficiently and provided a basis for genome editing.

We have also applied non-invasive imaging methods in marmoset research. The use of marmosets in such studies is limited to small numbers due to cost and ethical issues. Magnetic resonance imaging (MRI) is a non-invasive imaging technique to visualize various organs in detail. We have adapted a number of MRI techniques, including diffusion tensor tractography (DTT; Fujiyoshi et al. 2007; Hikishima et al. 2015) and voxel based morphometric (VBM) analysis (Hikishima et al. 2011, 2015), and a new method for the visualization of myelin (Myelin Map; Fujiyoshi et al. 2016).

Transgenic Techniques and Genome Editing Technology for Marmoset Research

One of the strengths of the mouse model is the availability of powerful genetic tools, such as transgenic and knock-in/knock-out animals, that have given the mouse a central place in life sciences research over the past two decades. However, results from mouse genetics are not always directly relevant to humans. Particularly in the neurosciences, there are considerable interspecies differences in brain anatomy and physiology, behavioral control mechanisms, and life span, and some mouse disease models do not recapitulate human symptoms. For example, neurofibrillary tangles, the neuropathological hallmarks of Alzheimer's disease, cannot be recapitulated in mice showing amyloid plaques (Chin 2011; Games et al. 1995; Hsiao et al. 1996; Sturchler-Pierrat et al. 1997; Tanzi and Bertram 2005; Walsh and Selkoe 2004). It is also known that mice in which *parkin*, the gene associated with familial Parkinson's disease in humans, has been knocked out do not show parkinsonism.

Despite the scientific demand for research in non-human primates, efforts to generate transgenic non-human primate animals have been unsuccessful until recently. In 2008, Yang et al. (2008) reported a transgenic rhesus macaque expressing the human huntingtin (*HTT*) gene with a CAG-expansion encoding the poly glutamine as a model of Huntington's disease. However, despite the genomic insertion of the human *HTT*-transgene in the founder monkeys, germline transmission of the transgene has not been confirmed. Our group independently generated transgenic common marmosets expressing the enhanced GFP (*EGFP*) gene and we reported the first germline transmission in a non-human primate (Sasaki et al. 2009).

While the establishment of transgenic marmosets enables the generation of marmoset models of diseases caused by overexpression of a relatively small mutant gene, such as Parkinson's disease, Alzheimer's disease, and amyotrophic lateral sclerosis (ALS), transgenic techniques limited our ability to genetically modify non-human primates. Transgenic technologies available at the time could only randomly insert only <8 kb of exogenous genes into the genome (Sasaki et al. 2009). Moreover,

transgenes were segregated and suppressed across generations, expression levels could not be controlled, and the techniques were only suited to gain-of-function, not loss-of function, studies. Most human genetic diseases are caused by either point mutations or deletions of endogenous genes, which highlighted the need for new gene modification technologies for use against endogenous genes.

Remarkable recent advances in genome editing technology have now made it possible to overcome these previous limitations (Sato et al. 2016). Genome editing tools, i.e., engineered nucleases, bind to a target genome sequence and introduce specific double-strand breaks. Double-strand breaks initiate cell-endogenous repair mechanisms such as homology-directed repair (HDR), non-homologous end-joining (NHEJ), and microhomology-mediated end-joining (MMEJ). Mutagenesis against endogenous genes can be introduced by taking advantage of such mechanisms. Zinc finger nucleases (ZFNs), transcription activator-like effector nucleases (TALENs), and the clustered regularly interspaced short palindromic repeat (CRISPR)/Cas system are mainly used as engineered nucleases. A number of genetically modified animals have already been generated using such restriction enzymes (Bedell et al. 2012; Geurts et al. 2009; Hauschild et al. 2011; Mashimo et al. 2010; Ochiai et al. 2010; Sung et al. 2013; Suzuki et al. 2013; Wang et al. 2013; Yang et al. 2013). Among these, the CRISPR/Cas system was developed the most recently and is particularly promising (Cong et al. 2013; Mali et al. 2013).

Using these genome editing technologies, we recently generated X-linked SCID model marmosets by knock-out of interleukin-2 receptor subunit gamma gene (Sato et al., 2016). Currently, we are now seeking to generate marmoset models of autism spectrum disorders, including Rett syndrome (Chahrour and Zoghbi 2007; Kishi and Macklis 2005) and tuberous sclerosis complex (Ess 2010; Fig. 1). Although a mouse model (male hemizygous *Mecp2* mutation) is available for Rett syndrome, it does not necessarily mimic the critical symptoms. For example, while male hemizygous mice (*Mecp2*^{-/-}) are used as model mice, Rett patients are exclusively female heterozygous in human. It is likely that males with *MECP2* mutations are embryonic lethal in human, but not in mice. Furthermore, phenotypes appear at adult stages in mouse models, whereas symptoms become evident by 1 year of age in human Rett syndrome patients. New primate models that more closely mimic the

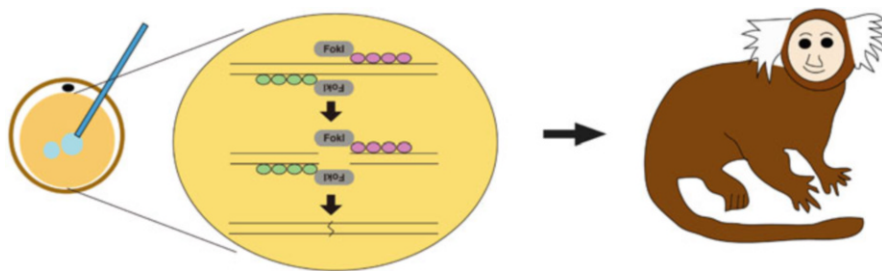


Fig. 1 Generation of a knock-out marmoset by genome editing with ZFN or TALEN

clinical course of human disease may thus contribute to a better understanding of the pathogenesis and future treatments for neurodevelopmental disorders.

Future Perspectives

Genome editing has developed rapidly in recent years, leading to the production of genetically modified animals in many species. This technology has also been applied to non-human primates, and some groups have begun to report genetically modified macaques (Niu et al. 2014; Liu et al. 2014). Macaques offer a number of advantages, but it is difficult to expand colony size within a reasonable research period. We suggest that the common marmoset is thus a highly suitable alternative model primate for many areas of study, and the creation of knock-in/knock-out marmosets would help to introduce the benefits of this model to a larger community of researchers. Since germ-line-competent marmoset embryonic stem cells are not currently available, it is necessary to perform genome editing in one-cell stage embryos (fertilized eggs) to obtain knock-in/knock-out marmosets efficiently. The emergence of more sophisticated genome editing techniques will facilitate and accelerate the development of new gene manipulation technologies in marmoset. Marmoset models of disease generated using genome editing may contribute to the development of new therapeutic strategies for currently incurable neurodegenerative diseases and mental disorders.

Acknowledgments This work was supported by a grant from Brain Mapping by Integrated Neurotechnologies for Disease Studies (Brain/MINDS) from the Ministry of Education, Culture, Sports, Science, and Technology of Japan (MEXT) and the Japan Agency for Medical Research and Development (A-MED).

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