Methods for creating transgenic primates

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Non-human primates (NHPs) have been a subject of biological research for many years, and have been essential to treating disease and alleviating human suffering. Because of their similarities to humans in behavioral, anatomical and physiological properties, they are indispensable for studying drug and vaccine efficacies, psychiatric disorders, brain function, periodontal disease and aging, among other conditions. Historically, NHPs have been studied without extensive genetic manipulation, and have been used as behavioral or physiological models to complement genetic studies done in rodents. However, in the past twenty years genetic engineering techniques have advanced to allow the creation of transgenic primates. By definition, transgenic primates contain DNA from another organism integrated into their own genome. The type of transgene can range from the Channelrhodopsin-2 gene found in the small algae *Chlamydomonas reinhardtii*¹, to the integration of *HTT*², the human gene mutated in Huntington's disease. Given the growth of transgenic techniques, the power of NHPs as a disease model is only beginning to be realized.

Creation of transgenic primates by lentiviral transduction

The first efforts at creating transgenic primates used delivery of a transgene through a lentiviral vector. In this method, viral genomes are modified to contain a gene of interest in place of genes that encode replication machinery. In this way, the transgene can integrate into the host genome without the danger of viral replication. The modified viruses are injected into oocytes, followed by sperm injection. The resulting transgenic embryo is then implanted into a surrogate mother (Fig. 1). This method was first successfully used in 2001 with the

FIGURE 1 | Schematic illustration of a viral-mediated strategy for creating a transgenic primate. Illustration by Kim Caesar.

creation of “ANDi,” a transgenic rhesus macaque containing the GFP gene³.

Lentiviral transduction has also been used to establish models of human disease in NHPs. In 2008 a mutant human *HTT* gene, which causes Huntington's disease, was successfully introduced into rhesus monkeys. The young monkeys displayed traditional symptoms of Huntington's disease, including dystonia (slow repetitive movements or abnormal postures), and chorea (jerky, involuntary movements)². The authors were able to conduct a two-year longitudinal study on one of the five offspring, and found further cognitive decline and brain abnormalities⁴. In reaching these conclusions, the authors adapted assessments normally used for human Huntington's patients, demonstrating the power of NHPs to model human disease.

Most recently, a research group established a transgenic NHP line to model autism spectrum disorders (Fig. 2)⁵. In this work, researchers used lentiviral transfection in order to express the human MeCP2 (methyl CpG binding protein 2) gene in cynomolgus monkeys. Duplications in this gene are known to cause *MECP2* duplication syndrome, which shares symptoms with many autism spectrum disorders⁶. The adult transgenic monkeys showed aberrant locomotion, as demonstrated by repetitive circling around their cages (Fig. 3). By quantifying the amount of time the transgenic monkeys sat together with other monkeys—an established metric of socialization—the authors showed that transgenic monkeys interacted

less compared to their wild-type counterparts, and that this phenotype was worse in males. Notably, this model is of particular value because of the ability to capture changes in social interaction, which have been difficult to assess in mouse models.

Work using lentiviral delivery has brought invaluable progress to establishing NHP models of human disease. However, there are certain limitations with this method that have been difficult to overcome. First, lentivirus-based gene delivery is generally limited in size to an 8.5 kb gene, precluding expression of larger cassettes. Additionally, it is not possible to control the frequency or location of integration of the lentivirus into the host genome (Fig. 4), which can lead to variable copy number and expression, and in some cases lethality. Furthermore, it is not possible to perform site directed mutagenesis using this technique. Several new and improved genetic-engineering technologies, however, provide a potential avenue to circumvent these issues.

FIGURE 2 | Image of infant *MECP2* transgenic monkeys. Adapted from *Nature* 530, 98–102; 2016.

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FIGURE 3 | Movement trajectories (red lines) in cages holding wild-type and transgenic *MECP2* monkeys. Note the increased repetitive circular movements of the transgenic monkey, interpreted by the authors as an 'autism-like' behavioral phenotype. Adapted from *Nature* **530**, 98–102; 2016.

Targeted gene integration to create transgenic primates

Previous NHP models relied on gene expansion or increase in gene copy number in order to establish a disease state. However, using a recently developed technique known as CRISPR (clustered regularly interspaced short palindromic repeats), gene deletions, or point mutations, can now be introduced. The CRISPR/Cas9 system is harnessed from an ancient defense mechanism used in bacteria to fight off viral infection. In this method, a synthetic guide RNA (sgRNA) complementary to the gene target is co-injected with mRNA coding for a nuclease, Cas9. Cas9 induces double-strand breaks at the location directed by the guide RNA. The host's DNA repair machinery then repairs this break using non-homologous end joining, an error-prone process that results in site-specific mutations. This technique has been

used successfully by co-injecting sgRNA targeting *Rag1* and *ppar-γ* and Cas9 mRNA into single-cell stage embryos of cynomolgus monkeys and subsequently implanting the embryos into surrogate mothers⁷. The resulting infant monkeys contained mutations in both target genes, demonstrating not only that CRISPR/Cas9 is an effective strategy for genetic mutation in NHPs, but also that it can be used to target multiple genetic loci simultaneously. Owing to its efficiency and flexibility, CRISPR/Cas9 will likely become the preferred technique for creating transgenic NHPs in the near future.

Modifying NHP-derived embryonic stem cells

One disadvantage of the CRISPR/Cas9 system is the possibility of 'off-target effects'. Since the guide RNA responsible for directing Cas9 to its target is only 20 base pairs in length, it is possible that lesions can be created in the genome in places other than the site of interest. An additional method for creating transgenic animals is the genetic modification of embryonic stem cells (ESCs) *in vitro*. ESCs are pluripotent cells derived from the inner cell mass of embryos in the blastocyst stage. These cells can be cultured *in vitro*, genetically modified using homologous recombination, and injected into the inner cell mass of developing embryos. *In vitro* modification provides the advantages of using antibiotic selection to screen for cells that have specifically incorporated the genetic construct and screening for off-target insertions before the cells are incorporated into the embryo. Successful incorporation of modified cells produces a 'chimeric' animal, meaning that the animal contains genetic information from the parent and the genetically modified stem cells. A proportion of the germ-line cells in the resulting adult will contain these genes of interest, and progeny can be screened to allow for the generation of a stable transgenic line.

Embryonic cell lines have been established in several species of primates^{8,9}, but until very recently genetic modification and subsequent injection of these cells into blastocysts failed to result in the development of a chimeric organism¹⁰. However, by manipulating the cell culture conditions, Chen and colleagues were able to breakdown this barrier and successfully incorporate ESCs into a developing embryo, resulting in chimeric

fetuses¹¹. When Chen *et al.* terminated the pregnancies after 100 days and examined the fetal tissue for cells expressing GFP, which they used as a transgenic marker, they found all three germ layers contained contributions from the injected stem cells, demonstrating the feasibility of this technique. This finding represents a major step forward for the field and the establishment of adult chimeric primates seems imminent.

Conclusions

Recent developments in the creation of transgenic primates have relied mostly on lentiviral delivery of the genes of interest. While these models have been important in advancing our understanding of human disease, new innovations in CRISPR/Cas9 and stem cell technologies sets the stage for greatly improved genetic manipulation of NHPs. As some of our closest evolutionary ancestors, NHPs have been an important source for scientific and medical discoveries. With new genetic tools in hand, their importance as models for complex human diseases will likely continue to grow.

1. Han, X. *et al.* Millisecond-timescale optical control of neural dynamics in the nonhuman primate brain. *Neuron* **62**, 191–198 (2009).
2. Yang, S.H. *et al.* Towards a transgenic model of Huntington's disease in a non-human primate. *Nature* **453**, 921–924 (2008).
3. Chan, A.W.S., Chong, K.Y., Martinovich, C.C. & Schatten, S.G. Transgenic Monkeys Produced by Retroviral Gene Transfer into Mature Oocytes. *Science* **291**, 309–312 (2001).
4. Chan A.W. *et al.* A two years longitudinal study of a transgenic Huntington disease monkey. *BMC Neurosci.* **15**, 1–11 (2014).
5. Liu, Z. *et al.* Autism-like behaviours and germline transmission in transgenic monkeys overexpressing *MECP2*. *Nature* **530**, 98–102 (2016).
6. Ramocki, M.B. *et al.* Autism and other neuropsychiatric symptoms are prevalent in individuals with *MECP2* duplication syndrome. *Ann. Neurol.* **66**, 771–782 (2009).
7. Niu, Y. *et al.* Generation of gene-modified cynomolgus monkey via Cas9/RNA-mediated gene targeting in one-cell embryos. *Cell* **156**, 836–843 (2014).
8. J.A. T. *et al.* Isolation of a primate embryonic stem cell line. *Proceedings of the National Academy of Sciences of the United States of America* **92**, 7844–7848 (1995).
9. Sasaki, E. *et al.* Establishment of novel embryonic stem cell lines derived from the common marmoset (*Callithrix jacchus*). *Stem Cells* **23**, 1304–1313 (2005).
10. Tachibana, M. *et al.* Generation of chimeric rhesus monkeys. *Cell* **148**, 285–295 (2012).
11. Chen, Y. *et al.* Generation of cynomolgus monkey chimeric fetuses using embryonic stem cells. *Cell Stem Cell* **17**, 116–124 (2015).

FIGURE 4 | Image indicating genome-wide distribution of *MECP2* transgene using lentivirus-based transgenic strategy. Dots indicate sites of insertion distributed across multiple chromosomes. Dots are color coordinated by monkey (transgenic monkeys T04–T11, excluding sample failure for T08). Size of dots are proportional to the number of reads identified using deep-sequencing. Adapted from *Nature* **530**, 98–102; 2016.