PROBLEMS EDITING THE MONKEY GENOME

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By:

Thomas Cormier IQP-43-DSA-4459	Connor Darling IQP-43-DSA-4179	Zhidong He IQP-43-DSA-1145
Breahna Mattie IQP-43-DSA-3800	Dylan Pinnette IQP-43-DSA-6486	Chenwei Zhang IQP-43-DSA-8513

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APPROVED:

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Prof. David S. Adams, PhD
WPI Project Advisor

ABSTRACT

Scientists this year used new CRISPR and TALEN technologies to efficiently edit the non-human primate genome, which may allow us to create new disease models far more accurately than current mouse models. The overall goal of this project was to document and evaluate these new genome editing technologies, and assess ethical problems associated with their use in monkeys. We reviewed the published scientific literature on this topic and performed a series of interviews with academic researchers and bioethicists. Our findings indicate that the possibility of off-target mutations in the genome at sites other than the intended site to be edited was the single most important concern. But with proper genome monitoring (either by whole genome sequencing or by PCR of specific high-probability locations), careful guide RNA design (to avoid locations that could cross-hybridize), using new techniques for lowering off-target mutations rates, and screening the treated embryos prior to implantation into the uterus, the problem of off-target mutations is not likely to be a serious problem.

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Dr. Rodolphe Barrangou, Department of Food, Bioprocessing, and Nutrition Sciences, North Carolina State University, Raleigh, North Carolina 27695.

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Dr. Weizhi Ji, Kunming Primate Research Center, Chinese Academy of Sciences, Kunming 650223, China.

Dr. Yuet Kan, Department of Medicine, University of California, San Francisco, California 94143.

Dr. Ling Li, Atherosclerosis Research Unit, Division of Gerontology and Geriatric Medicine, Department of Medicine, University of Alabama at Birmingham, 1530 3rd Avenue South, BDB 658, Birmingham, Alabama 35294.

Dr. Wei Li, State Key Laboratory of Reproductive Biology, Institute of Zoology, Chinese Academy of Sciences, Beijing, China.

Dr. Scott W. Lowe, Memorial Sloan-Kettering Cancer Center, New York, NY 10065.

Dr. Prashant Mali, Department of Genetics, Harvard Medical School, Boston, MA.

Dr. Wenning Qin, Associate Director, Genetic Engineering Technologies, The Jackson Laboratory, 600 Main Street, Bar Harbor, ME 04609.

Dr. Erika Sasaki, Central Institute for Experimental Animals, 1430 Nogawa, Miyamae-ku, Kawasaki, Kanagawa 216-0001, Japan.

Dr. Young Sung, Department of Biochemistry, College of Life Science and Biotechnology, Yonsei University, Seoul, Republic of Korea.

Dr. William E. Van Nostrand, Department of Neurosurgery, Health Sciences Center, T-12/086, Stony Brook University, Stony Brook, NY 11794.

Dr. Julien Valton, Cellectis SA, 8 rue de la croix Jarry, 75013 Paris, France.

Dr. Bing Yang, Department of Genetics, Development, and Cell Biology, Crop Bioengineering Consortium, Iowa State University (Ames, Iowa).

Dr. Shang-Hsun Yang, Department of Physiology, College of Medicine, National Cheng Kung University, 1 University Rd, Tainan 70101, Taiwan.

Dr. Jiing-Kuan Yee, Department of Virology, Beckman Research Institute of City of Hope, Duarte, CA.

Dr. Lianfeng Zhang, Key Laboratory of Human Disease Comparative Medicine, Ministry of Health, Institute of Laboratory Animal Science, Chinese Academy of Medical Sciences, Beijing 10021, China.

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EXECUTIVE SUMMARY

This year, scientists used two new gene editing technologies (CRISPRs and TALENs) to edit the genomes of transgenic monkeys, an achievement that may allow us to create new disease models far more accurately than with rodent models. But this new technology comes with ethical concerns of permanently altering the genome of a species very similar to humans, and it comes with technical problems that might introduce off-target mutations into the animal's genome which could harm the animal. The overall goal of this project was to document and evaluate these new genome editing technologies, and assess ethical problems associated with their use in monkeys. A literature review was performed on the new CRISPR and TALEN editing technologies and on transgenic primates in general to identify initial problems, then a series of interviews was performed with key scientists who use the technology and with bioethicists to further shed light on the problem. We then evaluated the information and made conclusions and recommendations based on the research.

The CRISPR technology uses a guide RNA to hybridize to a target sequence in the genome that is then cleaved on both strands by Cas9 nuclease. The TALEN system uses TAL type amino acid DNA-binding cassettes to bind specific target sequences. The binding of two TAL domains (and their associated half FokI nuclease domains) creates a function FokI nuclease that cuts the DNA. For either technique, the cut DNA is then filled in by the cellular DNA repair system, which makes mistakes, so mutations are placed at the target site to knock out its function. With modifications, the techniques can also be used to create gene knock-ins, and they appear to be applicable to almost any DNA sequence in any species. The techniques are far more efficient, easy to use, faster and more precise than the more traditional methods of genome editing such as homologous recombination or gene Floxing.

Based on the research performed for this project, our group was able to make several conclusions and recommendations. The single most important concern identified in this project with respect to making transgenic animals with the new CRISPR and TALEN methods was the potential for **off-target mutations**. These off-target effects could, in theory, inactivate a required gene in the animal's genome, activate an oncogene, or create a chromosomal inversion, which could harm the animal. Each scientist interviewed for this project agreed the CRISPR and TALEN techniques could, in theory, create off-target effects, but they disagreed considerably on whether such effects are truly a problem.

Some scientists argued that when editing very large numbers of cells, for example in a human patient's body, it would be likely that one cell out of the billions being treated could become cancerous. But this argument becomes moot when making transgenic animals, because 1) there are relatively few cells edited in the embryo used for implantation (so relatively few chances for mutations), and 2) some cells in the embryo could be pre-screened prior to implantation in the uterus to ensure no off-target effects. So, by screening the embryo prior to implantation, the process could be repeated until no off-target effects are observed.

When directly comparing the CRISPR and TALEN techniques for off-target rates, several scientists pointed out that direct comparison studies are lacking, so perhaps such comparisons should be performed. However, because new technique modifications are constantly being developed to lower the rates, perhaps it is too soon for such direct comparisons. Some of the interviewees thought that because TALENs use longer guide domains and require two of them (to dimerize the two FokI nuclease domains) the rates for the TALEN method are likely to be lower than with CRISPRs. Several new modifications to the original techniques have been published in the past year that appear to significantly lower the rates, and include using new software to ensure the guide RNA sequence is unique in the genome, using two guide

RNAs instead of one, altering the length of the guide RNA, or using an off-set nicking strategy. As the CRISPR and TALEN techniques continue to be used by more labs, we predict that even more modifications will be developed for further lowering off-target effects.

With respect to the best practice screening technologies to assay for off-target effects, the best practice appears to be whole genome sequencing. Although for this to be effective, the genome needs to be sequenced to about a 1000-fold depth (which is expensive) to distinguish the rare off-target effects from the normal cellular DNA mutation rates. And when whole genome sequencing is not affordable, the best practice appears to be using PCR to screen for mutations at specific locations most likely to be affected, i.e. those sites identified by bioinformatics as locations that could cross-hybridize with the guide RNA (for CRISPRs) or the TALN DNA-binding amino acids (for TALENs) to provide a more focused screening approach.

With respect to making transgenic animals in general, the interviews validated the preliminary findings of the Literature Review that the pronuclear microinjection method is the most popular method, and most of the interviewees who had directly created transgenic animals indicated they had performed the pronuclear (or single cell) injection method in their own labs. They indicated this method is simpler to accomplish than manipulating and growing embryonic stem cells, and it can be applied to *any* species even if ESCs cannot yet be grown for that species. Some interviewees indicated they no longer microinject specifically into the pronucleus but into the cytoplasm, which is easier to hit, and the DNA also enters the pronucleus.

When making transgenic monkeys, one scientist argued that marmosets are better suited than rhesus monkeys, so perhaps different monkey species should be tested. With respect to the future of transgenic primates, it is important that the genome editing be passed onto the offspring, otherwise the alteration dies out with the founder animal. Two interviewees best summarized how we might use transgenic monkeys in the future, saying that non-human

primates are relatively close to human beings (compared to rodents), especially with respect to "brain anatomy, brain function, neuro-pathological progression, and behavioral phenotypes, all of which are important to investigate" (Yang interview), and they are already preparing neuronal disease models and diabetic models (Sasaki interview). With respect to the ethical concerns about creating transgenic monkeys, one ethicist posed the question about what role we humans will eventually have as the creators of these new transgenic species, and what our moral obligation will be to these creatures. She suggested that the way we treat these new creations through our laws should be a reflection of our most noble aspirations (Glenn, 2003).

We conclude that with proper genome monitoring (either by whole genome sequencing or by PCR of specific high-probability locations), careful guide RNA design (to avoid locations that could cross-hybridize), using new techniques for lowering off-target mutations rates, and screening the treated embryos prior to implantation, the problem of off-target mutations is not likely to be a serious problem.

AUTHORSHIP

Author	Areas Covered
Thomas Cormier	Technologies for making transgenic animals
Connor Darling	Transgenic monkey applications and ethics
Zhidong He	Editing the monkey genome
Breahna Mattie	Introduction to genome editing and transgenic animals
Dylan Pinnette	Genome editing technologies
Chenwei Zhang	Editing the monkey genome

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PROJECT GOALS

Scientists this year used new TALEN and CRISPR technologies to edit the non-human primate genome. This achievement may allow us to create new disease models for testing vaccines or therapies, or may allow us to mimic human diseases far more accurately than current rodent models. But this new technology appears to come with technical concerns; in addition to editing the desired target gene it can introduce non-target mutations in the animal's genome which could disrupt a functional gene or cause cancer. Editing the genome of an animal so similar to humans also comes with ethical concerns. The overall goal of this project is to document and evaluate these new genome editing technologies and assess ethical problems associated with their use in monkeys.

The specific objectives are to:

1. **Develop** a comprehensive assessment of the scientific experiments that lead to the development of monkeys with altered genomes, and the potential applications of non-human primate transgenic models.
2. **Characterize** what key scientific stakeholders believe are the strengths and weaknesses of this new technology and the ethical concerns of working with monkeys.
3. **Evaluate** the obtained evidence for remaining problems.
4. **Recommend** potential solutions to any remaining problems.

LITERATURE REVIEW

Introduction (Breahna Mattie)

This year, scientists used a new gene editing technology to edit the DNA of a transgenic monkey (Niu et al., 2014). This achievement may allow us to create new disease models for testing vaccines or to mimic human diseases in an animal model far more accurately than can be achieved with current transgenic rodent models. But this new technology comes with ethical concerns when permanently altering the genome of a species very similar to humans, and it comes with technical problems that might introduce off-target mutations into the animal's genome.

WHAT IS GENOME EDITING?

The genome of an animal is its genetic material, the molecule(s) that contains an organism's "blueprint" for existence, and which encodes all the other molecules that constitute the organism. Although the genome of some viruses can be RNA, the genomes of all animals is deoxyribonucleic acid (DNA). Genome editing is the deliberate manipulation of the genetic material: repairing, modifying, deleting, adding, or exchanging pieces of DNA to create a genome different than normally found for that species in nature (Kowles et al., 1985). Genome editing can be done through plasmid insertion, viral integration, homologous recombination, TALENS, or CRISPRs, each of which will be discussed later. Genome editing might be done for the purpose of adding a new gene (knock-in) to a species which when expressed gives the species a new useful phenotype (visible trait), or the editing might be used to delete stretches of DNA (knock-out) to determine the effects of deleting specific genes.

WHAT IS A TRANSGENIC ANIMAL?

When the genome of an animal is altered, it creates a transgenic animal. The formal definition of a transgenic animal is an animal with genes added to their DNA (Parekh et al., 2004), but more recently the term also loosely applies to animals whose genomes have been edited to knock-out specific genes. So, the term is broadly used to describe animals whose genomes have been altered to create a new desired trait in the animal, or to study the effects of a specific gene knock-out or knock-in on the animal. When engineered successfully, the new trait is passed onto the founder animal's offspring and colonies of the animals can be created.

For thousands of years, before we understood what DNA was or how to edit it, we altered the genetic makeup of animals and plants by selective breeding, mating specific strains to each other to create new plant strains resistant to specific diseases, to create new strains of domestic animals that were stronger or faster, or to create new strains of yeast for fermentation. Through the advancement of recombinant DNA and genome editing technology, we now have more precise tools for altering an organism's genome.

The world's first transgenic organism was a bacterium *E. coli* engineered to contain plasmid DNAs (as cloning vector) containing other cloned bacterial genes (Cohen et al., 1973). This study was followed in 1974 with the plasmid cloning of various eukaryotic genes in *E. coli* (Cohen and Chang, 1974). In 1974, the technology was applied to animals by altering a mouse's genome by transferring bone marrow cells from specific strains of mice into blastocyst embryos of Swiss albino mice. The injected blastocysts were allowed to develop in the uteri of foster mothers, and the pups were analyzed for evidence of incorporating the genetically different cells. 137 adult mice resulted from the impregnation. Their data indicated that the transferred cells were able to establish small colonies in the embryos and that some of these cells persisted into the adult mice. In the same year, Jaenisch and Mintz (1974) created a mouse containing foreign

SV40 viral DNA by injecting the DNA into the blastula, although in this case the foreign DNA was not expressed. The world's first transgenic animal that *expressed* its foreign DNA to create a new visible phenotype was "supermouse" (Palmiter et al., 1982). These mice contained a rat growth hormone gene driven by a mouse metallothionein-I promoter (always on), and were much larger than their non-transgenic littermates. The mice were created by pronuclear microinjection of the foreign DNA, creating 21 mice, 7 of which were transgenic.

Since the early transgenic experiments, the technology has been applied to thousands of transgenic animals. The vast majority of the animals are mice due to their fast breeding, extensive genetics, and easy culture. The rodent models have vastly served biomedical science by providing models for specific gene knock-in's and knock-outs, and allowing therapies to be tested in a complex physiological environment. But rodent physiology is not closely related to humans. The human diseases being modeled in simple animals, such as mice, are sometimes inadequate for complex diseases. So, scientists have researched making transgenic non-human primates whose physiology more closely matches humans.

MONKEY GENOME EDITING

The first published account of creating a transgenic monkey was in 2001 with the creation of the rhesus monkey ANDi (Chan et al., 2001). ANDi was created by pronuclear microinjection of DNA encoding a marker gene (green fluorescent protein, GFP). The GFP gene was delivered to the genome using retroviral insertion. The impregnation resulted in three offspring, but only ANDi showed evidence of being transgenic (transgene incorporation). However, his GFP transgene was not expressed. But ANDi provided evidence that transgenic primates can be created, and he provides hope for creating disease models more closely related to humans.

The first transgenic primate disease model was created in 2008 when a rhesus monkey was genetically modified to express the human Huntington disease gene "huntingtin" (Yang et al., 2008). Several "hallmark features of HD, including nuclear inclusions and neuropil aggregates, were observed in the brains of the HD transgenic monkeys" (Yang et al., 2008). This experiment showed that it is possible to use primates to replicate human diseases, and the models may be used in the future for screening potential therapies. But one of the downfalls of creating genetically altered monkeys came to light in this study, their birthrate was incredibly low. This was speculated to be due to the freezing and thawing of the embryos which reduced embryo quality, so newer procedures are being developed.

In 2010, a group of scientists used a modified simian immunodeficiency virus (SIV) vector to deliver a transgene to the blastocyst cells of rhesus monkey embryos (Niu et al., 2010). The pregnancy rate and the fetal survival rate increased with the use of the SIV-based vector. They infected 81 1-to-8-cell embryos. After 3 to 5 days in culture, the embryos were tested to see which expressed the fluorescent gene implanted days earlier. The results showed that seventy embryos expressed the transgene, which is a drastically better transgenic rate than the attempts done in the past. Once implanted into the surrogate mother, the overall pregnancy rate was 62%, and the birth rate was 27% (Niu et al., 2010). This vector may provide a striking improvement in efficiency when making transgenic primates, and perhaps the SIV vector is less harmful to the embryos. This method may be used in the future for creating primate disease models that will further benefit the research of different human diseases.

Although the use of viral vectors (HIV and SIV) proved relatively efficient methods for creating transgenic monkeys, the techniques are not without their problems. Viral vectors integrate randomly in the host genome, which can inactivate needed host genes. But this potentially serious problem might be overcome with the recent use of new gene editing

technology such as TALENs or CRISPRs. CRISPR was used in 2014 as a precise genome editing tool for creating transgenic monkeys (Niu et al., 2014), and this recent success was the primary focus of our project.

TRANSGENIC CONSEQUENCES

But the new genome editing technology may also have its own problems, such as mutations induced in the host genome off-site from the desired target gene. Do all scientists see these problems? Do all scientists assay for these problems? What is the best way to assay for off-site mutations? Do scientists see off-site mutations as a potential problem for creating accurate primate disease models? How can the off-site mutations be reduced? The manipulated embryos are usually frozen, which some scientists say reduces the embryo quality and success rate, do all scientists use this technique? Will becoming efficient at altering a monkey genome eventually lead to trying to alter the human genome? The benefit of altering a primate genome so similar to humans is that testing medical devices and medications might predict a more accurate outcome for a human subject. But once we get good at it, will we try and alter the human genome? These questions were investigated in this project through a series of interviews with various stakeholders, including scientists performing the technology, and ethicists who can comment on the main ethical problems.

The applications for transgenic animals is vast and varied. From medicinal uses to agricultural, it seems that transgenic animals are the future for a growing world population. What promises does making transgenic monkeys hold for medical research?

Genome Editing Technology (Dylan Pinnette)

The previous section of this Literature Review introduced the concept of editing the monkey genome and gave a brief background on genome editing, transgenic animals, and potential consequences. This section will discuss various techniques for editing genomes. Much of the technology for editing genomes uses biological processes which exist in nature but have been adapted for use in genetic engineering. The techniques discussed below include plasmids, retroviruses, homologous recombination, and the new TALEN and CRISPR systems.

PLASMIDS

Cloning vectors are molecules used to amplify DNA and transfer it into cells. Plasmids are one of the most common methods for cloning DNA. Plasmids are self-replicating circular DNA molecules that can that can replicate independently of chromosomal DNA in a cell. Plasmids in nature are often found in bacteria, and can carry genes for antibiotic or bacteriophage resistance. Plasmids were first used in genetic engineering about 1972 (Cohen et al., 1973; Cohen and Chang, 1974). After the discoveries of restriction enzymes (which recognize specific sequences of DNA to cut DNA) and DNA ligase (which seals the ends of cut DNA molecules together), scientists were able to splice together DNAs into plasmids (Pray, 2008).

Engineered plasmids normally have several features, including an origin of replication (Ori), an antibiotic resistance gene (to select for positive cells), and the cloned gene inserted into a multiple cloning site (MCS), is a short sequence of DNA containing many sites recognizable by restriction enzymes. By containing a gene for antibiotic resistance, the bacteria in which the plasmid has entered can be identified by growing on plates containing the antibiotic. Cells lacking the plasmid die (Pray, 2008). Plasmids alone do not generally integrate their DNA into

the host chromosome, but when combined with nuclease-based methods that encode enzyme for integration, plasmids can integrate.

RETROVIRUSES

Viruses vary considerably in their composition, but the ones used most frequently as cloning vectors are retroviruses which contain an RNA genome, a protein coating of the RNA, and an enveloping lipid bilayer (Anson, 2004; Yi, 2011). Retroviruses are able to convert their RNA into DNA through the enzyme reverse transcriptase, then integrate the DNA into the host chromosome. An engineered retrovirus can insert itself into a chromosome and the desired cloned gene contained by it can be expressed. There are several advantages to using retroviruses for cloning. Retroviruses can cause lower toxicity than plasmid methods, as usually only one retrovirus will enter the cell, as opposed to numerous plasmids (Yi, 2011). And there usually is only one edit to the cell's genome (although it is random and can harm the cell). The retrovirus also doubles as a way to both transfer genetic material to the cell and to editing the genome. It can also be used on mature organisms, giving it therapeutic value.

Retroviral therapy was a strongly favored gene delivery technique until a 2003 study showed an increased risk of cancer when using retroviral therapy (Yi, 2011). Most natural retroviruses cause some form of cancer (although these genes are usually edited out when doing therapies), but the risk of the retrovirus transducing to non-target cells still remains. Targeted retroviral treatments show the potential for a safe genome editing technique, but until they are developed, such treatments are considered a last resort (Yi, 2011).

HOMOLOGOUS RECOMBINATION

Homologous recombination is a natural process used by a variety of organisms to recombine DNA between two DNA molecules. In eukaryotic cells during meiosis, this process is used to mix gamete chromosomal DNA. In mitotic eukaryotic cells, homologous recombination is used to repair double-stranded breaks. More rarely, homologous recombination is also used for somatic hyper-mutation, replicating homing endonucleases, and mating-type conversion in yeast (Porteus, 2007).

Using homologous recombination to target specific genes in a cell for engineering first started with yeast experiments in the late seventies. The first step is to create a DNA with the transgene surrounded by DNA identical to that of the target gene loci. When the foreign DNA with homologous regions flanking both sides of the transgene enters the cell, it can align with the native DNA in the corresponding region, and homologous recombination can occur at both surrounding regions of the DNA. It is also possible that non-homologous binding can occur, with the transgene bonding at an undesired location. To ensure that only homologous bonding of DNA has the occurred, negative selective markers can be placed in the invasive DNA molecule on either side of the transgene and the surrounding homologous region. If homologous bonding occurs, the selective markers will not enter into the native DNA molecule, as they were not from the homologous region. If non-homologous bonding occurs, the negative selective marker becomes part of the chromosomal DNA. Addition of an antibiotic then allows those cells not containing the desired DNA to be killed (Campbell, 2014).

Homologous recombination varies greatly between different organisms. In some organisms, such as yeast, it occurs easily and predictably. In humans and most mammalian cells the rate of gene targeting is quite low. In mammalian cells, the use of homologous recombination is limited, “For every successful gene-targeting event, there will 10,000-20,000

random integrants in mammalian cells" (Porteus, 2007). With respect to transgenic animals, usually embryonic stem cells (ESCs) are targeted, as they have higher gene targeting rates than differentiated cells, and the transgenic DNA includes a selective marker to determine which stem cells have been modified by the transgene (Porteus, 2007). Then the ESC lines containing the marker can be bred together to create transgenic lines. Homologous recombination can be used most effectively in mammalian cells when combined with nuclease-based methods such as TALENs and CRISPRs (discussed below). Nuclease-based methods can be used to induce the process of homologous recombinant through double-stranded break repair. Thus, for making transgenic monkeys, homologous recombination is most useful when combined with CRISPRs or TALENs.

Genome Editing by CRISPRs and TALENs

Recently, two new powerful genome editing techniques have taken the biological research area by storm: CRISPRs and TALENS. These two techniques guide tailor-made DNA endonucleases to specific targeted sites in the DNA resulting in double-stranded breaks. Following the cutting, mutations in target site can be inserted by error prone DNA repair mechanisms, by homologous recombination, or by designer templates. The edited cut sites are then ligated together using cellular DNA ligation processes. These new genome editing techniques are revolutionizing genetic engineering due to their extremely high efficiency, speed, ease of use, and applicability to almost any species. Each of these features is an extremely important advantage relative to the more traditional labor-intensive methods of targeted genome editing such as homologous recombination. CRISPR and TALEN techniques can be used to create gene deletions (usually by inserting mutations at the site), gene insertions, or gene

corrections. Both of these techniques have recently been used to edit the non-human primate genome, so they are of direct interest to this IQP project.

CRISPRs

CRISPRs are **c**lustered **r**egularly **i**nterspaced **s**hort **p**alindromic **r**epeats that are present in the DNA of many bacterial species (reviewed in Barrangou, 2014). CRISPRs are found in approximately 40% of all sequenced eubacteria and in 90% of the sequenced archaea (Grissa et al., 2007). The repeat sequences form an array that participates in a type of adaptive immunity to protect bacteria from invading viruses and plasmids (Barrangou et al., 2007). In this crude immune system (**Figure-1**), an invading bacteriophage (bacterial virus) for example attaches to the surface of a bacterial cell (diagram upper left) and inserts its double-stranded DNA into the bacterial cytoplasm. The viral DNA is then cleaved by a CRISPR-associated (Cas) nuclease (diagram upper right) to create a viral spacer DNA that is inserted into the CRISPR array in the bacterial genome (diagram center) to become a part of a series of repeat sequences. Each viral DNA spacer is followed by a short spacer from a previous viral exposure to make the "array". Transcription of the viral repeat sequence creates a new guide RNA (sgRNA) or CRISPR RNA (crRNA) (diagram lower center) which associates with Cas nuclease (lower left). The guide RNA targets the Cas nuclease to other entering molecules of viral DNA to cleave and inactivate them, thus protecting the bacterial cell from the virus.

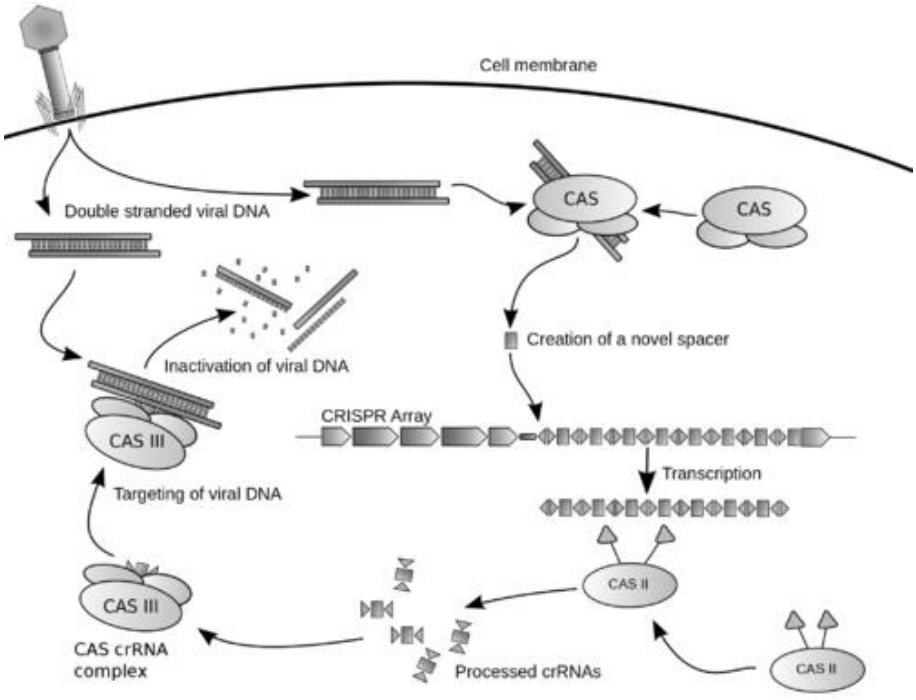

Figure-1: Diagram of the CRISPR-Cas9 Immune System in Bacteria. Shown are: the invading virus (bacteriophage) (diagram upper left) attached to the surface of a bacterial cell (cell membrane); the double-stranded viral DNA being cut into a short spacer DNA by Cas nuclease (diagram upper right); the viral spacer DNA being incorporated into a CRISPR array in the bacterial genome (diagram center); the short spacer viral DNA being transcribed into a short guide RNA or CRISPR RNA (crRNA) (diagram lower center); and the use of the guide RNA to guide CAS nuclease to other invading bacteriophage DNAs complementary to the guide RNA to cleave them (diagram left center). Diagram is from: http://en.wikipedia.org/wiki/CRISPR

With respect to its use as a tool for genetic engineering, the CRISPR bacterial immune system has been nicely adapted for performing gene editing in almost any species at almost any DNA location (reviewed in Mali et al., 2013). The technique has become even more popular than TALENs because the targeting is provided by a single RNA molecule, so it is simpler to design (**Figure-2**). The proteins encoded by CRISPR-associated (Cas) genes (such as Cas9) (blue in the diagram) are guided to specific locations in the DNA by short CRISPR RNAs (crRNAs) also known as guide RNAs (gdRNAs) (dark brown line, diagram upper right). The guide RNA contains sequences complementary to the desired target sequence to be edited plus a short proto-

spacer adjacent motif (PAM). PAM sequences are NGG, where N represents any nucleotide, and are widely present randomly throughout most genomes. The guide RNA binds a PAM sequence (diagram center) adjacent to the DNA site complementary to the guide RNA target sequence. This binding unzips the DNA duplex. Two different Cas9 nuclease domains (HNH and RuvC) (diagram center) then nick one strand each to generate a double-stranded break.

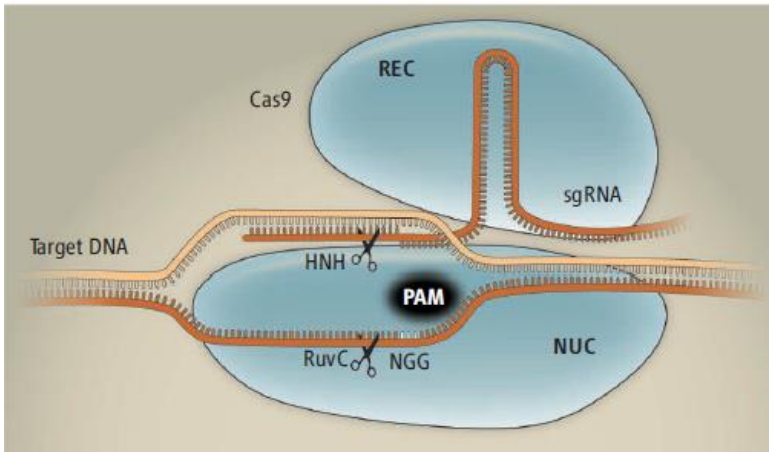

Figure-2: Diagram of the CRISPR-Cas9 System for Editing DNA. Shown are the two Cas9 nuclease domains (blue) attached to DNA via a short guide RNA (sgRNA, upper right). The binding occurs at PAM sequences (NGG, where N is any nucleotide) in the DNA adjacent to the target sequence complementary to the guide RNA. The cutting occurs at two locations (HNH and RuvC, shown as scissors in the diagram) to create a double-stranded break. The break can then either be filled in with mutations using an unfaithful DNA repair system, or can be inserted with other desired sequences. Diagram from Barrangou et al., 2014.

TALENs

TALENs (transcription activator-like effector nucleases) are artificial restriction endonucleases engineered to cleave DNA at specific sites (reviewed in Boch, 2011; Valton et al., 2014; Wright et al., 2014). In nature, TALE nucleases are secreted by *Xanthomonas* bacteria, but the system has been shown to be active in a large variety of species. Different than CRISPRs, the endonuclease in this case is not Cas9 but consists of two FokI dimers that must associate together to cut the DNA (**Figure-3**, diagram center). There is no guide RNA, but instead engineered TALE amino acid repeat domains (shown as colored boxes in the figure) bind to

specific DNA locations to target the nuclease. Specific domains bind to specific nucleotides to create a "one-repeat-one-nucleotide DNA recognition code" that can be altered to bind almost any DNA sequence (Sun and Zhao, 2014). Because two different DNA-binding domains are usually used (that flank the site to be cut), some scientists think that TALENs are more specific than CRISPRs and have fewer off-target effects. In a recent advance, the two FokI dimers are encoded by one DNA-binding domain, which can be simpler to design when editing genomes (Sun and Zhao, 2014).

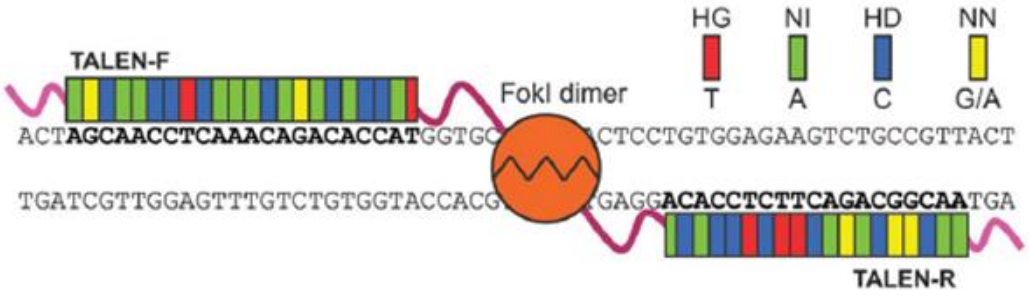

Figure-3: Diagram of a Typical TALEN. The FokI nuclease dimer is shown in orange in the center of the diagram. The TALE DNA-binding domains are shown as colored boxes. The N-terminal segments (NTS) are shown in pink (left and right side), and the C-terminal segments (CTS) are shown in darker pink (diagram center). Figure is from: Sun and Zhao, 2014.

When doing genome editing, the DNA target sequence is identified, then two TALEN sequences are designed to bind DNA upstream and downstream from the target. The TALEN sequences and the FokI nuclease-encoding domains are cloned into a plasmid DNA, which is then inserted into a cell using any of a variety of techniques, such as electroporation. Inside the cell, the plasmid is translated into a functional TALEN protein which enters the nucleus to cut the DNA at the target site. The process can be used to introduce an error to knock out a target gene (if the error prone DNA repair process is used which can insert nucleotides, delete

nucleotides or induce chromosomal rearrangements). Alternatively, the process can be used to introduce a new DNA sequence into the target gene.

Potential Problems with TALENS and CRISPRs

In spite of their unprecedented usefulness for genome editing, some scientists think that these techniques have the potential to introduce unwanted mutations at locations other than the target site (off-target effects) (reviewed in Fu et al., 2013). If the DNA binding sites are not specific enough to bind only the target sequence, it can bind elsewhere in the genome and edit that site too. Such off-target cleavages may inactive a useful gene, activate an oncogene, or can lead to the production of enough double-strand breaks to overwhelm the repair machinery to induce chromosomal rearrangements and/or cell death. And because the cellular DNA repair or homologous recombination processes used to create the new DNA sequence can vary depending on the species, type of cell, target gene, or nuclease used, some scientists believe that potential off-target effects should be monitored when designing any new editing system.

Technology for Creating Transgenic Animals (Thomas Cormier)

The previous section of the Lit Review discussed the general purposes of making transgenic animals and the various methods used to edit their genomes. The purpose of this section is to discuss the main methods for making the transgenic animals themselves after the DNA has been edited. This information is important for understanding the subsequent Lit Review section on primate transgenics, especially since one method, listed below, seems to predominate when making them.

In general, there are two main methods for making transgenic animals (listed in their order of their development): 1) manipulation of embryonic stem cells (injection of genetically modified embryonic stem cells (ESCs) into the blastocoel cavity of a blastocyst), and 2) egg manipulation (microinjection of genetically modified DNA, usually into the male pro-nucleus of a fertilized embryo, or intra-cytoplasmic sperm injection into a genetically modified egg). These techniques will each be discussed below, especially in view of their pros and cons.

Manipulation of ES Cells

Historically, the world's first transgenic animal was created in 1974 (Jaenisch and Mintz, 1974) by infecting an *in vitro* fertilized (IVF) embryo grown to the blastocyst stage with SV-40 virus. The infected blastocysts were implanted into the uterus of a foster mother, and resulted in the birth of live pups transgenic with the SV-40 sequences; however this transgene was not expressed. Although the mice became adults with no signs of SV-40 tumors, after examination the mice were found to contain SV-40 DNA (i.e. were transgenic). Overall, 80 blastocysts were injected, producing 29 healthy mice that lived a full year until they were euthanized for tissue analysis. 25 of the surviving 29 mice were tested for SV-40 viral sequences. 7 of 25 livers and kidneys, and 4 of 15 brains tested positive for SV-40 sequences. With no visible manifestation of the SV-40 injections, the best explanation was the virus was integrated at an early developmental stage but it was a "cryptic transformant" which carries the virus but remains untransformed. So, the SV-40 transgene sequences were *not* expressed.

One of the early experiments that achieved successful expression of a transgene using ESC manipulation was performed in 1986 (Gossler et al., 1986). This experiment was one of the first to demonstrate that manipulating embryonic stem cells can successfully be used as a method for achieving transgenesis. Their transgene was neomycin phosphor-transferase (neo) which

confers resistance to the antibiotic G418, allowing selection of ESCs containing the transgene. The researchers were able to achieve transgenic yields nearly similar to the accuracy of the then developing pronuclear injection method. Although pronuclear injection on average leads to 1.5-6.0% of the injected eggs becoming transgenic, Gossler et al. discovered that injecting ESCs into a blastocyst leads to an average of 2% of transgenic mice. This method provided the advantage of being able to pre-screen the treated ESCs for those cells that successfully incorporated the Neo^r transgene prior to injecting the cells into the blastocyst. This helped improve the overall efficiency. However, the animals created using this technique are chimeric: some of the ESCs are transgenic while others (residing inside the blastocyst) are non-transgenic. As these cells develop to create the animal, some tissues are transgenic and others are not. Another disadvantage stated by these authors is the time it takes for the ESCs to transform with the viral treatment when introducing the DNA. But overall, the scientists were optimistic, stating that "the isolation and cloning of embryonic trans-acting regulators would be greatly facilitated by using ESCs as a source of material" (Gossler et al., 1986). Although they initially feared that the transmission of foreign DNA through blastocyst injection was unstable and unpredictable, the scientists did not experience these problems.

One of the primary advantages of using ESCs is their ability to undergo homologous recombination, a process that allows specific gene targeting. While viral treatments typically integrate randomly, homologous recombination allows target sequences to be replaced with the transgene. This process was successfully applied to ESCs and transgenic mice in 1994 (Bronson and Smithies, 1994). The most frequent use of this technology in mice is the famous gene knockout, whose purpose is to knock out the two copies of a particular gene in transgenic mice to study the effects of gene loss on the organism. The technique can also be used for gene insertions and gene replacements. This technique works by flanking a particular transgene with

host chromosomal sequences identical to those at the target site. As the cells undergo mitosis, the host sequences flanking the transgene hybridize with the host chromosomal sequences at the target site replacing them with the transgene. Until recently, homologous recombination was the only way to make a targeted gene alteration, but has now been supplemented with the new Talen and CRISPR methods (discussed in the previous Lit Review section). The effects on the transgenic mice depend on the particular gene being knocked out, and can range from fatal to non-observable. The authors conclude that the main advantages of working with ESCs include their easy culture, ready manipulation, and most importantly their ability to differentiate into a wide variety of tissues in the host.

The genetic manipulation of ESCs has proven to be a reliable technique for making transgenic animals, and the technology continues to improve. Scientists continue to improve our understanding of how the injected ESCs differentiate to make various tissues, and whether those developmental pathways are altered when using transgenic ESCs (Wobus and Boheler, 2005). They also continue to improve the methods for clonally expanding genetically manipulated ESC lines to produce larger number of cells for injection, and continue to design creative ways to avoid embryo lethality with knock-ins as well as knock-outs. Although foreign DNA can be introduced into ESCs by means of infection, transfection, or electroporation, those techniques typically insert at random positions. So, gene targeting has generally proved to be the more effective approach for transgenesis when using ECSs.

Pronuclear Injection

The technology that became the industry standard for decades when making transgenic animals was microinjection of genetically modified DNA into the male pro-nucleus of a newly fertilized embryo. The first successful use of the pronuclear microinjection method was in 1980

(Gordon et al., 1980) who inserted a transgene consisting of herpes simplex DNA and SV-40 viral DNA into the mouse genome, but the transgene was rearranged and it did *not* express.

Another early experiment that created a transgenic animal by pronuclear microinjection was performed by Gordon and Ruddle (1981), however their mice also did *not* express the transgene. They constructed a plasmid DNA made up of parts of herpes simplex thymidine kinase (TK) DNA and SV40 viral DNA, and injected it into the pro-nuclei of fertilized mouse eggs. Of the 78 mice born, only 2 showed signs of transgene incorporation. Their experiment shows that a recombinant plasmid can be transferred directly into nuclei, and the embryo will maintain the DNA throughout mouse development. Strong southern blot signals showed that most of the tissues of the two transgenic mice had some form of the foreign transgene integrated into their DNA, but there was a large amount of transgene rearrangement. One mouse had the SV40 and herpes virus TK sequences mostly removed, while the second mouse contained both recognizable transgenes.

The first transgenic animal created by *any* method that successfully *expressed* its transgene was created in 1982 using the pronuclear microinjection protocol (Palmiter et al., 1982). This experiment created "supermouse" by pronuclear microinjection of DNA encoding rat growth hormone under control of a mouse MT-1 promoter. The purpose of the experiment was to determine whether rat growth hormone is physiologically active in mice (and to attempt to get transgene expression). Of 21 mice that developed from the injected eggs, 7 were transgenic, and 6 showed successful expression of the growth hormone transgene and grew larger than their non-transgenic litter mates. So, supermouse was a transgenic success.

Some scientists have focused on improving the efficiency of pronuclear transgenic methodology. Brinster et al. (1985) analyzed the effects of DNA concentration, DNA size, DNA form (supercoiled vs. linear with a variety of different ends), injection site (male pronucleus,

female pronucleus, or cytoplasm), and buffer composition. Their most efficient protocol injected a few hundred copies of linear DNA into the male pronucleus of a one-cell embryo, and produced about 25% transgenic mice. They also determined that some hybrid mouse strains (such as C57/BL6 X SJL) express the transgene around 8-fold more efficiently than inbred strains (such as C57/BL6). Injecting too much DNA killed more embryos, although it had a higher integration rate. Injecting the DNA into the cytoplasm was less effective than injecting into the male pronucleus.

Other scientists have focused on improving the length of foreign DNA that can be integrated into the host animal. The traditional vectors, such as plasmids and viruses, have size limits on foreign DNA inserts, so Schedl et al. (1992) investigated using yeast artificial chromosomes (YACs) as cloning vehicles for creating transgenic mice by pronuclear microinjection. YACs have the capacity to clone 1,000 kilobases (kb) of DNA insert compared to the 40 kb limit with other methods. The researchers had to optimize the YAC cloning and purification processes, and their final protocol allowed a 15-fold YAC amplification in yeast. To evaluate the effectiveness of the YAC procedure for making transgenics, the scientists created a 35 kb YAC encoding the enzyme tyrosinase as a marker to rescue an albino phenotype in the recipient host. Approximately 50 YAC copies were microinjected per pronucleus, resulting in 35 mice born from 147 injected eggs. Of the 35 live mice born, 5 were transgenic (however were not albino but showed pigmentation). Despite a relatively weak expression of the transgene in the mice, this experiment gives hope to using long inserts in transgenic experiments, including complex gene clusters.

More recent experiments have focused on improving the efficiency of the transgenic success rates, especially when making transgenic primates that are difficult and expensive to maintain. Sun et al. (2008) developed an efficient procedure for making transgenic macaques

(*Macaca fascicularis*) using pronuclear embryo transfers. They compared the fertilization success rates for IVF eggs (33.3%) versus intra-cytoplasmic sperm injection (50.0%), and concluded the latter was more efficient. They showed that long-term culture of the embryo prior to implantation or freezing the embryo decreased the success rate. They acquired 174 oocytes from 22 super-ovulated female cynomolgus monkeys, and fertilized them through *in vitro* fertilization. Of the 174 fertilized oocytes, only 66 survived to the pronuclear stage (for injection), and they were placed inside of 30 different hosts. These 66 implanted embryos led to the birth of 7 baby cynomolgus monkeys. The scientists got their best results when the pronuclear embryos were transferred into the host 24 to 36 hours after the mother's estradiol peak (in the presence of new corpus luteum), so, it is important to consider the recipient's reproductive cycle to maximize success. Because of the similarities in physiology between monkeys and humans, these findings are extremely important for potentially making transgenic disease models for investigating vaccines and other treatments for human diseases prior to performing clinical trials.

Overall, both of the two main procedures for making transgenic animals have advantages and disadvantages that likely dictate which procedure should be used for a specific experiment. Pronuclear microinjection appears to be the most reliable and tested procedure, and is used by a majority of the labs. When that procedure works correctly, the transgene is disseminated to all tissues of the body. ESC manipulation allows for pre-screening of the genetically manipulated cells prior to injection into the blastocoel cavity, and it allows gene targeting with homologous recombination, but it produces chimeric animals containing only some transgenic tissues. The advent of the new gene editing technologies such as Talens and CRISPRs (discussed in the previous section), allows for specific gene targeting, and can be applied to either of the two main

methods for making transgenic animals. The next section of the report will discuss their applications for making transgenic primates.

Editing the Monkey Genome (Zhidong He and Chenwei Zhang)

Previous sections of this Literature Review discussed two main methods for making transgenic animals (egg manipulation and embryonic stem cell manipulation). We also discussed the various methods for editing animal genomes, including plasmids, viruses, homologous recombination, talens, and CRISPR-Cas9. In this section of the Literature Review, these interesting topics are combined to discuss several recent landmark experiments that lead to the production of transgenic non-human primates whose genomes were edited using the best of the new generation of genome editing tools, CRISPR. However, these landmark experiments are not without their problems, as the CRISPR technology is thought by some scientists to induce off-site mutations in the genome, which in theory could limit the use of these animals as disease models. These experiments and potential problems are introduced here, and will be further investigated in the Results section with follow-up interviews.

Since the 1973 construction of the world's first transgenic organism (a modified *E. coli*) (Cohen et al., 1973), recombinant DNA (rDNA) technology has been applied to thousands of transgenic animals. The vast majority of these experiments were performed with mice due to their ease of culture, fast breeding, and extensive genetics. These rodent models have vastly served biomedical science by providing *in vivo* studies that could not have been achieved otherwise. But because their physiology is not closely related to humans, rodents have limitations for studying some types of human diseases like Huntington's disease, Alzheimer's disease, Parkinson's, etc. (discussed in the next section of the Literature Review). On the other

hand, the physiology of nonhuman primates (such as monkeys) represents a closer match to humans, and may provide better models for studying human diseases (Sun et al., 2008). Some scientists believe that a primate model for testing vaccines should lead more quickly to treatments than using rodent models (Begley, 2001).

World's First Transgenic Monkey (ANDi)

The world's first transgenic monkey was created in 2001 in the laboratory of Dr. Gerald Schatten at the Oregon Regional Primate Research Center (Chan et al., 2001). The scientists cloned the gene for reporter green fluorescent protein (GFP) (whose expression can easily be detected) into a retroviral vector, and injected the virus into a mature oocyte. The egg was then fertilized by intra-cytoplasmic sperm injection, cultured to the blastocyst stage, and then implanted into the uterus of a foster mother. Five pregnancies resulted from 20 embryo transfers. Two pregnancies miscarried, a set of fraternal twins, at 73 days. The miscarriage is likely due to the twin pregnancy itself, which is rare and high-risk in rhesus monkeys (Chan et al., 2001). Three live males were born, one of which named ANDi (the initials for inserted DNA spelled backwards) was shown to be transgenic.

Southern blots and PCR were used to assay for the presence of the GFP transgene in various genomic DNA samples, RT-PCR was used to assay for transcription of the transgene, and GFP fluorescence was used to assay for the presence of the GFP protein. Several tissues from the two miscarried twins were shown to carry the GFP gene (including placenta and testes), and their hair follicles and toenails glowed green under fluorescent light indicating transgene expression (Chan et al., 2001). Of the three living monkeys, only one (ANDi) was shown to carry the transgene, which was present in in all of his tissues analyzed. But ANDi did not appear to express the transgene (his tissues did not glow under fluorescent light).

The researchers encountered several obstacles in their primate *in vitro* fertilization (IVF) procedures. For example, the monkey eggs proved difficult to collect, and primatologists do not know how to artificially control a monkey's reproductive cycle. And in addition to IVF problems, the expense and ethical considerations may limit the use of transgenic monkeys as medical models (Vogel, 2001). ANDi's birth raised several ethical and humanitarian problems. Will this lead to the same transgenic editing of the human genome? Will we try to replace a disease-causing gene with a healthy one to create a "genetically enhanced" baby? Those ideas initially appeared far in the future when ANDi was created, because viruses tend to insert the transgene in random places, which would seriously hinder doing human therapy experiments. But with the later advent of technologies for more precisely editing the genome, human experiments came back to the ethical forefront (Vogel, 2001).

Primate Human Disease Model

Huntington's disease (HD) is an autosomal dominant neurodegenerative disorder characterized by motor impairment, cognitive deterioration, and psychiatric disturbances (Davies and Ramsden, 2001). It is caused by the expansion of cytosine-adenine-guanine (CAG) tri-nucleotide repeats (which encode the amino acid glutamine) in the first exon of the human Huntington (HTT) gene (MacDonald et al., 1993; Yang et al., 2008). The mutant HTT protein with its expanded poly-glutamine domains is expressed in the brain and other tissues.

Transgenic rodent HTT models have been developed, but apparently they do not develop the full neuro-degeneration and behavioral changes associated with the disease, so scientists developed a non-human primate model for HD (Yang et al., 2008). 130 mature rhesus monkey oocytes were injected with lentiviruses encoding human HTT exon-1 containing 84 CAG repeats. The genetically modified oocytes were then fertilized by intra-cytoplasmic sperm

injection. 30 embryos were transferred to 8 surrogate mothers, resulting in 6 pregnancies, and 5 live births. All five live monkeys (rHD-1, rHD-2, rHD-3, rHD-4 and rHD-5) were shown by PCR and Southern blots to contain the transgene, and the HTT transgenes were of different lengths depending on how many CAG repeats were retained after integration. Monkey rHD-1 carried 29 CAGs (normal range), and the other 4 carried CAG repeats ranging from 27 to 88. Monkey rHD-3 died after one month, and rHD-4 and rHD-5 survived for less than a day and showed signs of HD motor impairments. rHD-2 displayed mild involuntary motor movement at week-1. Monkey rHD-1 having CAG repeats in the normal range appeared phenotypically normal (Yang et al., 2008). The early deaths of rHD-3, rHD-4, and rHD-5 seem to be related to the levels of mutant HTT. Different regions of the brain showed high expression levels of HTT, and the frequency and onset of involuntary movements seemed to depend on the length of the CAG repeats and the number of transgene integration sites (Yang et al., 2008).

These monkeys begin to provide good models for investigating Huntington's disease. They provide the opportunity to correlate a wide range of neurodegenerative and cognitive phenotypes with HTT expression patterns that appear to be identical or close to that observed with human HD patients (Yang et al., 2008). This experiment provided a "proof of principal" that monkey models for human diseases can be produced.

Improving the Efficiency of Primate Transgenesis

One of the main obstacles of creating transgenic primates is the low birth rates caused by assisted reproductive techniques. So, some scientists have attempted to improve the efficiency of the process. Sun et al. (2008) in Dr. Joseph Z. Tsien's lab at the Yunnan Banna Primate Model Research Center in Shanghai have determined that the use of intra-cytoplasmic sperm injection results in a higher egg fertilization rate (50%) than *in vitro* fertilization (33.3%). They

also determined that the highest rate of live births (64%) was achieved when the embryo transfer to the uterus was performed in the presence of new corpus luteum between 24-36 hours after the host's estradiol peak. Extensive *in vitro* culture of the zygotes into expanded blastocysts appeared to reduce the viability of the embryos. And freezing and thawing of cultured cleavage stage embryos further reduced embryo quality, so those steps likely should be avoided (Sun et al., 2008).

Germ line Transmission in Marmoset Transgenics

The Marmoset (*Callithrix jacchus*) has a relatively high reproduction rate for a primate, making it potentially suitable for transgenic modification, so some scientists have experimented with this species in biomedical research (Sasaki et al., 2009). This group of scientists at the Central Institute for Experimental Animals in Japan was the first to report non-human primate transgene expression in somatic tissues and germ-line transmission of the transgene with the full, normal development of the embryo. To introduce a gene encoding enhanced green fluorescent protein (EGFP) into the marmoset embryo, three kinds of self-inactivating lentiviral vectors were constructed, each carrying a different promoter (CAG, CMV, or EF1α) (to control transgene expression in different tissues). All lentiviral injections were performed at the earliest embryonic stage possible. After lentiviral injection, 4 of the IVF embryos and 12 of the naturally fertilized embryos were immediately transferred to recipient females.

Five transgenic marmosets were obtained, four of which expressed the transgene in several somatic tissues, such as hair root, skin fibroblasts, and peripheral blood cells. The remaining animal expressed the transgene only in the placenta. Two of the animals reached sexual maturity and showed the transgene insertion and expression in germ cells which would allow them to pass the transgene to their offspring (Sasaki et al., 2009). The results of this study

indicate that transgenic marmosets can be used as experimental animals for biomedical research, and may provide a better model than Rhesus monkeys.

Monkey Transgenesis Using SIV

Some scientists have tested viruses other than HIV for delivering the transgene to primates. Niu et al. (2010) at the Kunming Primate Research Center in China used simian immune-deficiency virus (SIV) to deliver a transgene encoding EGFP to rhesus monkeys. They injected the rSIV into early cleavage-stage embryos, at the 1-2 cell stage prior to genome activation, or at the 4-8 cell stage following genome activation. Neither the injection, nor the SIV infection, nor the time of activation appeared to alter the development of the embryo to the blastocyst stage. 30 embryos that showed EGFP fluorescence (transgene expression) were transferred to surrogate mothers, resulting in the birth of 4 infant monkeys from four singleton pregnancies. 2 of the 4 live monkeys expressed the transgene throughout the entire body, demonstrating the usefulness of using SIV-based vectors in primate transgenesis (Niu et al., 2010).

Precision Primate Gene Editing

As discussed above, viral vectors (HIV and SIV) have proved relatively efficient methods for creating transgenic monkeys. But viral vectors often integrate at *random* positions in the host genome, which can create problems in the host. This problem was apparently solved this year by applying the new CRISPR/Cas9 precise genome editing tool to transgenic monkeys (Niu et al., 2014). As discussed in a previous Lit Review section, the "clustered regularly interspaced short palindromic repeat" (CRISPR) system uses a guide RNA complementary to the gene targeted for excision, coupled with a gene encoding Cas-9 nuclease which cuts the targeted gene in half, to

cut out the targeted gene. Following gene excision, cellular DNA ligases then re-join the ends of the cleaved DNAs together. The precision of the system results from the use of the guide RNA which is designed to only hybridize to the target gene through base-paring (Jinek et al., 2012).

Niu et al. (2014) at the Yunnan Key Laboratory of Primate Biomedical Research in Kunming, China, co-injected Cas-9 mRNA and guide RNAs for two target genes (PPAR-γ and Rag-1) into one-cell stage Macaque embryos. The RNAs were transcribed *in vitro* using T7 RNA polymerase. PPAR-γ encodes a key protein in fat cell production. RAG-1 is central to the development of B and T cells, so a monkey with a defective RAG-1 gene could provide a valuable primate model for immunodeficiency disease. The team first determined whether the CRISPR system could alter the primate genome in embryos (with no implantation). 22 one-cell embryos were injected with the Cas-9 and guide RNAs. 15 embryos appeared to develop normally to the morula and blastocyst stages. Double gene disruptions (the desired outcome) occurred in 6 out of 15 embryos.

To create transgenic monkeys using the CRISPR technology, 198 oocytes were collected and fertilized by intra-cytoplasmic sperm injection (ICSI). The Cas9 and guide RNAs were then injected into one-cell embryos. 83 out of 186 injected embryos were transferred to 29 surrogate mothers, resulting in 10 pregnancies. Of the 10 pregnancies, 1 miscarried, 3 appear to be twins, 3 appear to be triplets, and 4 appear to be singlets. So far, a set of twin live babies have been delivered, and the others remain in gestation. Three tissues (placenta, umbilical cord, and ear punch tissues) of the two live born monkeys were collected for analysis. CRISPR-induced modifications (the desired outcome) were detected in both Rag-1 and PPAR-γ genes in the tissues from both of the live monkeys, proving the CRISPR approach worked. Importantly, the DNA from the placentas were screened for potential off-target mutations at 84 sites. This is a potentially serious problem associated with the CRISPR technology, so the authors wisely chose

to assay for it. Although such off-target mutations are frequently found in CRISPR treated mice, no off-target mutations were observed in the 84 sites tested in this study. This finding provides strong optimism for using the CRISPR system for creating transgenic monkeys (Pennisi, 2014).

Overall, our Lit Review of the various transgenic primates showed a general flow of experiments from creating the very first transgenic monkey in 2001, to developing the first transgenic primate disease model in 2008, to improving the efficiency of the primate transgenic techniques, to applying the highly specific CRISPR technology to transgenic primates in 2014. Because monkeys have similar complex cognitive and social abilities as humans, these transgenic primates may provide models for studying complex disorders such as autism, schizophrenia, and Alzheimer's. However, monkeys are not as cheap, and they take a longer time to reach sexual maturity produce the next generation, so if used these models will come with a high price (Shen, 2013).

Transgenic Monkey Applications and Ethics (Connor Darling)

The previous sections of the Lit Review discussed the basics of gene editing technology (CRISPRs and TALENs), different vectors that are used to insert foreign genes into an animal's genome (plasmid and viruses), and the different ways for making animals transgenic by manipulating embryonic stem cells or manipulating one-cell embryos. The purpose of this section of the Lit review is to discuss the potential applications of transgenic monkey models for studying genetic and neurological diseases, and some of the key ethical issues of using nonhuman primates as models.

Transgenic Monkey Applications

Scientists have long desired to create transgenic monkeys to help advance neurological research. Genetic disorders such as Alzheimer's, autism, and schizophrenia cannot be fully replicated in lab mice which lack the complex cognitive and social abilities of primates. This important difference has caused some drugs to test positive in mice but later fail in human trials. Now that scientists are able to perform gene insertions and edits in monkeys, some researchers believe these models will offer a more robust testing ground for new drugs, gene therapy and modified stem cells.

For example, MIT Neurologist Guoping Feng wishes to disrupt a gene called SHANK3 which has been implicated in some human genetic cases of autism. Autistic-like social behavior has been observed in mice engineered to contain Shank2 mutations (Won et al., 2012) that mimic some human cases (Berkel et al., 2010), but researchers argue similarly engineered primates would be a better match for human symptoms. In another example, mice and primates have been engineered to mimic Huntington's disease (HD). Huntington's disease is a neurodegenerative disorder associated with motor and cognitive impairment. HD is caused by the expansion of cytosine-adenine-guanine (CAG) trinucleotide repeats in the gene encoding human huntingtin (HTT) protein (MacDonald et al., 1993). CAG encodes the amino acid glutamine, so HD is sometimes referred to as a polyglutamine disease. The non-functional HTT protein strongly impacts the striatal neurons that affect motor function. When searching for an HD treatment, Emerich et al. (1997) investigated whether treatment with ciliary neurotropic factor (CNTF) can help prevent the degradation of striatal neurons in a non-transgenic HD monkey model. Quinolinic acid was injected into the striatum of cynomolgus monkeys to induce degeneration, and this was followed by treatment with fibroblast cells engineered to secrete CNTF. Their data

showed that the CNTF had a neuro-protective effect on several striatal cell populations and prevented the atrophy of neurons in the motor cortex.

Shang-Hsun Yang et al. (2008) extended this work to generate a transgenic non-human primate HD model. They injected 130 mature rhesus oocytes with lentiviruses expressing exon-1 of the human huntingtin (HTT) gene or lentiviruses expressing a marker green fluorescent protein (GFP). Then, 30 of the embryos that formed were implanted into eight different surrogates, and 6 pregnancies occurred within those 8 monkeys. Of the 6 pregnancies, five live births were completed to term, including two sets of twins. Each of the five live birth monkeys expressed both mutant HTT and GFP. 3 of the 5 transgenic monkeys showed higher levels of mutant HTT expression and died only a few months after birth. These monkeys were also found to have a larger number of CAG repeats, so a high number of repeats may correlate with a high level of mutant HTT expression and early death. One of the monkeys, designated rHD-1, although recombinant had the same low number of CAG repeats as a non-HD monkey, and showed no symptoms. The HD monkeys that survived showed some symptoms that are common in HD patients such as chorea (abnormal involuntary movement disorder) and dystonia (sustained muscle contractions cause twisting and abnormal postures). Thus, these HD transgenic monkeys could provide an opportunity for a large range of behavioral and cognitive tests that are similar (or nearly similar) to those done in humans. The two surviving HD monkeys will be used as founders to create subsequent generations of transgenic monkeys. Their sperm will be taken and cryopreserved. In the future, other non-human primate models may be created to mimic other important neurodegenerative diseases to help understand how these diseases develop, to aid early detection, and to develop methods for treating them.

Transgenic Monkey Ethics

Although the use of transgenic monkeys for research would allow the creation of more accurate models of neurological disorders than current rodent models, their use has setbacks. When considering animal welfare in general, the "moral status" describes what an animal (or human) is owed in its own right instead of its instrumental values for others. The moral status assessment is not an all-or-nothing assessment, but carefully weighs the benefit to society (i.e. the amount of medical advancement) against the detriment to the animal (i.e. potential suffering, chance of escape into the environment, etc.). How the general public reacts to using specific animals for tests depends on how high the animal occurs in a "socio-zoological scale" which rates how high animals are valued by humans (Arluke, 2010). At the top of the scale are companion animals such as cats, dogs, and horses, and nonhuman primates (Arluke, 2010). Due to the complex brains that monkeys have, and their very close >90% genetic ties to humans, there is more resistance by activists and the general community to prevent or discourage their use in research compared to mouse models.

Some individuals argue that the best way to proceed is to ban the use of monkeys in all research, and then perform highly targeted human clinical trials. In this case, a small number of human patients would first be used to test drug safety, and if safe this would be followed by another small trial for drug effectiveness, with none of the above using monkey models. But for some types of experiments, the risk to humans is too great for any type of clinical trial, so models must be created first to help minimize the risk to humans. If testing is to be performed on monkeys, we must gauge whether the testing is ethically justifiable by weighing the scientific benefits (different for each case) against the harm to the animals (also different for each case) (Coors et al., 2010; Olsson and Sandøe, 2010). What are the goals of the research? What are the expected effects on the animals? How will animal suffering be measured and by whom? Are

there any alternatives to this type of research? Why won't mouse models work here? Are monkey models needed prior to any human clinical trials? One relevant case as an example pertains to the potential use of genome-edited monkeys to test the effects of knocking out human lineage-specific (HLS) DNA sequences that monkeys share with humans. Such experiments cannot be done in humans because we do not know the function of those sequences, or the effects of knocking them out, until we actually do it. So, some individuals argue doing these tests in monkeys. But for precisely this same reason (we don't know the effects) some argue we should not do that type of experiment in monkeys either (Coors et al., 2010).

Some individuals argue that developing efficient technologies for editing the monkey genome will get us one step closer to *editing the human genome*, so the former should not be done. Some envision a future of genetically altered "designer" children. The ethics and morals surrounding human genome editing would be much stricter than animal genome editing because it could generate humans that would pass any accidentally induced mutations to their children. When such mutations occur in animal models, the animals can be euthanized if the mutations are harmful, but this would not be an option if an editing mistake is made in humans.

Overall, scientists in favor of using transgenic monkeys seek to use them mostly for modeling complex neurodegenerative diseases where primate behavior more closely mimics human behavior. The models would be used to test potential treatments before proceeding into human clinical trials. The models could also be used to obtain a more detailed understanding of how complex neurological disorders affect the brain than can be obtained with mouse models. But it is important to weigh the potential benefits to society (the medical advances) for each experiment versus the potential suffering of the animal, and to have protocols in place for monitoring the animal's health. And such models should only be used if no other alternatives are available. If a mouse model would suffice for a particular study, or if there is a feasible way to

perform a limited closely monitored human clinical trial, then the primate model should not be used in that case.

METHODS

To accomplish objective-1, we performed an extensive review of the current research literature, including reputable academic journal articles, relevant books, scholarly websites, and other pertinent materials. **To accomplish objective-2**, we conducted a set of semi-structured, in-depth interviews with various academic researchers who had performed primate genome editing or who had significantly contributed to the development of genome editing technology, and with bioethicists with experience with transgenic animals, to determine their range of opinions on the strengths and weaknesses of this new technology and whether other techniques that do not involve genome editing could accomplish the same goal of making disease models for testing.

Who: The stakeholders were mostly academic experts on primate genome editing, genome editing in general, or transgenic animals in general. The interviewees also included some academic bioethicists to help us discern the main ethical issues associated with primate genome editing. Some of the stakeholders were initially identified by referral from the project advisor, Dave Adams, but other subjects were identified from the literature as corresponding authors on key scientific papers, or sometimes by referral from the initial interviewee.

Where and When: Once contact had been made with a potential interviewee (see below), if he or she was close to WPI, a time and place was set up for the interview to be performed at the interviewee's workplace. Whenever possible, interviews were conducted in person, but most were conducted by email, phone, or Skype.

How: Initially, our interviewees were contacted by email and/or phone. If no response was received within a few days, we used follow-up emails and/or phone calls. We developed an initial set of interview questions based on our background Literature research (shown in the

Appendix). Based on his/her responses to our initial questions, we sometimes tailored more specific subsequent questions to best obtain information from that person. The Appendix also covers the range of topics needed to fully cover our project, and the methods used to ensure interviewee confidentiality if necessary. At the start of the interview, we informed the interviewee about the purpose of our project, and asked for permission to quote them (see **Interview Preamble** in the Appendix). We explained how we would protect their confidentiality if necessary, by giving them the right to review any quotations used in the final published report, explaining that the interview is voluntary, and explaining that they may stop the interview at any time or refuse to answer any question. After the interview, we asked each interviewee for permission to follow-up with them at a later date, if needed, to fill in any gaps in the information. And sometimes, as mentioned above, we asked the interviewee to recommend other potential stakeholders we might interview. With respect to the total number of interviews needed for our project, we no longer continued interviewing additional subjects after we obtained sufficient information to represent all sides of the problem, and when all unclear points had been clarified.

To accomplish objectives-3 and 4, the group synthesized the information obtained from the Literature Review, interviews, and follow-up interviews, to ascertain the strength of the evidence in favor or against editing primate genomes, and then created recommendations for future research.

RESULTS / FINDINGS

Results: General Topics on Genome Editing (Breahna Mattie)

The Literature Review for this section identified some potential problems associated with monkey genome editing, including the potential to create off-target mutations in the animal's genome that could be deleterious to the animal, and problems associated with poor efficiency. These problems were pursued further in a series of interviews with scientists who had used the CRISPR or TALEN genome editing technologies themselves or who had created transgenic monkeys. This new information was then used to help clarify the problems and to design future experiments. Five scientists responded to my interview requests.

Dr. Prashant Mali of the Department of Genetics, Harvard Medical School, Boston, MA, was first author on a 2013 review article published in *Nature Methods*, entitled "Cas9 as a Versatile Tool for Engineering Biology" (Mali et al., 2013). When asked the general question of whether he felt that off-site mutations might be a problem when using the CRISPR technology for therapies, he stated "Off-site mutations are indeed a potential problem, and where feasible, whole genome sequencing is an ideal assay to pick the highest fidelity clones [those cells containing no undesirable mutations]. For *in vivo* gene therapy it may not be feasible to do so [select for non-mutated cell clones], so one will have to ultimately engineer an improved high precision genome targeting system or methodology (like the new off-set nicking strategy discussed later in the Results). So, Dr. Mali clearly believes that off-target mutations might be a problem when using the new genome editing technology, the mutations should be assayed for using whole genome sequencing, and new modifications to the technology should be developed to decrease off-target effects.

The next interview was with **Dr. Jennifer A. Doudna**, a scientist in the Department of Molecular and Cell Biology, University of California Berkeley. Dr. Doudna was corresponding author on a 2013 paper published in *eLife*, e00471, entitled "RNA-programmed genome editing in human cells". They determined that using shorter guide RNAs and extending their 3' ends appears to enhance DNA targeting. When asked how using shorter guide RNA's produces fewer off-site mutations she stated, "The shorter guide RNAs are less efficient overall [they cut a fewer percentage of the DNA molecules], so they produce fewer off-target cleavages for this reason. She did not directly comment on how extending the guide RNA at its 3' end enhanced DNA targeting, although this makes sense for a more specific hybridization reaction.

The third interview was performed with **Dr. Rodolphe Barrangou** of the Department of Food, Bioprocessing, and Nutrition Sciences, North Carolina State University, Raleigh, North Carolina 27695. Dr. Barrangou was sole author on a recent 2014 paper published in *Science*, entitled "Cas9 Targeting and the CRISPR Revolution" (Barrangou, 2014). When asked whether he thought that off-target mutations might be a problem when using the CRISPR system for gene editing, he said "Off-target cleavage issues have been overblown. Provided you design your guide RNA well, and are mindful of potential homologies to non-targeted sequences, you should be fine." So, obviously Dr. Barrangou does not think off-target mutations are a problem when using the new CRISPR technology.

The fourth interview was with **Dr. George M. Church**, a Professor in the Department of Genetics, Harvard Medical School (Boston). Dr. Church has been an author on numerous papers using the CRISPR technology, and in particular was senior author on a 2013 article published in *Science*, entitled "RNA-Guided Human Genome Engineering via Cas9" (Mali et al., 2013). Dr. Church was asked a similar question about whether he thought that off-target mutations might be

a problem when using the CRISPR technology, and he stated "The off-target mutation rate [exists but] is low (less than 5% chance that a clonal edited cell population will have even a single off-target hit). Several methods published since our 2013 *Science* paper have reduced that rate further by factors of 1000-fold each. Using these new methods together could give multiplicative improvement (million or billion fold), but tests of such combinations have not been published yet. Also, since cancer is the main concern, most off-target sites [which would cause cancer] are very low impact. Double null mutants [knocking out the function of both copies of a gene] in tumor suppressor genes [which could cause cancer] constitute a tiny fraction of the genome. Sites can be computationally and empirically chosen to have high specificity and low impact on such genes. This is in contrast to the early days of retroviral insertion gene therapy where viral vector transcriptional promoters could activate oncogenes at some distance and only a single hit was required". Thus, Dr. Church agrees that off-target mutations can be induced with the CRISPR technology, but the chances of actual oncogene activation is extremely low, and new modifications to the procedure have already greatly reduced the chances of off-target effects. So, some of the interview responses indicate that mutations are not as big an issue as some articles make them out to be.

The last interview was with **Dr. Kevin Esvelt**, a Technology Development Fellow at the Wyss Institute for Biologically Inspired Engineering, Harvard University. Dr. Esvelt was middle author on the same Mali et al. (2013) article discussed above. He was asked to comment on his thoughts on the CRISPR technology issue and replied, "Great question! It depends on the application you're interested in. For gene therapy, off-site mutations may hit an oncogene or a tumor suppressor and lead to cancer. If you need to modify a lot of cells in a lot of patients, it's inevitable that an off-site mutation will occur eventually. That's the case even if you employ all of the methods developed in the past year to increase specificity, such as using pairs of nickases

instead of nucleases (which turns off-target cuts into far less mutagenic nicks) and truncating the guide RNA spacer sequences from 20 base pairs to 17 (so there are fewer close matches in the genome)". So, he thinks that off-site mutations could theoretically be a problem in therapies.

He went on to explain an interesting point that it is impossible to test for all the off-target sites when using therapies on a million patients. He stated that, "Suppose you plan to edit a billion cells (which is not that many relative to how many cells we have in our bodies) in each of a million patients. That's 10^{15} potential mutational events [10^9 x 10^6]. It's impossible to test whether a given set of conditions will result in an off-target nick when you have that many events, which in turn means you can't engineer or evolve Cas9 to ensure it becomes that specific. The only way to conceivably avoid this is to use a recombinase enzyme that holds on tightly to all the DNA ends when editing and reliably stitches them back together. But we can't target recombinases to new sequences anywhere near as well as we can Cas9, so using [recombinases] just isn't feasible for most applications. He concluded by saying that "if the patient is suffering from a serious illness with few alternative options, then the risk of cancer stemming from gene therapy using CRISPR is almost certainly lower than the risk of doing nothing." So, although it might not be possible to completely eliminate all off-target mutations due to the high number of potential cell targets, the likelihood of cancer formation remains low relative to the help that could be provided to the patient by the gene editing.

He also reminded us that "not all therapeutic applications require lots of cells to be edited in each patient. For example, you might edit only one cell, expand that cell in culture (possibly growing them into an organ if we can do that), and only then [if no off-target mutations are found] infuse them into a patient. Right now we're not great at expanding cells in culture (among other things it's super-expensive), but for some applications (such as for HIV therapy)

editing a few stem cells might be enough." So, expanding a small number of correctly treated cells might be a feasible approach to avoid off-target effects.

When making transgenic animals, he stated that "off-target mutations that occur when making a transgenic organism could cause unexpected side-effects that may interfere with the experiment. But since making transgenics requires many fewer cellular editing events (because you only need to edit the [embryo] cells that will contribute to the new organism) the risk is fairly low. If the (many) ethical issues are ever worked out, this means that even humans might be edited using CRISPR with reasonable safety. That said, this will always be problematic for people who believe life begins at conception and consequently object to IVF [manipulations], because the main safety net here would involve making several embryos, letting them divide a few times, removing one cell, sequencing its whole genome, and then implanting the one that doesn't have any off-target mutations (or indeed other potentially problematic mutations that may have occurred spontaneously or been inherited from one or both parents). But this method could be applied to making transgenic monkeys with fewer ethical issues".

As for assaying for off-target mutations, Dr. Esvelt stated "yes, whole-genome sequencing is typically the most reliable assay we have. Other options include making a library of all closely related sequences in the target genome and testing whether CRISPR will cut them *in vitro*. You could also deliver that library into, say, mouse cells and use sequencing to test whether CRISPR will cut them there. But since neither of those options measures cutting in the relevant context (i.e. in the nucleus of cells of the target species), they're not as reliable as whole-genome sequencing. So, indeed there are several different assays in addition to whole genome sequencing that could be done to test for off-target mutations.

Overall, the interview responses above all indicate that genome editing using CRISPR technology is less harmful than portrayed in some journals. Each respondent agreed that off-

target mutations exist with the new CRISPR technology, but they can be assayed using whole genome sequencing or by making a library of human sequences and testing them for cutting. Several interviewees pointed out several very new CRISPR modifications (such as designing the guide RNA carefully to not cross-hybridize with key sequences in the genome, using an off-set nicking strategy, using two guide RNAs, or altering the length of guide RNAs) that have already shown strong decreases in off-target effects. One respondent indicated that even if off-target mutations are induced, the chances of that actually hitting an oncogene to cause cancer is relatively low compared to the potential benefit to the patient of knocking out the problematic gene. One interviewee thought it would be impossible to ever design a technique to completely rule out off-target effects due to the very large number of target cells and target sequences, especially if millions of patients are treated, but the likelihood of a harmful event still remains relatively low compared to the potential benefit. When making transgenic animals such as transgenic primates, one interviewee reminded us that only a very small number of cells need be tested for off-target effects, such as the genome-edited embryo prior to implantation. So, by assaying the embryo prior to implantation, the risk can be made to be very low when making transgenics, and the screening process can be repeated until no off-target effects are observed in the embryo to be implanted.

Results: Genome Editing Technology (Dylan Pinnette)

The literature review for this part of the project focused on the techniques for editing genomes. Genetic engineering techniques capable of editing the genome (with insertions, knock-outs, or edits) include plasmid insertion, viral integration, homologous recombination, TALENs, and CRISPRs. The TALEN and CRISPR techniques are the most recently developed, and appear

to be far more efficient than the other three traditional editing methods. Both have recently been used to create transgenic primates, and thus were the focus of the interviews conducted. Both TALEN and CRISPR techniques have shown rising popularity because of their efficiency and ease of use, but have risks for creating off-site mutations in edited genomes. It was unclear from the literature review which technique likely creates the least off-target mutations, and what can be done to minimize their effects.

To help determine some of the potential risks of off-site mutations and to gauge how to minimize their effects, several scientists were interviewed who had direct experience with TALEN and CRISPR techniques. The first interviews were with **Dr. Ignacio Agenon** (a scientist at INSERM UMR 643, Nantes, France) and **Gregory Cost** (a scientist at Sangamo Biosciences, Richmond, CA). Drs. Agenon and Cost were authors on a 2011 paper published in *Nature Biotechnology*, entitled "Knockout rats generated by embryo microinjection of TALENs" (Tesson et al., 2011). When asked whether TALENs and CRISPRs would cause fewer off-site mutations, Dr. Agenon replied that there was not enough research done with non-biased methods to make a clear comparison of the two techniques, but "TALENs recognize longer guide sequences than CRISPRs, which favors less off-site effects". Dr. Cost replied that it would be "difficult to measure" a direct comparison between the two methods, and it depends on the specific chosen target site, but "CRISPRs are probably worse with respect to off-target cleavage. However, CRISPRs are so easy to use that most people will use them anyway." So, these two scientists hypothesize the potential superiority of TALENs, but acknowledge there is not enough direct evidence to claim this with certainty.

The third interview was with **Dr. Yuet Kan**, a scientist in the Department of Medicine, University of California, San Francisco, California 94143. Dr. Kan was the corresponding author on a very recent 2014 paper published in *Genome Research* entitled, "Seamless gene

correction of β-thalassemia mutations in patient-specific iPSCs using CRISPR/Cas9 and piggyBac" (Xie et al., 2014). Their data indicated that when correcting the beta-thalassemia mutation in human iPSCs prepared from patients, no off-target mutations were observed, and the stem cells retained full potency. When Dr. Kan was questioned on why his use of the CRIPR/Cas9 method showed no off-site mutations while others had, he explained that they had only checked for off-site mutations in locations where there were genetic similarities to the target site, the locations most likely to have shown such mutations, and that checking the entire genome for off-site mutations would require deep genomic sequencing which he did not do. To help accomplish a focused hybridization, he explained that they were "careful in choosing a gRNA that stayed away from the delta-globin gene sequence, unlike one published paper on targeting the beta-globin gene". Dr. Kan was also asked what the next steps for human treatment were, and replied that when human applications were performed "we would whole genome sequence a number of individually derived cell clones and use those clones that show the least known deleterious changes". So, Dr. Kan's testimony supported the earlier claims that off-target effects can be avoided, in his case by carefully designing the guide RNA to not cross-hybridize with other known related sequences.

The fourth interview was with **Dr. Young Sung**, a scientist in the Department of Biochemistry, College of Life Science and Biotechnology, Yonsei University, Seoul, Republic of Korea. Dr. Sung was one of two corresponding authors on a 2013 paper published in *Nature Biotechnology*, entitled "Knockout mice created by TALEN-mediated gene targeting" (Sung et al., 2013). When asked how he compares the off-target mutation rate between TALEN versus CRISPR protocols, he responded that they could not find "mutations in the predicted off-target loci when we produced mice with TALENs….however, other reports have shown off-target effects". He went on to state that the off-target problem was still under debate. Dr. Sung said

that to know the answer would require "whole genome sequencing data that is not available [to our lab] at present". He added that the risk of off-target mutations is not always important. For example, in mice, the off-target mutations likely "will disappear when we successively cross these mutant mice to wild-type mice to produce F1 pups or to safely maintain the mutant mouse lines, unless they exist on the very chromosome containing the on-target mutation." So, Dr. Sung's answers were in line with the previous responses that off-target effects might be a problem, but likely not long-term if the transgenic animals are bred over many generations. And they assayed those sites most likely to be affected (those most closely mimicking the guide RNA sequence) to show those sites remained unaffected.

The fifth interview was with **Dr. Thomas Auer**, a scientist at the Institut Curie, Centre de Recherche, Paris F-75248, France. Dr. Auer was first author on a recent 2014 paper published in *Methods* in the April 1 issue, entitled "CRISPR/Cas9 and TALEN-mediated knock-in approaches in zebra fish" (Auer and Del Bene, 2014). After learning from the previous interviewees about the importance of fully sequencing the genome, if possible, to search for off-target mutations, Dr. Auer was asked whether he had performed this technique on his edited zebra fish genomes, and responded that while he had not, other labs had done so. In comparing TALEN and CRISPR methods, Dr. Auer claimed that the shorter binding site of CRISPR/Cas9 led to more off-target cleavages, and the "required dimerization of the FokI nuclease subunits reduces the amount of off-target events in the case of TALENs." So, Dr. Auer predicts that the TALEN method likely will show fewer off-target problems than the CRISPR method.

The sixth interview was with **Dr. Bing Yang**, a scientist in the Department of Genetics, Development, and Cell Biology, Crop Bioengineering Consortium, Iowa State University (Ames, Iowa). Dr. Yang was second author on a recent 2014 review article published in the *Biochemical Journal*, entitled "TALEN-mediated genome editing: Prospects and perspectives" (Wright et al.,

2014). When asked to compare TALENs and CRISPRs for off-target effects, Dr. Bing stated that "TALENs provide greater specificity due to the longer sequence of the target sites," while "the CRISPR specificity is largely defined by about 16 base pairs of guide RNA and are presumably less specific." Dr. David Wright, the first author on the same study, also responded, saying that most "TALEN targets in the literature tend to be 16 to 20 bases long, although they can be longer, and most CRISPR/CAS9 targets tend to be 20 bases". So, presumably using longer target sequences would provide fewer off-target effects. Dr. Wright extrapolated on the properties of TALENs saying, "There is some evidence to suggest that the 3' end of a TALEN target is not very important because once a TALEN begins to bind a site it becomes less discriminatory as binding progresses. So, shorter TALEN targets may be just as effective as longer ones". Dr. Wright further extrapolated the importance of the target site in determining off site mutations saying "what makes a good target is uniqueness in the genome and a good mix of bases in the target, but no one know really knows what makes a good target beyond these basics. Two targets that may not look very similar may actually bind a particular nuclease with similar affinity, and two targets that look similar may differ substantially in binding affinity". Dr. Wright was somewhat contradictory to the previous interviews as he leaned towards the CRISPR method being safer due to lower toxicity, and opened a the line of thought that the *execution* of the method is more important than *which* method is used. It is also not clear that using longer guide RNAs will always provide more specific targeting, and it more likely depends on the exact gene being targeted and how similar it is to others in the genome.

Overall, the interviewees consistently pointed out that direct comparisons of the CRISPR and TALEN methodologies is lacking in the literature, and that off-target mutations in theory can be a problem with both techniques. Complete full genome sequencing information to make the direct comparisons is not readily available, so we recommend doing those types of studies.

When assaying for off-target effects, those labs that did not perform whole genome sequencing usually screened the genomic locations most likely to be affected (i.e. those locations predicted by bioinformatics that loosely match the guide RNA sequence). One interviewee noted that the shorter binding sites of CRISPR/Cas9 guide RNAs are one reason that technique might induce more off-target mutations than TALENs which use longer guide RNAs. And the requirement for two dimerized FokI nuclease subunits in the TALEN method likely reduces the number of off-target events for that technique. But one interviewee cited higher toxicity when using TALEN methods, and applauded the ease of use and efficiency of the Cas9 method. Some interviewees pointed out that instead of focusing on *which* editing technique is better, it is far more important to carefully design the guide RNAs to hybridize to very unique locations in the genome that will not cross-hybridize to off-target sites.

When creating transgenic animals, one interviewee made the excellent point that it is relatively easy to test the small number of cells that would be implanted in the uterus for potential off-target mutations in advance before doing the implantation (i.e. testing the embryonic stem cells prior to implantation into the blastocyst). So perhaps off-target effects will not be a major problem when creating transgenic animals, especially when the animals are created using embryonic stem cell lines that can be screened in advance.

Results: Technology for Creating Transgenic Animals (Thomas Cormier)

The Literature Review created for this section focused on the technology used to create transgenic animals which pertains to the procedures used after the transgene (the DNA to be inserted) has been edited. This is an important topic as it relates to the type of animal created (chimeric versus pure transgenic), and it relates to the applicability towards different species (including monkeys, the topic of this IQP). Our Literature Review identified two main

procedures for creating transgenic animals: 1) embryonic stem cell (ESC) manipulation, and 2) egg (or one-cell embryo) manipulation. In the former technique, genetically modified ESCs are injected into the blastocoel cavity of a blastocyst prepared by *in vitro* fertilization (IVF), the embryo is implanted into the uterus of a foster mother, and the pregnancy is continued to hopefully produce transgenic pups. In the latter technique, modified DNA is microinjected into the pro-nucleus or cytoplasm of a newly fertilized IVF embryo, or a genetically modified sperm is injected into the egg (intra-cytoplasmic sperm injection), the embryo is grown to the blastocyst stage, and then the embryo is implanted into the uterus as in the former technique.

Our review of the literature identified several potential *pros* and *cons* of each technique. The technique using genetically manipulated ESCs produces *chimeric* animals, a situation where some tissues are transgenic while other tissues are not. This occurs because some of the ESCs in the embryo represent the original cells of the inner cell mass (ICM) which are not transgenic, and these cells mature into non-transgenic tissues in the offspring alongside the genetically manipulated cells. In some cases, the literature indicated these chimeric animals were in-bred further to create more fully transgenic tissues. In other cases, the experiment remained valid as long as a specific key tissue tested positive as being transgenic. The ESC procedure had the potential advantage of allowing the genetically modified ESCs to be screened for the presence of the transgene prior to injection, which increased the chances of successfully making the animal transgenic. But the procedure requires the culture and expansion of ESCs which adds complexity that some labs might not be familiar with, so this potential problem was analyzed further in interviews.

The egg manipulation procedure had the advantage of making pure transgenics when it worked, but the disadvantage that it was not very efficient. The success rates for making transgenics were as low as 0.1% which required substantial screening to identify the transgenic

pups from among the larger numbers of normal pups. But in spite of this potential disadvantage, egg manipulation appeared to be the most popular of the methods, and might be the most reliable overall. So this question was also investigated in the interviews.

To investigate these issues further, interviews were performed with several researchers that had direct experience creating transgenic animals. The interviews focused on verifying whether our initial finds from the Literature Review were correct, and if so, why.

The first interview was performed with **Dr. Xingxu Huang**, a scientist at the Key Laboratory of Model Animal for Disease Study, Model Animal Research Center of Nanjing University, National Resource Center for Mutant Mice, Nanjing 210061, China. Dr. Huang was senior corresponding author on a 2014 paper published in the *International Journal of Biochemistry and Cell Biology*, entitled "One-Step Generation of Different Immunodeficient Mice with Multiple Gene Modifications by CRISPR/Cas9 Mediated Genome Engineering" (Zhou et al., 2014). His lab created several strains of transgenic mice by microinjecting genetically modified DNA (Cas9 DNA encoding and several guide RNAs directed against immune genes to aid their knockout) directly into one-cell embryos ("egg" manipulation). This point was verified by Dr. Huang who said, "We achieved the gene targeting by microinjection of Cas9 and sgRNA into one-cell embryos, instead of ESCs into blastocysts." From the interview we learned they injected the DNA into *both* the egg cytoplasm and pronucleus, and they prefer this technique because it is more convenient than manipulating ESCs and it can be applied to any species even if their ESCs cannot yet be grown.

The second interview was performed with **Dr. Lianfeng Zhang**, a scientist at the Key Laboratory of Human Disease Comparative Medicine, Ministry of Health, Institute of Laboratory Animal Science, Chinese Academy of Medical Sciences, Beijing 10021, China. Dr. Zhang was senior corresponding author on a 2014 paper published in *Cell Research*, entitled

"Generating Rats with Conditional Alleles Using CRISPR/Cas9" (Ma et al., 2014). During the interview, Dr. Zhang clarified that they microinjected their modified DNA into one-cell fertilized eggs (pronucleus and cytoplasm simultaneously), which can potentially increase the efficiency of the genetic modification. He also stated that his lab does not like the use of embryonic stem cells to create transgenic animals especially when creating knockouts because "It takes about 12 months to produce a floxed rat or a knockout rat, while the CRISPR/cas9 takes only 2-3 months." He stated that the new CRISPR system for creating knockouts is so efficient, his lab has created 50 knockout lines in only 2 years.

The next interview was performed with **Dr. Ling Li**, a scientist at the Atherosclerosis Research Unit, Division of Gerontology and Geriatric Medicine, Department of Medicine, University of Alabama at Birmingham, 1530 3rd Avenue South, BDB 658, Birmingham, Alabama 35294. Dr. Li is senior corresponding author on a 2006 paper published in *Neurobiology of Aging*, entitled "Lack of LDL Receptor Aggravates Learning Deficits and Amyloid Deposits in Alzheimer's Transgenic Mice" (Cao et al., 2006). From the interview we learned that Dr. Li doesn't always create the transgenic animals in his own lab but mostly breeds previously existing transgenic mice created in other labs with other strains of mice to create the specific models he needs for his work. But he agrees that "most labs prefer to use the pronuclear microinjection method when introducing new genes into mice (knock-ins), while many labs use ESCs when creating knockout mice". So, this interview indicates that the method used for creating the transgenic animal may depend on the *type* of genetic manipulation (gene knock-in or knock-out) required for the model.

The next interview was performed with **Dr. Wenning Qin**, Associate Director, Genetic Engineering Technologies, The Jackson Laboratory, 600 Main Street, Bar Harbor, ME 04609. The Jackson Laboratory has prepared hundreds of strains of transgenic mice, so is very

experienced with the technology. From the interview we learned that Dr. Qin uses both the new gene editing technologies (CRISPR, TALEN, ZFN) and the more traditional gene targeting technology [homologous recombination] in embryonic stem cells. He argues that the new gene editing technologies are faster, cheaper, and more flexible than the more traditional methods of genetic manipulation. When using the new gene editing technologies, he prefers to edit ESC DNA instead of pronuclei especially when doing "large-scale manipulations", although he argues "this may change as we learn more about the new nuclease-based gene editing technologies and what they can accomplish". Dr. Qin also stated that when editing DNA using the new CRISPR or TALEN methods, he worries about potential off-target mutations, but they minimize this by carefully designing their guide RNAs (that specifically hybridize to the target gene to be edited). And most importantly, they perform "whole genome sequencing to understand the scope of the [off-site] issue". He provided the qualifier that his primary goal is "to create the transgenic models, while the researchers are the ones who will determine whether any off-target mutations exist, and if so are they a problem".

The next interview was performed with **Dr. William E. Van Nostrand**, a professor in the Department of Neurosurgery, Health Sciences Center, T-12/086, Stony Brook University, Stony Brook, NY 11794. Dr. Van Nostrand was senior corresponding author on a 2004 article published in the *Journal of Biological Chemistry* that described the creation of transgenic mice expressing the Swedish or Dutch/Iowa mutant form of amyloid-β to make several types of Alzheimer's mouse models (Davis et al., 2004). In the interview, Dr. Van Nostrand said that his lab "uses the pronuclear injection method for making all his various transgenic Alzheimer's mouse strains", although he said that he "relied on collaborators for these methods". He said has no experience with the ESC method for making transgenic animals, so could not directly compare the two methods.

The final interview was performed with **Dr. Yongxiang Zhao**, a scientist at the Biological Targeting Diagnosis and Therapy Research Center, Guangxi Medical University, Nanning, Guangxi, China. Dr. Zhao was a corresponding author on a 2013 article published in *Nature Biotechnology*, entitled "Heritable Gene Targeting in the Mouse and Rat Using a CRISPR-Cas System" (Li et al., 2013). Dr. Zhao verified that he used the pronuclear microinjection protocol for making the mice described in their 2013 paper, and said that he *always* uses that technique. He also said he has never worked with embryonic stem cells to make transgenic animals.

Overall, the interviews performed for this portion of the project validated the preliminary findings of the Literature Review, that the pronuclear microinjection method for making transgenic animals is the most popular method. All 6 interviewees indicated they had performed the pronuclear injection method in their own labs. They indicated this method is simpler to accomplish than manipulating and growing ESCs, and it can be applied to *any* species even if ESCs cannot yet be grown for that species. Several interviewees also indicated they no longer microinject specifically into the pronucleus but into the cytoplasm (which is easier to hit and the DNA also enters the pronucleus). For some scientists, the choice of method depended on the *type* of genetic manipulation, with gene knock-outs easier to accomplish with ESC manipulation (homologous recombination or CRISPR), and gene knock-ins easier to perform with pronuclear microinjection. The scale of the manipulation also mattered, with one interviewee saying he prefers using ESCs for large scale experiments.

With respect to methods for DNA manipulation (although this is also the subject of another section of the report), one scientist indicated the new CRISPR system takes only 2-3 months to work, while the older technology of preparing a floxed gene for a knockout takes about a year. So, scientists are excited about the newer methods of genetic manipulations.

However, they agreed with our assessments from the Literature Review that off-site mutations might be a problem with the new CRISPR and TALEN methods, so in a best practice scheme some scientists are carefully designing their guide RNAs (that dictate which gene is targeted for deletion) to more precisely hybridize only to the target gene, and are performing whole genome sequencing to identify any off-site mutations that may occur.

Results: Editing the Monkey Genome (Zhidong He and Chenwei Zhang)

The Literature Review performed for this part of the project focused on the experiments conducted to date with transgenic non-human primates. The review covered key experiments from the creation of the world's first transgenic monkey in 2001, to the 2014 application of the highly specific CRISPR genome editing technology to transgenic primates. The long-term hope is that transgenic monkeys will provide models for studying complex disorders such as autism, schizophrenia, and Alzheimer's. However, monkeys are not easy to make transgenic, nor are their embryos easy to manipulate. And the CRISPR technology recently used to edit their genomes comes with potential problems, such as off-target mutations, which could hinder the use of the primates as models. In addition, low birth rates caused by assisted reproductive techniques also prevents primate models from being widely used in biomedical research. Some scientists believe that marmosets might be better suited than rhesus monkeys for cloning techniques.

To shed more light on the risks associated with the new CRISPR technology and the problems encountered when making transgenic monkeys, a series of interviews was performed with key scientists in the field who had direct experience with genome-edited monkeys. The first interview was performed with **Dr. Xingxu Huang**, a scientist at the MOE Key Laboratory of

Model Animal for Disease Study, Model Animal Research Center of Nanjing University, National Resource Center for Mutant Mice, Nanjing 210061, China. Dr. Huang was a corresponding author on a 2014 article published in *Cell* entitled "Generation of Gene-Modified Cynomolgus Monkey via Cas9/RNA-Mediated Gene Targeting in One-Cell Embryos" (Niu et al., 2014). His lab first applied the CRISPR/Cas9 system to target monkey genomes by editing two target genes simultaneously (PPAR-gamma and Rag-1). When questioned about whether off-site mutations were a problem, he verified that their lab indeed checked for off-site mutations induced by the CRISPR system, and found none. "We did check the off-target effects, and fortunately, we didn't find any detectable off-target mutations." He said that their data indicates that off-target mutations when they do occur, do so at specific locations, not randomly, so this would help tremendously if we move forward into other monkey models or with human therapies, especially if they know those sites are harmless. "Based on our experiences, the off-target effects are site-dependent, and more approaches will be developed to overcome this obstacle". When questioned about the future of transgenic primates, he thinks they will be used in health (medical) studies. "Because of the similarity between humans and non-human primates, in my opinion, the transgenic primates will be more important [than rodents] for human health studies."

The second interview was performed with **Dr. Erika Sasaki** of the Central Institute for Experimental Animals, 1430 Nogawa, Miyamae-ku, Kawasaki, Kanagawa 216-0001, Japan. Dr. Sasaki was first author on a 2009 paper published in *Nature*, entitled "Generation of transgenic non-human primates with germline transmission" (Sasaki et al., 2009). Her lab created the world's first monkeys that *expressed* their transgene and it was also inherited to offspring. When questioned about the key reason for their success, she thinks it was because they developed a new efficient protocol for inserting the transgene that included lentiviral injection and sucrose.

The new method received a patent in several countries, including the U.S., Japan, Australia, and Singapore. Second, they pre-selected GFP-expressing embryos (that contained lentivirus) prior to embryo transfer into surrogate mothers to select in advance for embryos expressing at least the GFP portion of the transgene. All their infants became GFP-transgenic marmosets, and 4 out of 5 animals showed germline transmission. Third, they believe that the marmoset monkey is more suitable in general for producing transgenic animals than rhesus monkeys (like ANDi) because of their high rate of proliferation compared to rhesus monkeys. With respect to where she believes transgenic monkey experiments are headed in the future, she indicated they are trying to establish marmoset neuronal disease models to understand neuronal cell functions. "We are also trying to produce diabetic marmoset models."

The third interview was performed with **Dr. Weizhi Ji** from the Kunming Primate Research Center, Chinese Academy of Sciences, Kunming 650223, China. Dr. Ji was a senior author on a 2010 article published in the *Proceedings of the National Academy of Sciences, USA,* entitled "Transgenic Rhesus Monkeys Produced by Gene Transfer into Early-Cleavage-Stage Embryos Using a Simian Immunodeficiency Virus-Based Vector" (Niu et al., 2010). When questioned about whether he had any problems delivering the transgene using viral vectors, Dr. Ji responded that he saw no genetic problems when doing so. When questioned about his observations of off-site mutations using the new CRISPR system, he also indicated that when he uses the CRISPR gene editing technology, he sees no off-target mutants. He also brought up an interesting point that the extent of off-target mutations observed may depend on the gene being targeted. So, perhaps some target genes are very unique and their guide RNA does not recognize any off-target sites for that particular target. He agreed that we need more data on off-target mutations for a variety of target genes to know whether they truly will be a problem. It was not

clear whether he had used the best practice technology of whole genome sequencing to look for off-target mutations, so this is our recommendation.

The fourth interview was performed with **Dr. Wei Li** of the State Key Laboratory of Reproductive Biology, Institute of Zoology, Chinese Academy of Sciences, Beijing, China. Dr. Li was a corresponding author on a 2013 paper published in *Nature Biotechnology*, entitled "Simultaneous generation and germline transmission of multiple gene mutations in rat using CRISPR-Cas systems". Their lab showed that the CRISPR system in rats not only can be used to create multiple gene alterations simultaneously, but the edited genes can also be passed to offspring through germline transmission. When asked whether they have tested germline transmission of CRISPR-induced edits in any other species besides rat, he said they have also verified it for mice. Although they have also prepared CRISPR genome-modified pigs and monkeys, due to the long maturation times in those species, they have not yet verified transmissibility. However, they believe the gene modifications likely will be inherited because the modifications occurred in both alleles (both copies of the target gene), so it should be inherited. Inheritance of the edited gene in monkeys would make the models far more useful and easy to disseminate to other labs, so this was a key finding.

The fifth interview was performed with **Dr. Shang-Hsun Yang**, an Associate Professor in the Department of Physiology, College of Medicine, National Cheng Kung University, 1 University Rd, Tainan 70101, Taiwan. Dr. Yang was the first author on a 2008 article published in *Nature*, entitled "Towards a Transgenic Model of Huntington's Disease in a Non-Human Primate" (Yang et al., 2008). When asked the advantage of making transgenic primates, she said that non-human primates are relatively close to human beings, especially with respect to "brain anatomy, brain function, neuro-pathological progression, and behavioral phenotypes, all of which are important to investigate". She also said that although she used viruses to deliver the

transgene in the original 2008 paper, she now has recent experience with both TALEN and CRISPR gene editing systems, and pointed us towards two of her published papers (Liu et al., 2014; Niu et al., 2014). The latter paper was discussed earlier in this section of the report. In the former paper, the authors used TALEN-mediated mutagenesis to alter the methyl-CpG binding protein 2 (MECP2) in both rhesus and cynomolgus monkeys. Microinjection of the targeting TALEN plasmids into rhesus and cynomolgus zygotes resulted in an effective gene editing of the target gene with no detected off-target mutagenesis. So, the TALEN system appears to also be suitable for editing the monkey genome.

Overall, the interviews performed for this section of the report show that most of the interviewees worry that off-target mutations might be a problem when using the new genome editing technologies, so they assayed for those mutations. Some scientists saw no off-target mutations, but it was not clear whether they had used the best practice method of whole genome sequencing, so we generally recommend that best practice be followed. One scientist indicated the off-target mutations might occur only at specific locations. If those sites are harmless this would help the new technology gain acceptance, so this should be investigated further. Another scientist indicated the extent of off-target mutations might depend on which gene is being targeted. This may depend on how unique the target sequence is in the genome, and whether the guide RNA will find off-target sites that resemble the target, so this point should also be investigated further. Our findings indicate that both the CRISPR and TALEN technologies have been used to edit the monkey genome, but it is too early to know which is best or produces the least off-target effects.

Another best-practice technology appears to be the pre-selection of transgenic-positive embryos prior to implantation, such as combining the transgene with a second marker gene such as GFP and then selecting for the marker. One scientist argues that marmosets are better suited

than rhesus monkeys for making genome edited models, so different monkey species should be explored. With respect to the future of transgenic primates, it is important that the genome editing be passed onto the offspring, otherwise the technique is far less useful. Scientists have proven transmissibility with rats and are working to verify it for monkeys. Two interviewees best summarized how we might use transgenic monkeys in the future, saying that non-human primates are relatively close to human beings (compared to rodents), especially with respect to "brain anatomy, brain function, neuro-pathological progression, and behavioral phenotypes, all of which are important to investigate" (Yang interview), and they are already preparing neuronal disease models and diabetic models (Sasaki interview).

Results: Transgenic Monkey Applications and Ethics (Connor Darling)

The Literature Review for this section focused on potential applications of transgenic monkeys and the ethical concerns resulting from their close genetic link to humans (>90%) and their similarly complex brains. Although transgenic rodent models exist for diseases such as Parkinson's and Huntington's, scientists would benefit from models that more precisely mimic the human condition as therapies would more accurately be tested. The review discussed our need for transgenic monkeys in research, especially for complex diseases such as Huntington's and Alzheimer's which lack current effective treatments, where we are forced to make the hard decision to test therapies on monkey models instead of on humans. However, in some cases small highly focused human clinical trials might suffice, and would prevent needless testing on monkeys.

To investigate these issues further, interviews were performed with several researchers who either had created transgenic animals or who had commented on their ethics. The first

interview was conducted with **Dr. Linda Macdonald Glenn (JD, LLM)**, a Bioethics lawyer at the Alden March Bioethics Institute, Albany Medical Center, 47 New Scotland Avenue, MC 153, Albany, NY 12208. Dr. Linda Glenn was the author of a 2013 article published in *ActionBioscience* entitled "Ethical Issues in Genetic Engineering and Transgenics" (Glenn, 2013). When asked about her views on the ethics of creating transgenic monkeys, she commented that we must view using transgenic animals in a "larger context; that is the moral status of humans and our relationship to all creatures with which we share the planet." She also proposed a question as to "what moral obligation would we have to the new animals as their makers"? She discussed several hypothetical cases in which the line between a simple test subject and a fully sentient being is blurred. One scenario included genetically altering a chimpanzee so it could communicate with the same proficiency as a five year old. In that scenario we would have to perform the evaluation almost as if the chimpanzee were a child (Glenn, 2003). She also discussed how scenarios such as these will force courts and scientists to think outside current laws to rework our definition of a "sentient being" to decide what will apply to them.

The second interview was conducted with a scientist (who wishes to remain anonymous) at a well-known U.S university who used TALENs in his research. When asked about his research and whether he had seen any off-site mutations while using TALENs, he replied that "We have sequenced numerous genomes by now and revealed off-targeting to be below the natural mutation rates". This indicates he does not think those mutations will be a problem when doing editing to human genomes any more than normal mutation rates would. So, he feels that off-site mutations are less of a worry than previously thought.

The third interview was conducted with **Dr. Scott W. Lowe**, a scientist at the Memorial Sloan-Kettering Cancer Center, New York, NY 10065. Dr. Lowe was the corresponding author

on a 2012 paper published in *Cell*, 148: 1099-1109, entitled "Life in the Fast Lane: Mammalian Disease Models in the Genomics Era" (Dow and Lowe, 2012). When asked to comment on where he thinks that primate models are headed, he responded "…there has been an explosion in the development of genome editing technology, particularly based on the CRISPR/Cas9 system involved in the innate bacterial immune system. This system makes producing specific genetic changes quite easy, avoiding the production of targeting vectors for homologous recombination, and can be done directly by injection of plasmids into the embryo. Such a system can be readily adapted to embryos from any species, including primates." This shows that Dr. Lowe thinks that with the development of the CRISPR system, the formation of more primate models will be more feasible. He went on to warn us about some potential of the new editing technologies, and the possible mutations that may occur. "…these new systems are so efficient they can produce unintended [off-site] mutations, so more research is needed to minimize this." So, this disagrees with the earlier interview that off-site mutations are not be problem and occur at less than natural mutation rates. Dr. Lowe also expressed his concern that there are ethical issues that need to be addressed as the use of primates as models expands, and he mentioned the financial limitations of monkey models. "The use of primates in research is extraordinarily expensive, and federal funding for research is at an all-time low." Although the cost could eventually decrease as more models are developed, the funding of controversial research will only be possible if there is a greater push for it within either the scientific community or within the government.

A fourth interview was conducted with **Dr. Jiing-Kuan Yee**, a Professor in the Department of Virology, Beckman Research Institute of City of Hope, Duarte, CA. Dr. Yee was the corresponding author on a recent 2014 paper published in *PLoS One*, April 1 issue entitled, "Precise gene modification mediated by TALEN and single stranded oligodeoxynucleotides in human cells" (Wang et al., 2014). This article described how they had devised a new system that

used both TALENs and single-stranded oligodeoxynucleotides (ssODNs) to maximize the cutting efficiency of TALENs. Their data showed that deletions of up to 7.8 kilobases could be made in human cells at high efficiencies. When asked if he had assayed for off-target mutations using the new ssODN system, Dr. Yee indicated that had indeed assayed for off-target mutations for some of their TALEN pairs, and found no off-target cutting. They also stated that they had submitted the manuscript for publication, and it is currently under consideration by a journal. That could mean that their new ssODN technique could someday be used to maximize TALEN cutting efficiency while producing no off-target problems.

The final interview was conducted with **Dr. Julien Valton**, a Project Leader in the R&D Department, Cellectis SA, 8 rue de la croix Jarry, 75013 Paris, France. Dr. Valton is first author on a recent 2014 paper published in the journal *Methods*, July 15 issue, entitled "Efficient strategies for TALEN-mediated genome editing in mammalian cell lines" (Valton et al., 2014). The authors of the paper concluded that screening strategies for off-target effects need to be implemented based upon what the specific application of genome editing will be. When asked about the specific case of editing a specific gene during human therapy, and whether in this case sequencing the whole genome was the best assay, he indicated that he would use the PROGNOS software program to help select the final guide RNA sequence as it would help identify potential off-target sequences, and if present he would analyze just those sites by PCR. In those cases where *multiple* genes are being edited, he would also analyze for potential chromosomal translocation events plus the specific genes identified by PROGNOS. He also had this to say about deep sequencing multiple genomes, "the drawback of deep-seq is that you just "shine light" onto known off-sites without looking at them genome-wide. Genome sequencing has a VERY poor sensitivity, so you would have to sequence at least 1000 genome-depths to be able to detect a potential offsite that occur at 10E-3 frequency. If you do the math, it could be very

expensive to do so." So, he believes that for the most part, deep-sequencing is too expensive to perform routinely, especially when you need to sequence 1000 genome depths to detect potential offsite mutations occurring at a low frequency. So, maybe if money is not a problem deep-seq could be done, but using PCR on potential off-target sites is money better spent.

Overall, the interviews conducted for this section showed that the interviewees think off-site mutations do not occur often, and even when present likely occur the same as natural mutation rates. When screening for potential off-target effects, one scientist mentioned using software such as PROGNOS to identify potential off-target sites based on the target RNA sequence, and identifying just those sites by PCR, which is far more cost effective than doing deep sequencing. Some interviewees mentioned new techniques that are being developed to minimize off-target problems by increasing the cutting efficiency in conjunction with using ssODNs. One interviewee mentioned the financial problems facing transgenic research, both due to the expensive nature of the research and the general lack of funding right now from the federal government. In regards to the ethical concerns about creating transgenic animals, one ethicist posed the question about what role we humans will eventually have as the creators of new transgenic species, and what our moral obligation will be to these creatures. The interviewee suggests in her writing that the way we treat these new creations through our laws should be a reflection of our most noble aspirations (Glenn, 2003).

CONCLUSIONS / RECOMMENDATIONS

Based on the research performed for this project, our group is able to make several conclusions and recommendations. The **single most important concern** identified in this project with respect to making transgenic animals with the new CRISPR and TALEN methods was the potential for **off-target mutations**. These off-target effects could, in theory, inactivate a required gene in the animal's genome, activate an oncogene, or create a chromosomal inversion. Each scientist interviewed for this project agreed the CRISPR and TALEN techniques could, in theory, create off-target effects, but they disagreed considerably on whether such effects are truly a problem.

Some scientists observed no off-target mutations in their experiments, but it was not clear whether they had used the best practice method of whole genome sequencing to identify the mutations. Some interviewees argued that even when off-target effects do occur, the likelihood of hitting an oncogene or a functional gene in the genome is relatively low, so that most off-target mutations will have no observable effects on the animal. On the other hand, other scientists argued that when editing very large numbers of cells for example in a human patient's body, it would be likely that one cell out of the billions being treated could become cancerous. This argument becomes moot when making transgenic animals, because 1) there are relatively few cells edited in the embryo used for implantation (so relatively few chances for mutations), and 2) some cells in the embryo could be pre-screened prior to implantation into the uterus to ensure no off-target effects. So, by screening the embryo prior to implantation, the process could be repeated until no off-target effects are observed. However, this cannot be done when treating large numbers of cells in human patients.

With respect to directly **comparing the CRISPR and TALEN techniques**, several scientists pointed out that studies directly comparing the off-target rates for CRISPRs and TALENs is lacking, so perhaps such direct comparisons should be done. However, because new technique modifications are constantly being developed to lower the rates, perhaps it is too soon for such direct comparisons. Some of the interviewees thought that because TALENs use longer guide domains and require two of them (to dimerize the two FokI nuclease domains) the rates for the TALEN method are likely lower than with CRISPRs. But another scientist cited a higher cellular toxicity when using TALEN methods, and applauded the ease of use and efficiency of the Cas9 method. So, instead of focusing on *which* editing technique is better, perhaps it is far more important to carefully design the guide RNAs and use new modifications for lowering the rates than to choose one technique over the other.

With respect to **new techniques for lowering the rates of off-target effects**, several new technique modifications have already been developed in the past year that appear to significantly lower the rates. These include the use of new software for ensuring the guide RNA sequence is unique in the genome, the use of two guide RNAs instead of one, altering the length of the guide RNA, or using an off-set nicking strategy. As the CRISPR and TALEN techniques continue to be used by more labs, we predict that even more modifications will be developed to increase specificity and further lower off-target effects.

With respect to the **best practice screening technologies** identified in this project for assaying for off-target effects, the best practice appears to be whole genome sequencing. However, for this to be effective the genome needs to be sequenced to about a 1000-fold depth (which is expensive) to distinguish the rare off-target effects from the normal cellular DNA mutation rates. When whole genome sequencing is not affordable, the best practice appears to be using PCR to screen for mutations at specific locations most likely to be affected, i.e. those

sites identified by bioinformatics as locations that could cross-hybridize with the guide RNA (for CRISPRs) or the TALN DNA-binding amino acids (for TALENs) to provide a more focused screening approach.

With respect to making transgenic animals in general, the interviews validated the preliminary findings of the Literature Review that the pronuclear microinjection method is the most popular method. Most of the interviewees who had directly created transgenic animals indicated they had performed the pronuclear (or single cell) injection method in their own labs, and indicated it is simpler to accomplish than manipulating and growing embryonic stem cells, and it can be applied to *any* species even if ESCs cannot yet be grown for that species. Some interviewees indicated they no longer microinject specifically into the pronucleus but into the cytoplasm, which is easier to hit and the DNA also enters the pronucleus. And the scale of the manipulation mattered, with one interviewee saying he prefers using ESCs for large-scale experiments.

When making transgenic monkeys, one scientist argued that marmosets are better suited than rhesus monkeys for making genome edited models, so different monkey species should be explored. With respect to the future of transgenic primates, it is important that the genome editing be passed onto the offspring, otherwise the technique is far less useful, and would die out with the founder animal. Two interviewees best summarized how we might use transgenic monkeys in the future, saying that non-human primates are relatively close to human beings (compared to rodents), especially with respect to "brain anatomy, brain function, neuro-pathological progression, and behavioral phenotypes, all of which are important to investigate" (Yang interview), and they are already preparing neuronal disease models and diabetic models (Sasaki interview). With respect to the ethical concerns about creating transgenic monkeys, one ethicist posed the question about what role we humans will eventually have as the creators of

these new transgenic species, and what our moral obligation will be to these creatures. She suggested that the way we treat these new creations through our laws should be a reflection of our most noble aspirations (Glenn, 2003).

We conclude that with proper genome monitoring (either by whole genome sequencing or by PCR of specific high probability locations), careful guide RNA design (to avoid locations that could cross-hybridize), using new techniques to lower off-target mutations rates, and screening embryos prior to implantation, the off-target effects are not likely to be a serious problem.

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APPENDIX

LIST OF INTRODUCTORY INTERVIEW QUESTIONS

Example Questions for Researchers Who Have Achieved Primate Genome Editing:

1. How strong do you think your lab's evidence is that you have achieved primate genome editing?
2. What were some of the major problems you overcame to accomplish this feat?
3. What applications do you envision for such animals?
4. Do you think that transgenic or genome edited primate models will be more accurate than rodent models? Does genome editing create mutations elsewhere in the genome (off-target editing)?
5. Do you think that there are other technologies for testing vaccines or for studying human diseases that are as good as primates?
6. What experiments do you think need to be done prior to using transgenic or genome edited primates as disease models?
7. Do you think that the funding for primate genome editing will increase or decrease?

Example Questions for Academic Bio-Ethicists:

1 Are you familiar with this year's discovery of the ability to efficiently edit the primate genome using the new CRISPR technology? If not, we will explain this briefly to them.
2 What do you think are the main ethical concerns with editing primate genomes?
3 Do you think researchers should try to find alternatives for this?
4 Do you think that more research should be performed to more fully understand genome editing (including understanding the probability of off-target mutations) prior to using such animals as models for testing vaccines?

Example Questions for General Genome Editing Researchers:

1. Which technique do you currently use in your lab to edit genomes, and why?

2. How strong do you think the evidence is that primate models more accurately test vaccines than other animal models?
3. What do you think were the key technical improvements that led to the success?
4. What were the main problems that prevented earlier success?

INTERVIEW PREAMBLE

We are a group of students from the Worcester Polytechnic Institute in Massachusetts, and for our research project we are conducting a series of interviews to investigate technical and ethical problems associated with using the new CRISPR and TALEN technologies for editing the monkey genome.

Your participation in this interview is completely voluntary, and you may withdraw at any time. During this interview, we would like to record our conversation for later analysis. We will also be taking notes during the interview on key points. Is this okay with you?

Can we also have your permission to quote any comments or perspectives expressed during the interview? This information will be used for research purposes only, and we will give you an opportunity to review any materials we use prior to the completion of our final report, which will be published on-line in WPI's archive of projects.

If the subject does not agree to be quoted, we will respond as follows: "Since you would not like to be quoted during this interview, we will make sure your responses are anonymous. No names or identifying information will appear in any of the project reports or publications."

Your participation and assistance is greatly appreciated, and we thank you for taking the time to meet with us. If you are interested, we would be happy to provide you with a copy of our results at the conclusion of our study.