ABSTRACT

KANAVY, DONA MICHELLE. Genetic Pest Management Technologies to Control Invasive Rodents (Under the direction of David Threadgill and John Godwin).

Many strategies exist to manage invasive pests on islands, ranging from poison to trapping, with varying degrees of success. Genetic technologies are increasingly being applied to insect pests, but not so far to vertebrates. We are developing a genetic strategy to eradicate invasive mouse populations as another tool for pest control.

*Mus musculus*, the common house mouse, is one of the most widespread invasive species. Mice threaten human health, agriculture, and biodiversity on many islands, particularly of seabirds. Rodenticides are the most common method of eradicating mice, but their use leads to poisoning of non-target species and has limited efficacy against mice. An approach that could eliminate impacts on non-target species would be daughterless mice with super-Mendelian inheritance for self-sustained propagation. For this project, we have investigated exploiting a naturally occurring meiotic distorer, the t-haplotype. Using the $t^{\mu2}$ haplotype, we have observed a transmission distortion ratio of approximately 95%.

The daughterless phenotype is being generated by inserting the *Sry* (male sex-determining) gene into an autosome containing the $t^{\mu2}$ haplotype via CRISPR/Cas9 gene editing. The presence of *Sry* will induce testis formation, regardless of sex chromosomes. When in the t-haplotype, the male sex determining gene should spread through the population, eliminating female offspring. This model system will support studies to evaluate the effectiveness of eradicating an invasive population without adversely affecting other species. This is a novel idea, and though the t-haplotype is only present in mice, once this method has been perfected it will advance genetic pest management in mammals using a super-Mendelian inheritance strategy for the eradication of other invasive mammal species.
Genetic Pest Management Technologies to Control Invasive Rodents

by
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BIOGRAPHY

Dona Kanavy was born on April 22, 1987 in New Brighton, PA. Though she started life as a Yankee, she moved down south to North Carolina in 2001 and quickly adapted to life as a southerner, drinking all the sweet tea she could. While Dona’s first career choice was to become a cheerleader, she quickly realized the error of her ways and decided in first grade she was going to grow up and look at things under a microscope.

This love of science matured throughout Dona’s undergraduate years at the University of North Carolina at Chapel Hill. While there as a brief moment of insanity while she contemplated attending medical school, she settled on a degree in Clinical Laboratory Science after she realized you could have a job working in a laboratory. This was an amazing program, but the reality of being a technician was not challenging enough. During a microbiology rotation at Duke Hospital, Dona was inspired by the Medical Microbiology Fellow she met in the lab. This experience, coupled with the trend towards genetic testing in hospitals, lead Dona to pursue a PhD in genetics at North Carolina State University with the ultimate goal of becoming a director of a clinical genetics laboratory.

Partway through graduate school, Dona’s graduate advisor Dr. David Threadgill moved to Texas A&M and Dona followed him to the wild west, where disappointingly they did not have sweet tea. She did discover Buc-ee’s, however, and considered the move worth it. After graduation, Dona plans to move back to North Carolina to continue the journey towards becoming a Clinical Genetics Fellow.
ACKNOWLEDGMENTS

The saying it takes a village to raise a child is very true, though in this case it takes a lab to raise a scientist. The work done during this PhD would not have been possible without the support of friends, family, and lab mates.

I am eternally grateful for all of the love and support I received while on this journey. The Threadgill lab became my family in Texas and helped me to learn and grow as a scientist. My family in North Carolina wanted nothing but the best for me and waited patiently for me to return home. I could not have gotten this far without them.
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CHAPTER 1

INTRODUCTION
ISLAND Biodiversity

Environmental and biodiversity conservation is of utmost importance due to the detrimental effects of human activity on ecosystems. In 2000, the United Nations made environmental sustainability one of its millennial goals, with Target 7a being the reduction of biodiversity loss by 2010 (1). However, the rate of biodiversity loss has not slowed despite increased public awareness. Based on data from the International Union for Conservation of Nature (IUCN) Red List, an average of 52 vertebrate species move one category closer to extinction every year (2).

Islands are especially sensitive to biodiversity and habitat loss. Islands contain a large percentage of the world’s biodiversity, with many species endemic to specific islands due to genetic isolation. Not only do islands typically contain a higher density of species compared to the mainland, they also contain a larger percentage of extinct and endangered species (3). When comparing the surface area of an island versus the mainland, islands have 30 times more extinct species and 14 times more endangered species, primarily due the presence of invasive species and habitat loss. Species on islands are at increased vulnerability due to smaller populations and home ranges, less genetic diversity, and limited adapted defenses as many island species evolve in the absence of predators. With 92% of highly threatened vertebrates on islands breeding solely on islands and not the mainland, and 70% breeding on only one island in the world, conservation interventions targeting these at-risk islands should be prioritized in order to preserve these rare species and delicate ecosystems (4).
CONSERVATION AND INVASIVE SPECIES

The native plants and wildlife found on many of these islands are threatened by invasive species, which are the largest contributor to extinctions of amphibians, reptiles, and mammals. It is estimated that 86% of all extinctions caused by invasive species have occurred on islands (5). Invasive rats and cats are involved in many mammalian and bird extinctions, disease is the cause for many herbivore, bird, and amphibian extinctions, and invasive plants lead to the majority of plant extinctions. Birds and mammals seem to be particularly vulnerable in Pacific islands, while amphibians are most threatened in the Americas (5). Rats, cats, and ungulates such as feral pigs are the most commonly found invasive vertebrates on islands. Human activities play a major role in the spread of invasive species, often unknowingly providing transportation to new islands (4).

The house mouse, *Mus musculus*, is also a widespread alien species present on many islands. Because the presence of other mammalian invasive species can limit their numbers, it can be difficult to detect the presence of *Mus musculus* (6). Eradication of other invasive mammals, such as rats, can lead to an explosion in mouse populations, resulting in little to no measureable benefit to the island ecosystem (7) (7).

Mice often have a negative impact on native plants, insects, and seabirds due to their adaptability to new environments. As opportunistic feeders, the mouse diet varies from leafy vegetation and seeds to invertebrates and vertebrates. They can directly impact birds by preying on young chicks or eggs, or indirectly by consuming food that is part of the bird’s food chain, or by attracting predators (6). There is direct evidence of mice on Gough Island attacking albatross, shearwater, and Atlantic petrel chicks. Even though the birds are about 300 times the size of the mice, the birds have no defense against these attacks (8). Invasive
rodents definitively associated with the extinction of 75 species, 52 of which were bird species, making bird conservation of high importance (9).

Since invasive rodents are the major cause of ecological disruption, their removal can translate into significant rebound of native species populations. Islands that have undergone invasive species removal have seen improvements in the endangered populations (9). For example, invasive black rats on Anacapa Island near California prey on the nests of the Spripps’s murrelet (*Synthliboramphus scrippsi*). After a successful rat eradication, hatching success increased from 30% to 85%, helping to spare the bird from an “endangered” listing. A literature review by Jones et al indicated that of the species that experienced a population rebound after a successful invasive species campaign, 69% were birds, followed by reptiles, mammals and finally invertebrates (9). The eradication of invasive mammals is predicted to prevent 41-75% of vertebrate extinctions from islands (4).

**FARALLON ISLANDS**

The Farallon Islands, located off the coast of San Francisco, are one such example of the negative impacts caused by invasive mice. The Farallon Islands are part of the Farallon National Wildlife Refuge, which is comprised of South Farallon, Middle Farallon, North Farallon, and Noonday Rock Islands. Approximately 25% of the seabirds of California breed on the Farallon Islands, which is home to the world’s largest breeding colonies of Petrels, Brandt’s cormorants, and western gulls (10). About 85% of the breeding population of the Ashy Storm Petrel (*Oceanodroma homochroa*) is located on the Farallon Islands (11).

There are 20 species of storm petrels and little is known about them, as they spend most of their time at sea. They primarily come to land to breed, which is often at night on isolated
islands, and they nest in burrows. The eggs are incubated for 1-2 months, followed by 8-11 weeks of nesting. During this period the petrels are most vulnerable, as the majority of predation occurs while at the nesting grounds (11).

Invasive mice were most likely introduced to the Farallon Islands in the 19\textsuperscript{th} century aboard ships visiting the islands. The mouse population on the Farallon Islands is variable; it is nearly undetectable during the winter followed by a nearly exponential population growth during the spring and summer. Peak densities can reach 500 mice per acre, one of the highest island densities in the world (12). The mice are indirectly responsible for the population decline of the endangered Ashy Storm Petrel, as migrating burrowing owls remain on the islands to prey on the mice. When the mouse population crashes during the winter months, the owls resort to preying on the young and nesting Petrels as a primary food source (10). Eradication of invasive mice from the Farallon Islands is expected to result in a significant recovery of the endangered Ashy Storm Petrel population.

**PEST MANAGEMENT**

**Non-genetic methodologies**

Following concerted efforts from government and private organizations, rodents have been eradicated from 577 islands with a 80% success rate (13). The eradications have been focused primarily on small, uninhabited islands. Current eradication techniques use second-generation anticoagulants to poison rodents, with brodifacoum, a vitamin K antagonist, being the most widely used. However, about 5% of rat eradications and 19% of mouse eradications fail using this method (14).
Poison can be distributed through bait stations or by hand or helicopter, depending on the terrain. Bait stations allow for easier monitoring of the amount of rodenticide being consumed and can prevent some non-target species ingestion of the poison. However, bait stations are more practical for smaller, more easily accessed islands (without cliffs), and they require close monitoring. For larger islands, aerial broadcast from a helicopter is preferred due to time and cost savings. However, spreading the poison evenly over the entire island is difficult to accomplish with this method, and it might not reach all of the mouse population. Missing one pregnant female would cause the eradication to fail. Proper timing of this approach is imperative, since the bait is spread over a much shorter time period compared to the bait station approach (14).

Ariel broadcast for inhabited islands requires significant planning to keep pets and livestock, away from the bait for the duration of the eradication campaign. Another consideration would be residual rodenticides present in marine species could end up in the human food supply. However, less than 6% of tested marine invertebrates and fish had detectable levels of brodifacoum when examined after 10 eradication campaigns in New Zealand and the United States from 1997 to 2011 (15), and the amounts present would not pose a great risk to the marine wildlife or humans consuming them. The rodenticide levels also dissipate over a period of a few weeks, and banning fishing for a period of time following the eradication can minimize human risk (15).

While anticoagulants are the most effective tool currently, there are many islands where this technique would not be feasible. One reason is an increase in rodent resistance to the anticoagulants. Several mutations in the vitamin K epoxide reductase (Vkorc1) gene have been associated with increased resistance to warfarin-related anticoagulants by decreasing the
binding of the anticoagulants to the VKOR enzyme (16-18). There is also growing resistance from society concerning the use of rodenticides, with the EPA eliminating the availability of second-generation anticoagulants for personal use (19).

**Genetic methodologies**

Genetically engineering of pest species could be an alternative for eradications from islands. There are several advantages to using these methods over toxicant-based approaches, but there are additional considerations and risks.

Genetic pest management techniques have been used successfully with several insect species beginning in the 1960’s. One of the first insects eradicated was the screwworm fly (*Cochliomyia hominivorax*) from the southern US to Central America using the **sterile insect technique** (SIT). In SIT, male insects are sterilized through radiation exposure. They are then released in large numbers, with a target ratio of at least nine sterile males for every fertile male. The sterile males then mate with wild females but no progeny are produced. This technique is particularly suitable to insect populations in which females only mate once per lifetime as in the screwworm. A consistent release of sterile flies is necessary to drive the population to extinction (20).

SIT has also been successful with other species, including the Mediterranean fruit fly (*Ceratitis capitata*), a crop-damaging pest. SIT is most effective with pest populations that have little-to-no immigrant insects, have low native populations at the time of release, have the ability to be mass reared, and can readily be monitored in the wild. In addition, the radiation exposure itself can reduce the fitness of the insect, making this method untenable in some species (21).
A related but complementary approach known as underdominance was successfully used with the tsetse fly in a small area in Tanzania. In underdominance, the fitness of the offspring is greatly reduced compared to the fitness of the parents, which leads to a reduction in the population as the offspring are not fit to survive (22). One of the first applications of this method was in the mating of two tsetse fly species, *Glossina morsitans* and *Glossina swynnertoni*, in the 1940’s by Vanderplank (23). In this case, an underdominance system was used to produce offspring with low ecological fitness by breeding the two species. The original population, *G. swynnertoni*, mated with the introduced species *G. morsitans*. Because sterile progeny are produced in this mating, a population crash occurred as a result (23). Chromosome translocations (21) and toxin/antidote mechanisms (24) are other examples of underdominance. In translocations, offspring inherit either too few or too many chromosomes from the parent with the translocation due to asymmetric segregation of the chromosomes during meiosis, resulting in death or partial sterility (21). The toxin/antidote technique can also be used to suppress pest populations as the offspring must inherit the antidote to the toxin that is maternally produced (24).

Targeted mutagenesis was the next step in genetic pest management. Partial sterility could be induced through translocation of two chromosomes. Mating of heterozygotes with this translocation results in about half of the offspring having severe chromosome abnormalities, leading to death, while the other half are fertile and continue to pass on the translocation (25). Using specific translocations, it is possible to genetically separate the sexes to be used in a *female lethal technique* (26). In the Mediterranean fruit fly (also known as the Medfly), even sterile female flies cause damage to fruit by stinging. In mosquitoes, females
bite people and transmit disease. Therefore, in both cases, releasing sterile males would be acceptable, but females cannot be released (21).

A translocation in the Medfly results in the location of a gene necessary for pupae color on the Y chromosome, enabling physical sorting of male and female pupae. A further sexing step utilizes a temperature-sensitive lethal allele on the autosomes and the wild type allele for this mutation on the Y chromosome (21). While killing females during early development is more cost effective, it is sometimes necessary to rear them to maintain the population with the mutation. A method used for the Australian sheep blowfly is a field female killing system. A translocation is linked to the Y chromosome so that the females are unfit to survive in the wild, while the males are heterozygous for the mutation and pass it on. The blind females are able to survive in the laboratory setting, but they quickly die once released. Because the males are not blind, they survive and pass on their mutations, consecutively decreasing the overall population with each generation as fewer viable females are available for mating (27).

Currently, a lot of effort is focused on mosquitos to prevent the spread of malaria. Most attempts focus on either eliminating female mosquitos (28), since males do not transmit the disease, or by inserting a malaria resistant gene in the mosquito genome (29). As there were 445,000 deaths from malaria in 2016 (30), successful removal of the malaria vector would save hundreds of thousands of lives each year.

Disease control and population suppression or eradication are the main goals of genetic pest management. By allowing the pest itself to do the work, a genetic eradication strategy is expected to more effectively reach the entire population on an island. As mentioned above, SIT requires a constant release of genetically modified organisms (20). A transgenic approach would also require multiple releases since the transgene would likely be selected against and
lost from the population after several generations. To overcome these limitations, a gene drive system could be self-sustaining and result in disease resistance or population eradication (31).

**Super-Mendelian Inheritance**

Several selfish genetic elements exist, both natural and synthetically derived. They function by homing—copying themselves onto the second chromosome, or by conferring a fitness cost to the wild type allele so that the selfish allele is preferentially passed on (32).

*Transposable elements* are naturally occurring and function by excising themselves from one location in the genome and inserting into another (33). A desired gene could be incorporated into this element and spread through a population. However, this system is not the most predictable or effective because of low rates of mobilization and genetic instability (34).

*Homing endonuclease genes* (HEG) result in greater than the expected inheritance pattern of a locus. The HEG encodes an endonuclease that causes a double-stranded break in the sister chromosome. Through homologous repair, the HEG is then copied onto the other chromosome, resulting in homozygosity for the allele. If a desired gene, such as one that would confer disease resistance (29), could be incorporated into an artificial HEG construct, it too should be copied (31). Homing endonucleases can be naturally occurring, such as the LAGLIDADG family (35), or engineered, as with the Zinc Finger (36), TALEN (37), and CRISPR systems (38).

One method of conveying a fitness cost to the wild type allele is with a *gene drive* linked to the sex chromosomes. “Shredding” either the X (39) or the Y (40) chromosome in sperm cells can select for which sex chromosome is passed on to the progeny. This is done by
targeting endogenous repeat sequences in either the X or Y chromosome with an endonuclease to create several double stranded breaks, rendering the chromosome non-functional. This would skew the sex ratio and lead to a population crash over time (31). Another example of super-Mendelian inheritance is the t-haplotype in mice. The sperm carrying the t-haplotype are preferentially able to fertilize the egg over sperm carrying the wild type version (41).

THE t-HAPLOTYPE

The t-haplotype is a naturally occurring meiotic distorer found on chromosome 17 in some populations of Mus musculus. A series of four inversions covering about 30–40 Mb suppresses recombination, resulting in an accumulation of mutations over time that have mitotically isolated the chromosome. Most of the t-haplotype subgroups contain recessive embryonic lethal genes. A responder (Tcr) and up to three or possibly more distorer (Tcd) genes are present that influence the transmission ratio distortion of the t-haplotype to the next generation. Male mice carrying the t-haplotype pass this variant allele to greater than half of their offspring, with some variants transmitting to over 90% of their offspring (41).
Figure 1-1. Wild type and t-haplotype forms of chromosome 17. Four inversions with the associated genes are depicted. Smok$^Tcr$ is the responder gene and Tagap1$^{Tcd1a}$, Fgd2$^{Tcd2a}$, and Nme3$^{Tcd2b}$ are distorter genes. Adapted from (42).

The t allele impairs sperm motility of the wild type carrying sperm. The responder gene is a mutant form of the sperm motility kinase-1, Smok1$^{Tcr}$ (43). One of the distorters, Tcd1a, has been identified as a T-cell activation Rho GTPase-activating protein (TAGAP1) (44). Tcd2 has been identified as a FYVE, RhoGEF and PH domain-containing protein 2 (Fgd2) gene. Tagap1$^{Tcd1a}$ acts to inhibit SMOK1 while Fgd2$^{Tcd2}$ activates it(45). Both distorter genes target different Rho proteins (which play a role in sperm motility through actin formation), but act together to hyperactivate SMOK1 (46). Another distorter gene, Nme3, was later discovered in inversion 4 and in the same tcd2 region as Fgd2. In contrast to Tagap1$^{Tcd1a}$ and Fgd2$^{Tcd2a}$, which are hypermorphs, Nme3$^{Tcd2b}$ is a hypomorph, though the exact function is not known (42). The distorter genes act additively on all sperm to over phosphorylate SMOK1 targets. The responder gene, Smok1$^{Tcr}$, has reduced kinase activity and is able to nullify the effects of the distorters in the t bearing sperm, thus restoring sperm motility (47).
Figure 1-2. Mode of action of the t-haplotype on sperm motility. The distorter genes, Tcd1-3, act additively to hyperphosphorylate the sperm motility kinase 1, SMOK1, which leads to poor flagella motility. The mutant form of the gene, Smok1Tcr, is able to counteract the effects of the distorters and rescue the sperm’s motility, though only in sperm containing the t-haplotype. This leads to a disproportionate amount of t sperm fertilizing the egg and passing down the t-haplotype at high rates. Adapted from (48).

In the heterozygous state, only sperm carrying the t allele are able to function normally, which leads to an increased fertilization rate by t-haplotype sperm. Males homozygous for the t-haplotype are sterile because the Smok1Tcr gene is unable to overcome the deleterious effects of the distorter genes. Female mice have expected transmission of the allele and normal fertility (49).

While this selfish element would be expected to reach fixation, natural selection works against the t allele to keep population rates between 6-25% in the wild. Some of the reasons behind this are male competitiveness, female mate choice and sperm competition. In a study by Carroll et al. examining the spread of a non-lethal t-haplotype in semi-natural enclosures,
they saw a decrease in the transmission rate of the t from 88% in the laboratory to less than the 50% Mendelian expectation in the natural enclosures. The authors cite the lower fitness of the t/+ males and their inability to hold territory as one of the main factors in the low percentage of the t-haplotype (50). In another study, Lindholm et al. saw evidence of female mate choice, where heterozygous (t/) females were more likely to mate with wild type males (+/+) than heterozygous (t/) males. Wild type females (+/) did not show a mating bias (51). However, a more recent paper by Manser et al. cites sperm competition and polyandry as the major driving forces behind the low frequency of the t-haplotype. Since the meiotic drive functions by impairing the normal sperm within t males, these males have only half the amount of functional sperm as compared to a wild type male. A female mating with multiple males give the wild type males an advantage in fathering offspring (52).

**SEX BIAS**

For pest management, one of the main eradication strategies is to skew the sex ratio of a population to mostly males through a genetically engineered modification, resulting in a population crash. This is especially important with mosquitos and fruit flies, since the males do not have deleterious effects on health or agriculture (26, 28).

A number techniques can be used for this goal, such a female killing gene (20), an X shredder gene (39), or inducing the male pathway in chromosomally female mice (53-55). This rationale is easily extended to mice, as manipulating sex determination is relatively straightforward, and creating a daughterless phenotype would reduce the number of female mice giving birth and lead to a population crash (56).
MAMMALIAN SEX DETERMINATION

The Sry gene, the male sex determining gene located on the Y chromosome, is necessary and sufficient for development of a male phenotype (57). Sry is responsible for testes formation and ovarian suppression. The genital ridge forms in the mouse embryo at day 10 of gestation and contains precursor cells for both testis and ovarian development. If present, the Sry gene is expressed between 10.5-12.5 days post coitum (10.5-12.5 dpc) and initiates a signaling cascade leading to testis formation (Figure 1-3). SRY, the founding member of the SOX (SRY-related HMG box) family of transcription factors, leads to the upregulation of Sox9 in the male precursor (Sertoli) cells. SOX9 remains actively expressed throughout embryonic and postnatal development. SOX9 activates many male-specific genes and leads to testicular development, while suppressing differentiation into ovarian cells. While Sry is only active for a short period of time, the timing is crucial to ensure male development. If delayed, the female pathway will be initiated (58).
Figure 1-3. Sex determination in mice. When present, the *Sry* gene is expressed beginning at 10.5 days post coitum (dpc). *Sry* is only expressed for two days, but SRY activates expression of *Sox9*, thereby initiating the signaling cascades leading to testes development. In the absence of *Sry*, the female pathway is activated. Adapted from (58).

Koopman (59) was able to prove that the *Sry* gene is necessary and sufficient to direct male development by inserting the *Sry* gene onto the autosome of a chromosomally female (XX) mouse (Figure 1-4). The resulting phenotypically male XX mouse is sterile yet anatomically and behaviorally indistinguishable from an XY male. The XY males carrying the autosomal *Sry* gene will pass the gene to its offspring at the expected Mendelian ratio (53).
Figure 1-4. The effect of autosomal Sry on mouse sex determination. A) With the normal inheritance pattern of the sex chromosomes, 50% of offspring will be male (AAXY) and 50% will be female (AAXX), where A is an autosome and X, Y are sex chromosomes. B) If the Sry gene is inserted onto an autosome, 75% of the offspring will be male (AA_{Sry}XY, AAXY, AA_{Sry}XX) and 25% will be female (AAXX). If Sry is inserted into the t-complex, which has over a 95% inheritance rate, then the majority of offspring will be male (AA_{t,Sry}XY or AA_{t,Sry}XX).

GENETIC DAUGHTERLESS APPROACH

By combining the male determining gene with a gene drive, the sex ratio of a population can be skewed and lead to extinction over several generations. This approach would be isolated to Mus musculus, and would not impact off target or vulnerable species as a rodenticide eradication method would. A genetic method could also be more effective as the mouse itself drives the eradication process, whereas every mouse within the population would need to be targeted in the toxicant-based approaches.
While in theory one transgenic mouse could spread the t/Sry complex through a population, this is highly unlikely in practice. Modeling different scenarios will be crucial in developing an effective overall strategy. Also to be considered are the regulatory agency requirements, biosafety and ethical concerns, as well as public opinion (60, 61). With these considerations in mind, a naturally occurring meiotic distorfer like the t-haplotupe may be more likely to be approved for use in pest eradications than a synthetic gene drive, at least initially.
CHAPTER 2

DESIGNING THE t/Sry DAUGHTERLESS MOUSE
ABSTRACT

Invasive *Mus musculus* populations can lead to ecosystem alterations and the extinctions of native species, particularly in highly vulnerable island ecosystems. Our overall goal is to develop a genetic pest management approach for eradication of invasive mouse populations on islands for the purpose of conservation. Our strategy involves the insertion of the male determining gene (*Sry*) into a naturally occurring selfish element (the t-haplotype). The *Sry* gene, normally located on the Y chromosome, is necessary and sufficient to cause sex reversal of chromosomally (XX) female mice. By inserting this gene in a meiotic distorher locus, the t-haplotype, over 95% of the offspring will inherit the *Sry* gene independent of the sex chromosomes and develop phenotypically as a male. CRISPR gene editing tools are used to create the transgenic mouse.

INTRODUCTION

The current overall extinction rate is unprecedented, particularly in island ecosystems, primarily due to habitat loss and the effects of invasive species. These landmasses have a very high proportion of endemic species relative to continental areas, and they are particularly vulnerable to changes in their ecosystem (3). Invasive rodents are found on the majority of the islands worldwide due to their transport by humans and have flourished because of their very adaptable nature (14). These invasive mammals are responsible for the extinction of at least 75 species, the majority of which are birds (9). Eliminating invasive species has been shown to improve the survival of endangered species and is predicted to prevent future extinctions (4). By eliminating rats and mice from islands, the threatened species have the chance to rebound and recover their population (9).
While rodenticides are currently used for rodent eradications, there are many limitations to this method. Of great concern is secondary poisoning of non-target species, limiting rodenticide usage to primarily islands not inhabited by people and livestock. Rodenticides have lower efficacy rates for mice (8% failure rate for rats and 19% failure rate for mice (14)) due to several factors, including the difficulties in effectively spreading bait to reach an entire mouse population as well as the development of rodenticide resistance mutations within treated populations. Another consideration is the limited size of islands that current eradication methods are effective; eradication of invasive rodents in Australia and New Zealand is not possible with the current technologies (14). As such, a genetic-based approach, similar to the genetic methodologies that have been successful in eradicating livestock- and crop-disease-causing insects, could be more effective for eradication of invasive mouse populations compared to traditional toxicant-based approaches (31, 62).

One such approach is to skew the sex ratio of a population to mostly males through a genetically engineered modification, resulting in a population crash. This could be achieved by creating daughterless mice, which over several generations would lead to the extinction of the population (56). The Sry gene, the male sex determining gene located on the Y chromosome, is necessary and sufficient for development of a male phenotype (57). Sry is responsible for testes formation and ovarian suppression. While Sry is only active for a short period of time, the timing is crucial to ensure male development. If delayed, the female pathway will be initiated (58). Koopman (59) was able to prove that the Sry gene is necessary and sufficient to direct male development by inserting the Sry gene onto the autosome of a chromosomally female (XX) mouse. The resulting phenotypically male XX mice are sterile
yet anatomically and behaviorally indistinguishable from an XY male. The XY males carrying the autosomal Sry gene will pass the gene to its offspring at the expected Mendelian ratio (53).

Inserting the Sry gene onto an autosome will still result in enough females to prevent an eradication. Therefore it is necessary to take advantage of super Mendelian inheritance, which changes this ratio to one allele being inherited greater than 50% of the time. Several naturally occurring meiotic distorters and synthetically generated gene drives are available for this purpose (32). The t-haplotype, a naturally occurring meiotic distaster on chromosome 17 in mice, was chosen due to its 95% transmission distortion rate (63).

To address this need for better technology for conservation, we have designed a mouse to self-propagate a daughterless phenotype. This chapter presents the design process of the transgenic mouse, from choosing the t-haplotype genotype for the self-propagation and the Sry gene to cause sex reversal and CRISPR design for the gene editing process.

**METHODS AND MATERIALS**

**Mice**

Mice were maintained according at Texas A&M University and all procedures were performed in accordance to Texas A&M University IACUC approval. Mice were housed on a 12-hour light / 12-hour dark cycle and given water and food (Teklad Global 19% Protein Extruded Rodent Diet, Envigo, United Kingdom) *ad libitum*. The t<sup>w2</sup> mice were maintained on a mixed C57BL/6J and 129S1/SvImJ background. The original t<sup>w2</sup> stock was captured from the New York and Philadelphia area in 1946 and maintained in the laboratory of Dr. H. A. Schnieder at the Rockefeller Institute for Medical Research (64). Dunn and Morgan later identified the t-haplotype present in colony as t<sup>w2</sup> (63). The t<sup>w2</sup> mice were maintained at The
Jackson Laboratory (Bar Harbor, ME) before being cryopreserved at the Pasteur Institute (Paris, France). The Threadgill lab obtained three live mice from the cryopreserved stock. These three males were bred to C57BL/6J females, resulting in 34 male offspring, of which three were the t^w2/+ genotype. The colony has since been maintained primarily through brother/sister mating, with the occasional C57BL/6J and 129S1/SvImJ females from The Jackson Laboratory used to prevent genetic drift.

The t-haplotype was identified through PCR genotyping of the Hba-4ps locus. DNA was extracted from ear tissue punches. The protocol was adapted from Schimenti and Hammer (65) using the primers Hb.1: 5’-GAGTGACCTGCATGCCCAAGCTGTG-3’ and Hb.2: 5’-GAGCTGTGGAGACAGGAAGGGTCAGTG-3’ with the following changes: 20 uL reaction containing 1X Qiagen buffer, 1X Cresol Red, additional 2.5mM MgCl2, 0.2uM dNTPs, 0.3uM each Hb.1 and Hb.2 primers, and 1 unit Qiagen Taq polymerase. The reaction conditions were 94°C for 5 minutes, followed by 35 cycles of 95°C for 30 seconds, 66°C for 1 minute, and 72°C for 1 minute, followed by 72°C for 7 minutes. The PCR products were then run on a 3% agarose gel for 90-120 minutes. The t-haplotype contains a 16 bp insert at this locus, resulting in a 214 bp fragment, while the wild type fragment is 198 bp.

The region containing potential homology arms for a replacement construct was sequenced in both +/+ and t^w2/t^w2 mice in three overlapping amplicons. Amplicon 1 was sequenced using the primers t^w2 amp 1 F: 5’-CTAACTGCGAGCCCAAGAAT-3’ and t^w2 amp 1 R: 5’-GGAGACTCCAACAATGACATACA-3’ for a 963 bp fragment. Amplicon 2 was sequenced using the Sry cut site F and R primers described below for a 1 kb fragment. Amplicon 3 was sequenced using the primers t^w2 amp 3 F: 5’-CGTGAACATGCACACAC-
TTCATC-3’ and tw2 amp 3 R: 5’-AAGCTCCTGAGAGGCATCTA-3’ for a 991 bp fragment.

**Plasmid generation**

The *Sry* sequence was the same as described in (66), the 10 kb fragment produced from the digestion of the L741 fragment (53) with *Stu* I. The homologous arms were chosen from a site on chromosome 17 near the centromere, with each arm approximately 1.5 kb. The resulting 12,883 bp sequence was synthesized by GenScript (Piscataway). (See appendix A for sequence).

The plasmid was then transformed into *E. coli* 10-beta chemically competent cells (NEB, Ipswich) according to the manufacturer’s protocols. In short, 4 uL of plasmid DNA was added to thawed NEB 10-beta cells and placed on ice for 30 minutes. The mixture was then heat-shocked at 42°C for 30 seconds and then placed on ice for another 5 minutes. SOC media was added to the cell/DNA mixture and grown at 37°C for 1 hour. The cells were then plated on ampicillin agar plates and grown overnight at 30°C.

Positive colonies were detected with colony PCR using SapphireAmp Fast PCR master mix (Takara, Mountain View) with primers for the *Sry* gene, Sry-F: 5’-TTGTCTAGAGAGCATGGAGGGCCATGTCAA-3’ and Sry-R: 5’-CCACTCCTCTG-TGACACTTTAGCCCTCCGA-3’, producing a 273 bp fragment. For each reaction, 6 uL SapphireAmp mix, 2 uL dH2O, 1 uL of 10mM each Sry-F and Sry-R was used. A bacterial colony (or 2 uL of bacteria broth) was added and processed according to manufacturer’s protocol. The PCR product was then run on a 2% agarose gel for 30 minutes to detect positive colonies. Positive colonies were added to 5 mL LB broth with 5 uL ampicillin and grown...
overnight at 30°C while shaking. PRC was again used to confirm presence of the plasmid and then 0.5-1.0 mL were added to a large broth, 250 mL LB broth, 250 uL ampicillin, and grown at 30°C for 36-48 hours in a shaking incubator.

The plasmid was extracted using the PureYield Plasmid Maxiprep System (Promega, Madison) according to manufacturer’s protocol. The DNA concentration was determined using a Cytation3 spectrophotometer (BioTek, Winooski) and the 5’ and 3’ plasmid insertion sites were sequenced by Eton Bioscience (San Diego) using the primers, Sry-plasmid-F: 5’-TACAGTGTGTTCATATAATACAAATAAAT-3’ and Sry-plasmid-R: 5’-GAGTAAGG-GCTTCCAGAGATGACCAGTGAC-3’. Ethanol precipitation was used to purify and concentrate the plasmid. The plasmid was either left intact or was linearized by digesting with Bam HI enzyme for downstream applications.

CRISPR

The CRISPR design tool on http://crispr.mit.edu was used to design the gRNAs 1-1, 1-2, 2-1, and 2-2, which were obtained in plasmids from GenScript (Piscataway). Following transformation into One Shot ccdB Survival 2 T1 chemically competent cells, (Thermo Fisher, Waltham), the bacteria were grown on kanamycin agar plates and colonies were tested for successful integration of the plasmid with colony PCR. The reverse primer for all 4 gRNAs is 5’-AAAAGCACCGACTCGGTGCC-3’. The forward primers were each a unique 20 bp sequence, 1-1: 5’-GCAGGAGAGTGAGTGAGCT-3’, 1-2: 5’-GGGGTAAGAGGCAGCTT-3’, 2-1: 5’-GTATCAATACAGCTGTGAA-3’, and 2-2: 5’-TAAAGGTGGTAGCGG-3’. Positive colonies were cultured in small broths (5 mL LB broth, 25 uL kanamycin, positive colony) at 37°C overnight in a shaking incubator. The broths were
checked for positive presence of plasmid and then cultured in large broths (100 mL LB broth, 500 uL kanamycin, 200 uL positive broth) overnight at 37°C in a shaking incubator. The plasmid was extracted using the PureYield Plasmid Maxiprep System (Promega, Madison) and sequenced by Eton.

The gRNAs were then PCR amplified using Phusion PCR (NEB, Ipswich) and the primers described above though the T7 sequence (5’-TTAATACGAC-TCACTCAGTATTAGG-3’) were added to the beginning of the forward primer. The PCR products were purified and concentrated using ethanol precipitation. \textit{In vitro} transcription was performed using the MEGAshortscript T7 Kit according to the manufacturer’s protocol (Life Technologies, Carlsbad). The MEGAcleal kit (Thermo Fisher, Waltham) was used to purify the IVT product.

The gRNAs 1, 2, 3, and 4 were designed using CRISPOR (67), and, along with gRNA 2-2 and Cas9 nuclease, were obtained from IDT (San Jose). The crRNA portion of the gRNA contained the unique sequence that matches the t-haplotype and was combined with a universal tracrRNA.

\textbf{gRNA \textit{in vitro} screening}

The gRNAs were screened for cutting ability using the Guide-it sgRNA Screening System (Takara, Mountain View). A 1 kb region of the t-complex was PCR amplified using the primers \textit{Sry} cut site F: 5’-TGTTCTCTCCATGCCTTCTTTG-3’ and \textit{Sry} cut site R: 5’-CACAGCAGGTTCAGACAGTCTCTA-3’ according to manufacturer’s protocol with an annealing temperature of 57°C. The Cas9 cleave assay performed according to the manufacturer’s protocol.
RESULTS/DISCUSSION

$t^w_2$ Mouse Line Reestablishment

The mice being used for this project have a variation of the t-haplotype called $t^w_2$ on a 129/B6 mixed background (68). The heterogeneous background provides hybrid vigor and enables them to survive better (69). The $t^w_2$ haplotype was chosen because it is a complete t variant that results in sterile males when homozygous and does not cause embryonic lethality, which is more common for the different t variants. The t-haplotype is identified through a 16 base pair insertion in the alpha-globin pseudogene-4 (Hba-4ps) locus (Figure 2-1) (65).

![Figure 2-1. Genotyping the $t^w_2$ allele.](image)

This is an example of the three genotypes, homozygous wild type (+/+), heterozygous for the t-haplotype ($t^w_2$/+), and homozygous for the t-haplotype ($t^w_2/t^w_2$), with the PCR primers Hb.1 and Hb.2 used to detect a 16 base pair insertion in the Hba-4ps locus.

To date, almost 2,400 progeny of the original three $t^w_2/+ $ males in our colony have been genotyped at the t locus, confirming a transmission ratio distortion (TRD) of nearly 95\%. 

Table 2-1. Transmission ratio distortion (TRD) of the \( t^{w2} \) allele. On a mixed B6/129 background, the TRD is calculated to be over 94.3%.

<table>
<thead>
<tr>
<th>Number of mice</th>
<th>Genotype</th>
</tr>
</thead>
<tbody>
<tr>
<td>134</td>
<td>+/- (wild type)</td>
</tr>
<tr>
<td>1668</td>
<td>( t^{w2}/+ )</td>
</tr>
<tr>
<td>547</td>
<td>( t^{w2}/t^{w2} )</td>
</tr>
<tr>
<td>Total: 2349</td>
<td>TRD of ( t^{w2} ): 94.3%</td>
</tr>
</tbody>
</table>

**Sry Construct Generation**

Sex reversal in mice was first achieved in 1991 by inserting a 14 kb fragment of the Sry gene onto an autosome (53). This gene fragment was later shortened to 10 kb and still able to cause sex reversal (66), which was used to design the daughterless mouse. Because the expression of Sry is tightly regulated and only occurs in a 2-day window in the developing embryo, the endogenous promoter and regulatory sequences were preserved in the sequence used for the transgene (59). However, homologous recombination is very inefficient with fragments of this size. Inversion one in the t-haplotype was chosen as the Sry transgene insertion target site. This specific location is near actively expressed genes, increasing the likelihood of transgene expression, but it not expected to interrupt endogenous gene function (Figure 2-2).
Figure 2.2. The physical map of the t-complex located within chromosome 17. A) The location chosen for insertion was close to the centromere. B) The boxed region in A is expanded showing the target region is a gene-dense area. C) The boxed region in B is expanded confirming that the target insertion site should not interrupt endogenous genes.

Homology arms 1.5 kb in size were designed to flank both sides of the Sry sequence to increase the likelihood of the gene being inserted through homologous recombination (see appendix A for Sry construct sequence).
Figure 2-3. The Sry construct. The map of the 13 kb Sry construct shows the 10 kb Sry fragment flanked on both sides by 1.5 kb homology arms. The 10kb Sry fragment contains the endogenous promoter sequence and the protein-coding sequence.

The 13kb sequence was synthesized commercially by GenScript. This process was very challenging due to the overall size and the presence many repetitive sequences leading to high mutation rates (GenScript, personal communications). Once the construct was complete, the plasmid was transfected into DBH 10 E. coli, cultured, purified and sequenced. The large construct size again made this process difficult because the bacteria were more likely to take up the antibiotic resistant gene from the plasmid instead of the entire plasmid. Lowering the culture temperature enabled the plasmid-carrying bacteria with the burdensome genomic load to not be outcompeted by the bacteria containing only the antibiotic resistance gene from rearranged plasmids.

CRISPR Design

Using online tools (70), guide RNAs (gRNAs) were chosen to bind to the 1.5 kb homologous arms. Using the wild type chromosome 17 sequence of a C57BL/6J background, 4 gRNAs were chosen as pairs to be used with Cas9 nickase to create double-stranded breaks in the t-complex (Figure 2-4).
Figure 2-4. The location of the four gRNAs. A) The CRISPR target site is centrally located relative to the homologous arms and within amplicon 2. B) An amplified view of the 1000 bp amplicon 2 demonstrates the locations of the designed gRNAs.

Table 2-2. The sequence of the four gRNAs. 1-1 and 1-2 were one pair and 2-1 and 2-2 were another pair.

<table>
<thead>
<tr>
<th>gRNA</th>
<th>Sequence</th>
</tr>
</thead>
<tbody>
<tr>
<td>1-1</td>
<td>5’-GCAGGAGAGTGAGTGAGCT-3’</td>
</tr>
<tr>
<td>1-2</td>
<td>5’-GGGGTAAGAGGACCAGCTT-3’</td>
</tr>
<tr>
<td>2-1</td>
<td>5’-GTATCAATACAGCTGTGAA-3’</td>
</tr>
<tr>
<td>2-2</td>
<td>5’-TAAAGGGTTTAGAGAGCGG-3’</td>
</tr>
</tbody>
</table>
The gRNAs were screened *in vitro* for their ability to cut a 1kb PCR product corresponding to the t-complex using +/+, t\(^w2/+\), and t\(^w2/t\(^w2\) mouse DNA. While three of the gRNAs could cut the wild type DNA, only the gRNA 2-2 was able to cut the t-complex DNA (Figure 2-5).

**Figure 2-5. Ex vivo test of gRNA efficiency.** The cutting sites are validated after being chosen with the CRISPR software. A 1 kb region of the t-complex was amplified that contained the CRISPR cutting sites (amplicon 1). The gRNAs and Cas9 were combined to form ribonucloprotein (RNP) complexes, incubated with the PCR product, and subjected to gel electrophoresis. gRNA 1-1, 2-1, and 2-2 cut the wild type amplicon and gRNA 2-2 cut the t-haplotype variant. The boxes indicate where the cut bands for the t\(^w2/t\(^w2\) amplicion should be located, if present.

When the homologous arms and gRNAs were first designed, the thought was that the t-haplotype was the same sequence as the wild type, just inverted. This notion was proved false with the *in vitro* test of gRNAs 1-1, 1-2, 2-1, and 2-2 (Figure 2-7). There must be base pair differences if the gRNA is able to cut the wild type amplicon but not the t-haplotype amplicon. The 3 kb t-complex used as the homologous arms was then sequenced (Figure 2-6) and aligned to the corresponding wild type sequence to identify base pair differences between the t-haplotype and the wild type region (Figures 2-9, 2-10, and 2-11) to determine why two of the
gRNAs (1-1 and 2-1) could not cut the t-haplotype amplicon. While it has been demonstrated that 1-3 mismatches between the gRNA and the target DNA sequence can be tolerated, the specificity of the gRNA decreases with each mismatch. Mismatches near the 5’ end are more tolerated than the 3’ end, which is located near the PAM sequence. The gRNA/Cas9 complex first binds near the PAM sequence and then aligns to the target DNA like a zipper (71). Several missense mutations are present in the sequenced t-haplotype, as well as a large deletion of 92 bp in the t-haplotype in amplicon 1 (Figure 2-7) and a small deletion and insertion is present in the t-haplotype amplicon 3 (Figure 2-9). Amplicon 2 also has several mismatches and two small deletions (Figure 2-8), and gRNA 1-1, 1-2, and 2-1 all are present in these regions (Figure 2-12). gRNA 2-1 has 2 base pairs missing in the t-haplotype and 1-1 and 1-2 have mismatches. gRNA 2-2 is the same sequence for both wild type and t-haplotype and was therefore able to cleave both amplicons.

Figure 2-6. The region was sequenced with three overlapping amplicons. Each amplicon was approximately 1 kb in size.
**Figure 2-7. Amplicon 1 alignment.** DNA from a wild type mouse and a homozygous t<sup>w2</sup>/t<sup>w2</sup> mouse was sequenced, aligned, and then compared to the reference genome. Several base pair changes are present, and there is a 92 base pair deletion in the t-haplotype sequence (blue square).
Figure 2-8. **Amplicon 2 alignment.** Amplicon 2 contains the CRISPR cut site. Several base pair mismatches exist between the t-haplotype and the wild type t-complex, as well as two small deletions (blue squares) in the t-haplotype.
Figure 2-9. Amplicon 3 alignment. Again, several base pair mismatches are present, as well as a small insertion (red square) and deletion (blue square) in the t-haplotype compared to the wild type chromosome.

Given the fact that there are several base pair changes between the two sequences and deletions in the t-haplotype sequence, four new gRNAs were designed based on the t-haplotype sequence (Table 2-3). The new gRNAs were chosen to target the t-haplotype instead of the wild type t-complex.
Table 2-3. Sequences of the newly designed gRNAs. The gRNAs were designed based on the sequence of the t-haplotype instead of the wild type.

<table>
<thead>
<tr>
<th>gRNA</th>
<th>Sequence</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>5’-GCATAGTGATATCAATACAG-3’</td>
</tr>
<tr>
<td>2</td>
<td>5’-GGCCAAGCTTAAGCACCCAG-3’</td>
</tr>
<tr>
<td>3</td>
<td>5’-CCCGATGTTATGGAGACATT-3’</td>
</tr>
<tr>
<td>4</td>
<td>5’-GAGATTGCTCTGCCCATCAA-3’</td>
</tr>
</tbody>
</table>

Figure 2-10. Map of gRNAs 1-4 in relation to one another. The gRNAs are in the same amplicon 2 as the first four gRNAs.

The new set of gRNAs as well as gRNA 2-2 and Cas9 were ordered as synthetically made proteins from IDT after a recently published paper indicated they were more effective in this form, as well as less time consuming (72).

An in vitro screening assay was used to test the cutting ability in +/+ , t^{w2}/+, and t^{w2}/t^{w2} mouse DNA. Three of the four gRNAs were able to cut the PCR products (gRNAs 1, 2, and 4). gRNA 1 only cut the t-haplotype, while 2 and 4 cut both. In total, four out of the eight gRNAs were able to cut in t^{w2} DNA in vitro (Figures 2-5 and 2-11).
Figure 2-11. \textit{In vitro} gRNA validation of gRNAs 1, 2, 3, and 4. gRNA 1, 2, and 4 were able to cut the t-haplotype, with gRNA 4 being very efficient, as the entire 1 kb band has been cut, unlike with gRNA 1 and 2, where three bands can be seen. The square highlights the cutting that occurred in the t-haplotype PCR amplicon.
Figure 2-12. The aligned sequence of the wild type t-complex and the t-haplotype with the eight gRNA sequences outlined. The initial set of gRNAs contains mismatches compared to the t\textsuperscript{w2} sequence, except for 2-2. The second set of gRNAs was chosen with no mismatches (2 and 4) or mismatches to the wild type sequence to encourage only cutting in the t-haplotype sequence. All eight gRNAs are outlined in a different color box to show the sequence differences between the wild type and t-haplotype sequence.

CONCLUSION

One of the primary lessons learned from this experience is the importance of quality control. Evidence from the gRNA in vitro validation experiments shows that computer predicted sequences do not always work, though part of the problem with the first set of gRNAs stemmed from DNA sequence differences. While it is possible to have base pairing with
mismatched sequences, the validation experiments also showed that the efficiency is compromised, to the point where the three gRNAs from the first set that have mismatches did not function. The availability of synthetically made gRNAs makes the task of screening several possible gRNAs for cutting efficiency much easier as well as ensuring higher quality (72), as it is not dependent on in vitro transcription by individual laboratories, but is instead more consistently generated by a company. The second gRNA validation did have one gRNA (3) that still didn’t work, even with the sequence matching. After the gRNAs are screened for cutting ability, the next step is an in vivo validation in mouse embryonic fibroblast cells to test for cutting efficiency in the context of chromatin.
CHAPTER 3

GENERATING THE $t/Sry$ DAUGHTERLESS MOUSE
ABSTRACT

A lot of media attention has surrounded the introduction of the CRISPR/Cas9 system over the past several years, with many applications in a wide-range of organisms for immediate transgenic animal generation to the development of disease-resistant crops and precise editing for human gene therapy. CRISPR/Cas9 builds on the decades of homologous recombination technology, essentially significantly increasing the efficiency of the double-stranded break that initiates the homologous recombination event. Despite numerous successes, several hurdles, including off-target effects and low efficiency with large inserts, can result in significant project delays. This paper describes the process of developing a transgenic mouse with the Sry gene inserted into the t-haplotype through the use of the gene editing capabilities of CRISPR. The ultimate goal is to create a daughterless mouse phenotype for purpose of eradicating invasive mouse populations for conservation applications. Here we describe efforts to generate the transgenic mouse through microinjection of a large construct (13 kb) using a CRISPR/Cas9 editing system.

INTRODUCTION

The ability to precisely edit the genome is important for future gene editing endeavors. The ability to use genetic modification has the possibility of changing how we address crop and pest management and the prevention of vector-borne diseases and genomic editing for the treatment of human diseases. The discovery of the CRISPR/Cas9 system opened the door for more precise gene editing of many organisms that were previously out of reach with existing technologies. However, additional optimization of this system is still needed before it can be reliably used for many applications. The ability to use CRISPR to delete genes is fairly
straightforward, but inserting new DNA is significantly more challenging to achieve, especially when attempting to insert large constructs in targeted loci.

Our previous work focused on the reestablishment the t\textsuperscript{w2} mouse line and the development of the Sry construct. Here we detail the first attempts to achieve a 10 kb Sry insertion into the mouse t-haplotype using CRISPR. While the initial microinjection attempts were unsuccessful, we discuss the challenges and describe several validation steps implemented to optimize future rounds of microinjections.

METHODS AND MATERIALS

Mice

Mice were maintained at Texas A&M University and all procedures were performed with Texas A&M University IACUC approval. Mice were housed on a 12-hour light / 12-hour dark cycle and given water and food (Teklad Global 19% Protein Extruded Rodent Diet, Envigo, United Kingdom) \textit{ad libitum}. The t\textsuperscript{w2} mice were maintained on a mixed C57BL/6J and 129S1/SvImJ background. The original t\textsuperscript{w2} stock was captured from the New York and Philadelphia area in 1946 and maintained in the laboratory of Dr. H. A. Schnieder at the Rockefeller Institute for Medical Research (64). Dunn and Morgan later identified the t-haplotype present in colony as t\textsuperscript{w2} (63). The t\textsuperscript{w2} mice were maintained at The Jackson Laboratory (Bar Harbor, ME) before being cryopreserved at the Pasteur Institute (Paris, France). The Threadgill lab obtained three live mice from the cryopreserved stock. These three males were bred to C57BL/6J females, resulting in 34 male offspring, of which three were the t\textsuperscript{w2}/+ genotype. The colony has since been maintained primarily through brother/sister mating,
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site on chromosome 17 near the centromere, with each arm approximately 1.5 kb in size. The resulting 12,883 bp sequence was synthesized by GenScript (Piscataway).

The plasmid was then transformed into *E. coli* 10-beta chemically competent cells (NEB, Ipswich) according to the manufacture’s protocols. In short, 4 uL of plasmid DNA was added to thawed NEB 10-beta cells and placed on ice for 30 minutes. The mixture was then heat-shocked at 42°C for 30 seconds and then placed on ice for another 5 minutes. SOC media was added to the cell/DNA mixture and grown at 37°C for 1 hour. The cells were then plated on ampicillin agar plates and grown overnight at 30°C.

Positive colonies were detected with colony PCR using SapphireAmp Fast PCR master mix (Takara, Mountain View) with primers for the Sry gene, Sry-F: 5’TTGTCTAGA-GAGCATGGAGGGCCATGTCA-3’ and Sry-R: 5’-CCACTCCTCTGTGACACTTTAGC-CCTCCGA-3’, producing a 273 bp fragment. For each reaction, 6 uL SapphireAmp mix, 2 uL dH2O, 1 uL of 10mM each Sry-F and Sry-R was used. A bacterial colony (or 2 uL of bacteria broth) was added and processed according to manufacturer’s protocol. The PCR product was then run on a 2% agarose gel for 30 minutes to detect positive colonies. Positive colonies were added to 5 mL LB broth with 5 uL ampicillin and grown overnight at 30°C while shaking. PCR was again used to confirm presence of the plasmid and then 0.5-1.0 mL were added to a large broth, 250 mL LB broth, 250 uL ampicillin, and grown at 30°C for 36-48 hours in a shaking incubator.

The plasmid was extracted using the PureYield Plasmid Maxiprep System (Promega, Madison) according to manufacturer’s protocol. The DNA concentration was determined using a Cytation3 spectrophotometer (BioTek, Winooski) and the 5’ and 3’ plasmid insertion sites were sequenced by Eton Bioscience (San Diego) using the primers, Sry-plasmid-F: 5’-
TACAGTGTGTTCATAAATACAAATAAAT-3’ and Sry-plasmid-R: 5’-GAGTAAGG-GCTTCCAGAGATGACCAGTGAC-3’. Ethanol precipitation was used to purify and concentrate the plasmid. The plasmid was either left intact or linearized by digesting with BamHI enzyme for downstream applications.

**Mouse Embryonic Fibroblasts (MEFs)**

MEFs were derived from mouse embryos 12-14 days after a copulation plug was observed, using a previously-described protocol (73) with slight modifications. Briefly, embryos were either cultured together or separately, depending on need. Embryos were disinfected with 70% ethanol, the head and organs were removed, and scalpels were used to mince the tissue in 0.05% trypsin. The tissue/trypsin mixture was incubated at 37°C, 5% CO₂ for one hour, with the tube inverted every 15 minutes and more trypsin added after 30 minutes. The cells were then centrifuged, supernatant aspirated, and the pellet resuspended in MEF media (DMEM Glutamax Media, fetal bovine serum, penicillin, and streptomycin) and incubated overnight. Non-adherent cells were aspirated, and the media replaced. Cells were expanded and frozen in liquid nitrogen at passages 1 and/or 2.

**CRISPR**

The CRISPR design tool on http://crispr.mit.edu was used to design the gRNAs 1-1, 1-2, 2-1, and 2-2, which obtained in plasmids from GenScript (Piscataway). Following transformation into One Shot ccdB Survival 2 T1 chemically competent cells, (Thermo Fisher, Waltham), the bacteria were grown on kanamycin agar plates, and colonies were tested for successful integration of the plasmid with colony PCR. The reverse primer for all 4 gRNAs is
5’-AAAAGCACCAGACTCGGTGCC-3’. The forward primers were each a unique 20 bp
sequence, 1-1: 5’-GCAGGAGAGTGAGTGAGCT-3’, 1-2: 5’-GGGGTAAGAGGAC-
CAGC-TT-3’, 2-1: 5’-GTATCAATACAGCTGTGAA-3’, and 2-2: 5’-TAAGGTTTAG-
AGAGCGG-3’. Positive colonies were cultured in small broths (5 mL LB broth, 25 uL
kanamycin, positive colony) at 37°C overnight in a shaking incubator. The broths were
checked for positive presence of plasmid using colony PCR, and then cultured in large broths
(100 mL LB broth, 500 uL kanamycin, 200 uL positive broth) overnight at 37°C in a shaking
incubator. The plasmid was extracted using the PureYield Plasmid Maxiprep System
(Promega, Madison) and sequenced by Eton.

The gRNAs were then PCR amplified using Phusion PCR (NEB, Ipswich) and the
primers described above though the T7 sequence (5’-TTAATACGACTCACTCA-
CTATAGG-3’) was added to the beginning of the forward primer. The PCR products were
purified and concentrated using ethanol precipitation. In vitro transcription was performed
using the MEGAshortscript T7 Kit according to the manufacturer’s protocol (Life
Technologies, Carlsbad). The MEGAclear kit (Thermo Fisher, Waltham) was used to purify
the IVT product.

The gRNAs 1, 2, 3, and 4 were designed using CRISPOR (67), and, along with gRNA
2-2 and Cas9 nuclease, were obtained from IDT (San Jose). The crRNA portion of the gRNA
contains the unique sequence complementary to the t-haplotype and was combined with a
universal tracrRNA, either with or without the ATTO 550 fluorescent label.
**gRNA in vitro screening**

The gRNAs were screened for cutting ability using the Guide-it sgRNA Screening System (Takara, Mountain View). A 1 kb region of the t-complex was PCR amplified using the primers *Sry* cut site F: 5’-TGTCTCTGCCCAGTCTTTGT-3’ and *Sry* cut site R: 5’-CACAGCAGTCTTCACAGTTCTA-3’ according to manufacturer’s protocol with an annealing temperature of 57°C. The Cas9 cleave assay performed according to the manufacturer’s protocol.

**gRNA cutting efficiency**

The gRNAs were tested for cutting efficiency in t\(^{w2}\) MEF cells using the Alt-R CRISPR-Cas9 System: Cationic lipid delivery of CRISPR ribonucleoprotein complex into mammalian cells protocol (IDT, San Jose). The fluorescently-labeled tracrRNA was used to confirm ribonucleoprotein (RNP) uptake into MEF cells. The HPRT crRNA and crRNA #1 controls were used as positive and negative controls, respectively.

**Microinjections**

Microinjections were performed at the Texas Institute for Genomic Medicine (TIGM, College Station). Sperm from t\(^{w2/}\) males was used for *in vitro* fertilization of C57BL/6J eggs. This protocol required 1.5-2 ug of each gRNA and 50-70 ug of plasmid.
RESULTS/DISCUSSION

Microinjections

Four gRNAs (1-1, 1-2, 2-1, 2-2) were designed using online algorithms (Table 3-1) and then used in two rounds of microinjections. These gRNAs were generated from plasmid DNA.

Table 3-1. The sequence of the four gRNAs. gRNAs 1-1, 1-2, 2-1, and 2-2 used in first two rounds of microinjections.

<table>
<thead>
<tr>
<th>gRNA</th>
<th>Sequence</th>
</tr>
</thead>
<tbody>
<tr>
<td>gRNA 1-1</td>
<td>5’-GCAGGAGAGTGAGTGAGCT-3’</td>
</tr>
<tr>
<td>gRNA 1-2</td>
<td>5’-GGGGTAAGAGGACCAGCTT-3’</td>
</tr>
<tr>
<td>gRNA 2-1</td>
<td>5’-GTATCAATACAGCTGTGAA-3’</td>
</tr>
<tr>
<td>gRNA 2-2</td>
<td>5’-TAAAGGGGTAGAGAGCGG-3’</td>
</tr>
</tbody>
</table>

Following microinjection, the eggs were fertilized in vitro and implanted into pseudopregnant dams, resulting in 12 live pups at weaning age (Sry 1-12). The pups were genotyped, and 11 (or 92%) were t^w2/+ (Table 3-2). Only male mice (n = 5) were potential candidates for carrying the Sry construct, but initial testing revealed that none of the pups carried the Sry construct in the t-haplotype (data not shown).
Table 3-2. Pups from microinjection rounds 1-3. The 19 pups resulting from microinjections, with their dates of birth, sexes, and genotypes.

<table>
<thead>
<tr>
<th>Mouse Name</th>
<th>DOB</th>
<th>Sex</th>
<th>Genotype</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sry 1</td>
<td>12/11/16</td>
<td>Male</td>
<td>t^{w2}/+</td>
</tr>
<tr>
<td>Sry 2</td>
<td>12/11/16</td>
<td>Male</td>
<td>t^{w2}/+</td>
</tr>
<tr>
<td>Sry 3</td>
<td>12/11/16</td>
<td>Female</td>
<td>t^{w2}/+</td>
</tr>
<tr>
<td>Sry 4</td>
<td>12/11/16</td>
<td>Female</td>
<td>t^{w2}/+</td>
</tr>
<tr>
<td>Sry 5</td>
<td>12/19/16</td>
<td>Female</td>
<td>t^{w2}/+</td>
</tr>
<tr>
<td>Sry 6</td>
<td>12/19/16</td>
<td>Female</td>
<td>t^{w2}/+</td>
</tr>
<tr>
<td>Sry 7</td>
<td>3/15/17</td>
<td>Male</td>
<td>t^{w2}/+</td>
</tr>
<tr>
<td>Sry 8</td>
<td>3/15/17</td>
<td>Male</td>
<td>t^{w2}/+</td>
</tr>
<tr>
<td>Sry 9</td>
<td>3/15/17</td>
<td>Female</td>
<td>t^{w2}/+</td>
</tr>
<tr>
<td>Sry 10</td>
<td>3/15/17</td>
<td>Female</td>
<td>t^{w2}/+</td>
</tr>
<tr>
<td>Sry 11</td>
<td>3/15/17</td>
<td>Male</td>
<td>+/-</td>
</tr>
<tr>
<td>Sry 12</td>
<td>3/15/17</td>
<td>Female</td>
<td>t^{w2}/+</td>
</tr>
<tr>
<td>Sry 13</td>
<td>4/3/17</td>
<td>Male</td>
<td>t^{w2}/+</td>
</tr>
<tr>
<td>Sry 14</td>
<td>4/3/17</td>
<td>Male</td>
<td>t^{w2}/+</td>
</tr>
<tr>
<td>Sry 15</td>
<td>4/3/17</td>
<td>Male</td>
<td>t^{w2}/+</td>
</tr>
<tr>
<td>Sry 16</td>
<td>4/3/17</td>
<td>Male</td>
<td>t^{w2}/+</td>
</tr>
<tr>
<td>Sry 17</td>
<td>4/3/17</td>
<td>Female</td>
<td>t^{w2}/+</td>
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<tr>
<td>Sry 18</td>
<td>4/3/17</td>
<td>Female</td>
<td>t^{w2}/+</td>
</tr>
<tr>
<td>Sry 19</td>
<td>4/3/17</td>
<td>Female</td>
<td>t^{w2}/+</td>
</tr>
</tbody>
</table>
After the first two rounds of microinjections did not result in Sry construct integration, several quality control assays were performed on the gRNAs. In vitro screening was assessed using 1kb PCR product corresponding to the t-complex using +/-, t\textsuperscript{w2}/+, and t\textsuperscript{w2}/t\textsuperscript{w2} mouse DNA. While three of the gRNAs could cut the wild type DNA, only the gRNA 2-2 was able to cut the t-haplotype DNA (Figure 3-1).

**Figure 3-1. In vitro testing of gRNA efficiency.** A 1 kb region of the t-complex was amplified that contained the CRISPR cutting sites (amplicon 1). The gRNAs and Cas9 were combined to form ribonucleoprotein (RNP) complexes, incubated with the PCR product, and subjected to gel electrophoresis. gRNA 1-1, 2-1, and 2-2 cut the wild type amplicon and gRNA 2-2 cut both the wild type and the t-haplotype variant. The boxes indicate where the cut bands for the t\textsuperscript{w2}/t\textsuperscript{w2} amplicon should be located, if present.

With this result and new advances in CRISPR technology using a shorter synthetically generated crRNA and tracrRNA instead of producing the RNA from a plasmid (72), gRNA 2-2 was obtained and used for a third round of microinjections, resulting in seven more pups (Sry 13-19, Table 3-1).

To further examine the pup genomes for evidence of gene editing, the 1 kb region spanning the CRISPR target site was amplified for all 19 pups, as well as +/- (Figure 3-2), t\textsuperscript{w2}/t\textsuperscript{w2}+ (Figure 3-3), and t\textsuperscript{w2}/+ (Figure 3-4) control samples.
Figure 3-2. DNA sequence of the target region in a +/+ mouse. The wild type 1 kb region was amplified from a +/+ control mouse. The four gRNAs are outlined in red.
Figure 3-3. DNA sequence of the target region in a tw<sup>2</sup>/tw<sup>2</sup> mouse. The tw<sup>2</sup> 1 kb region was amplified from a tw<sup>2</sup>/tw<sup>2</sup> control mouse. The four gRNAs are outlined in blue. Only the complete gRNA 2-2 sequence is found in the t-haplotype. The bases GT are deleted from the beginning of gRNA 2-1, gRNA 1-1 has base pair change C→T at the second position and has a G deleted from the fourth position. The base G is deleted from the beginning of gRNA 1-2.
Figure 3-4. DNA sequence of the target region in a t\textsuperscript{w2/}+ mouse. The same region as depicted in figures 3-2 and 3-3 was sequenced in Sry 1, a t\textsuperscript{w2/}+ mouse. The wild type sequences that correspond to the gRNAs are underlined in red and the t-haplotype sequences are underlined in blue. The overlapping base pair calls are a result of the heterozygosity at this locus.

All 19 pups were sequenced at the CRISPR target site and examined for evidence of genomic cutting by the gRNAs. No insertions, deletions, or base pair changes were observed, indicating that the gRNAs did not successfully cut the target site. The next validation step involved an in vivo experiment to test the gRNA cutting efficiency in living cells, this time with four more newly designed gRNAs, 1-4 (Table 3-3) in addition to gRNA 2-2.
Table 3-3. Sequences of the newly designed gRNAs. The gRNAs were designed based on the sequence of the t-haplotype instead of the wild type sequence.

<table>
<thead>
<tr>
<th>gRNA</th>
<th>Sequence</th>
</tr>
</thead>
<tbody>
<tr>
<td>gRNA 1</td>
<td>5'-GCATAGTGATATCAATACAG-3’</td>
</tr>
<tr>
<td>gRNA 2</td>
<td>5'-GGCCAAGCTTAAGCACCCAG-3’</td>
</tr>
<tr>
<td>gRNA 3</td>
<td>5'-CCCCGATTTATGGGAGACATT-3’</td>
</tr>
<tr>
<td>gRNA 4</td>
<td>5’-GAGATTGCTCTGCCCATCAA-3’</td>
</tr>
</tbody>
</table>

Testing cutting efficiency in cells

gRNAs 1, 2, 3, 4, and 2-2 were tested for cutting ability inside living cells. A mixed population of mostly t\(^w2/+\) MEF cells was incubated with the gRNA/Cas9 RNPs for two days before DNA was extracted. A tracrRNA labeled with red fluorescent protein was used to visually confirm that the RNPs were able to get inside the cells through the use of lipofectamine (Figure 3-5).
Figure 3-5. QC validation of gRNAs entering MEF cells. The left 3 panels are bright field images and the right 3 panels are the Atto 550 fluorescence. Panel A2 and C4 are two experimental wells that are the cells incubated with gRNA 1 and 3, respectively, and panel H1 is a negative control well containing t\(^{w2/+}\) MEFs without CRISPR reagents.

Once the DNA was extracted from the cells, the same 1 kb region used to originally test the cutting ability of the gRNAs was PCR amplified. The enzyme T7E1 was then used to detect mismatches (74) that would occur from non-homologous end joining events after the Cas9 created double-stranded breaks at the designated loci. However, the inherent genomic differences between the wild type and t-haplotype genotypes made the detection of mismatching caused by cellular repair difficult to visualize in standard agarose gel electrophoresis (Figure 3-6). The additional bands in samples incubated with gRNA 4 and
2-2 indicate efficient cutting by the gRNAs in vivo. Capillary gel electrophoresis allowed for higher resolution of the bands (Figure 3-7).

Figure 3-6. T7E1 digestion assay for in vivo gRNA validation. A 1 kb amplicon of the CRISPR target site was with the T7E1 enzyme. The first well in each grouping is the PCR product alone, followed by the PCR product under digest conditions without the T7E1 enzyme (“No E”), which served as a negative control for digestion-related smearing. The next four wells are the experimental conditions repeated 4 times to ensure reliability. The bands in the negative gRNA (“- gRNA”) are due to the sequence differences in the t^w2 haplotype and wild type alleles.
Figure 3-7. Capillary gel electrophoresis separation of T7E1 digestion assay products. The red box in the PCR-only well denotes the uncut 1 kb amplicon. gRNA 4 and 2-2 cut the amplicon, denoted by red boxes in their respective wells.

To more clearly determine the cutting efficiency, separate primary MEF lines from were generated for each of the genotypes (+/+, t\(^{w2}/+\), and t\(^{w2}/t^{w2}\)). The T7E1 digestion assay was repeated in the genetically separate MEFs (Figure 3-8 and 3-9), more clearly demonstrating that gRNA 4 and 2-2 effectively cut the target site \textit{in vivo}. 
Figure 3-8. T7E1 digestion assay for *in vivo* gRNA validation using genotypically distinct MEF lineages. The same conditions from 3-7 were used here, with the first well containing the PCR amplicon alone, followed by the PCR amplicon under digest conditions without the T7E1 enzyme, followed by duplicates of each genotype, which is repeated for each gRNA. The bands in gRNA 4 and 2-2 are seen more easily in the +/+ and *t^w2/*t^w2 genotypes.
Figure 3-9. Details of the gRNA4 and 2-2 T7E1 digestion assay. The gRNA 4 and 2-2 regions from Figure 3-8 are enlarged to more easily observe the cut products, as denoted by the red boxes.

The Sry mice 13-19 were then examined for mismatched base pairing that would occur from nonhomologous end joining or partial insertion of the construct (Figure 3-10). The samples 13-19 were chosen since they were injected with the synthetically made gRNA 2-2, which was shown to effectively cut the target site in vitro and in vivo. However, no evidence of genomic editing was observed.
CONCLUSION

While CRISPR-based transgenic strategies are continually advancing, implementing efficient knock-in strategies of large donor plasmids are still very challenging. The first three rounds of CRISPR/gRNA microinjections failed to produce mice containing the Sry construct, which led us to perform in depth quality control assessment of our gRNAs. The first step taken, the in vitro experiments, indicated that minor sequence variation rendered a few of the gRNAs useless. While frustrating to have to design new gRNAs, it does indicate that off-target cuts should be minimal if faithfulness to the sequence is important. This also raises the possibility for resistance to gRNAs through polymorphisms at the CRISPR target site and something to take into consideration.

The next validation step was necessary to test cutting efficiency. The in vivo experiments were performed to ensure that the Cas9/gRNA RNPs were able to access the DNA in a live
cell to better mimic what would occur during microinjections. These steps lead to two gRNAs, 2-2 and 4, being identified as having high cutting efficiency. The next steps in the generation of the t/Sry mouse are to assess other techniques that can be exploited to increase the chance of homologous recombination during future microinjections.
CHAPTER 4

CONCLUSION AND FUTURE DIRECTIONS
INTRODUCTION

There is a long history of successful use of genetic strategies for insect pest management (20, 21, 25-27), but there have been no successful attempts in mammals to date. Generating the t/Sry mouse is one attempt to create a technique for the genetic toolbox for rodent eradications. While there have been successful eradication programs using rodenticides in isolated islands (9, 14), logistical and technical concerns (including off-target poisoning) severely limits the number of islands on which this method can be attempted or successfully implemented (75).

The t/Sry mouse, a “daughterless” transgenic mouse, has been designed and the necessary components have been validated. The next step is to optimize the rate of homologous recombination to increase the likelihood of successful insertion of the Sry gene in the t w2 haplotype, a naturally occurring meiotic distorer.

As our previous work shows, many of the components necessary for generating the t/Sry mouse have been validated. The Sry construct has been transformed successfully in E. coli competent cells and is available as a source of donor DNA. The gRNAs 2-2 and 4 have been validated to work in the t-haplotype of mouse embryonic fibroblast cells. The t w2 mouse line has a confirmed transmission ratio distortion rate of about 94%. After three failed attempts during the microinjections, the next steps forward are to optimize the microinjection technique or explore an alternative method of producing a transgenic mouse through cell line modality.
METHODS AND MATERIALS

Cell lines

Mouse Embryonic Fibroblasts (MEFs)

MEFs were derived from mouse embryos 12-14 days after a copulation plug was observed (E12-14). Embryos were either cultured together or separate, depending on need, using the Protocol (73) as follows: embryos were disinfected with 70% ethanol, the head and organs were removed, and scalpels were used to mince the tissue in 0.05% trypsin. The tissue/trypsin mixture was incubated at 37°C, 5% CO₂ for one hour, with the tube inverted every 15 minutes and more trypsin added after 30 minutes. The cells were then centrifuged, supernatant aspirated, and the pellet resuspended in MEF media (DMEM Glutamax Media, fetal bovine serum, and penicillin streptomycin solution) and incubated overnight. Non-adherent cells were aspirated, and the media replaced. Cells were expanded and frozen in liquid nitrogen at passages 1 and 2.

Induced Pluripotent Stem Cells (iPSCs)

iPS cells were derived from tw² MEF cells using the CytoTune-iPS 2.0 Sendai Reprogramming kit (Thermo Fisher, Waltham) according to manufacturer’s protocol. Cells were grown in 2i media (DMEM Glutamax media, fetal bovine serum, penicillin streptomycin solution, nonessential amino acids, β-mercaptoethanol, LIF, and the inhibitors PD and CHIR) on gelatinized plates. The iPSCs were either passaged by sub-cloning, where a single colony was picked up with a pipette, broken up with 0.05% trypsin, and transferred to a new plate, or the entire plate was detached using 0.05% trypsin and moved to a new plate.
FastLane Cell cDNA kit (Qiagen, Germany) was used to prepare first-stand cDNA which was then stored at -20°C for later reverse transcription verification of pluripotency.

**Embryonic Stem Cells (ESCs)**

Embryonic stem cells were derived from 129S1/SvImJ females mated to t<sup>w2</sup>/+ males. Blastocysts were collected at E3.5 and processed as described (73). Cells were grown in 2i media as described above.

**CRISPR**

**gRNA screening**

The gRNAs were screened for cutting ability using the Guide-it sgRNA Screening System (Takara, Mountain View). A 1 kb region of the t-complex was PCR amplified using the primers Ha1_gRNA F: 5’- CCTGGTCTCCCTGGTGCTAA-3’ and Ha1_gRNA R: 5’- CCTTCACACCCAAGCACACT-3’ and Ha2_gRNA F: 5’- AGCATCCATTGGGCTTCTT-3’ and Ha2_gRNA R: 5’- GTGACCACAGTGTGAGCTTTAG-3’ according to the manufacturer’s protocol. A total of ten gRNAs were tested, five that were designed to cut in homologous arm 1 (gRNA HA1_1, HA1_2, HA1_3, HA1_NA, HA1_NB) and five that were designed to cut in homologous arm 2 (gRNA HA2_4, HA2_5, HA2_6, HA2_TA, HA2 TB).
RESULTS/DISCUSSION

Optimization strategies of homologous recombination

Although target gene editing technology has existed since the early 1990’s (76), early methods were very difficult to engineer. The development of CRISPR-based methods (77) have greatly increased efficiency, enabling almost any DNA sequence in any organism to be targeted. However, while now easier to engineer, the rate-limiting step remains homologous recombination (HR). This is due in large part to the lower occurrence of the HR repair pathway, as compared to the non-homologous end joining (NHEJ) pathway (78). Since the HR repair mechanism depends on having the sister chromatid available to use as a template, this pathway is only available during the S and G2 phases of the cell cycle. In contrast, NHEJ can be used during all phases, and it competes with HR during S and G2 phase (78).

Several methods have been tested to increase the efficiency of the HR pathway when using CRISPR gene editing (79-85). One obvious choice is to inhibit the NHEJ pathway. However, deletion of the key enzyme in this pathway, a DNA ligase IV, results in embryonic lethality (86). Alternatively, the small molecule inhibitor SCR7 can be used to temporarily block the NHEJ pathway and has been shown to increase the rate of HR as compared to cells and embryos not treated (80). A recent study has suggested that the reagents (Cas9, gRNA, donor DNA) being inserted into the zygotes or ES cells cause toxicity to the cells. Wang et al was able to achieve higher rates of DNA insertion when using four fold less CRISPR reagents. When the authors combined this method with the SCR7 inhibitor, there was no observable difference in HR rate (83). Because SCR7 can also impede repair of non-targeted loci resulting in mutations elsewhere in the genome, methods that improve HR rates without inhibiting NHEJ may be preferred. Another innovation involved using two-cell embryos for the microinjection
process instead of the standard one-cell stage. The G2 phase is 10-12 hours long during the two-cell stage, giving a much longer window in which the HR pathway would operate (82).

Another approach involves optimizing the quality and delivery of Cas9, which can be delivered as a DNA plasmid, mRNA, protein, or be endogenously expressed. The Cas9 protein has been shown to be more effective in achieving homologous recombination than either the DNA or mRNA form (79, 87). When Cas9 is delivered as a protein, there is no need for it to be transcribed so it is immediately available for initiating the double-stranded break, thereby reducing the risk of premature degradation. Endogenous expression of Cas9 within the egg has also been shown to increase efficiency. In this case, the egg donor mouse either overexpresses Cas9 ubiquitously or in specific reproductive tissues. Cebrian-Serrano et al demonstrated that endogenous Cas9 was able to increase HR efficiency in some targets but was equivalent to Cas9 protein in others. However, both endogenous Cas9 expression and exogenous Cas9 protein outperformed Cas9 mRNA in HR efficiency (79).

Another consideration for knock-in strategies is the size of the insertion. Smaller DNA insertions are more efficiently and completely integrated than larger fragments. However, some strategies exist for increasing the likelihood of inserting larger DNA constructs (81, 85). Miura et al generated long single-stranded DNA (ssDNA) to act as the donor DNA with the premise that long ssDNA should be more efficiently inserted than double-stranded DNA (dsDNA). However, this method has only been successful with donor DNA up to 2 kb, and this strategy would likely not be useful for larger gene knock-ins (85). Yoshimi et al successfully inserted a 200 kb BAC sequence using single-stranded oligodeoxynucleotides (ssODNs) and a gRNA targeted to the donor DNA plasmid. The ssODNs act as homologous arms to avoid the step of generating homologous arms in BAC sequences. The gRNA targets
the circular DNA plasmid to prevent degradation of the construct until it has been inserted into the cell (81).

A description of how these approaches could be applied to the Sry construct is outlined in Table 4-1. Ultimately, using a gRNA targeting the Sry construct and performing microinjections with two-celled embryos will be used for the next round of microinjections.

Table 4-1. Possible experimental approaches to insert the Sry gene into the t-haplotype using the gRNA 4 and 2-2. Of the considered approaches, the two-cell embryo injection and the plasmid-targeted gRNA approaches (indicated with *) will be utilized for the next microinjection attempts.

<table>
<thead>
<tr>
<th>Method</th>
<th>Microinjection</th>
<th>Cas9 mode</th>
<th>gRNAs used</th>
<th>Plasmid Type</th>
<th>Inhibitor</th>
</tr>
</thead>
<tbody>
<tr>
<td>Standard</td>
<td>1-cell zygote</td>
<td>Cas9 protein</td>
<td>4, 2-2 gRNA</td>
<td>Linear plasmid</td>
<td>-</td>
</tr>
<tr>
<td>Endogenous Cas9</td>
<td>1-cell zygote</td>
<td>Endogenous Cas9</td>
<td>4, 2-2 gRNA</td>
<td>Linear plasmid</td>
<td>-</td>
</tr>
<tr>
<td>Two-cell*</td>
<td>2-cell embryo</td>
<td>Cas9 protein</td>
<td>4, 2-2 gRNA</td>
<td>Linear plasmid</td>
<td>-</td>
</tr>
<tr>
<td>NHEJ Inhibitor</td>
<td>1-cell zygote</td>
<td>Cas9 protein</td>
<td>4, 2-2 gRNA, gRNA to cut plasmid</td>
<td>Linear plasmid</td>
<td>Scr7-inhibit NHEJ</td>
</tr>
<tr>
<td>Plasmid-targeted gRNA*</td>
<td>1-cell zygote</td>
<td>Cas9 protein</td>
<td>4, 2-2 gRNA</td>
<td>Circular plasmid</td>
<td>-</td>
</tr>
<tr>
<td>Lower concentrations of reagents</td>
<td>1-cell zygote</td>
<td>4-fold less Cas9 protein</td>
<td>4 fold less 4, 2-2 gRNA</td>
<td>Linear plasmid</td>
<td>-</td>
</tr>
</tbody>
</table>
**Plasmid-targeted gRNA development and in vitro validation**

Several gRNAs were chosen to cut near the 5’ end of homologous arm 1 (gRNA HA1_1, gRNA HA1_2, gRNA HA1_3, gRNA HA1_NA, and gRNA HA1_NB) or the 3’ end of homologous arm 2 (gRNA HA2_4, gRNA HA2_5, gRNA HA2_6, gRNA HA2_TA, and gRNA HA2_TB) (Figure 4-1 and Table 4-2).

*Figure 4-1. Location of the 10 gRNAs designed to cut the Sry plasmid.* The gRNAs were targeted to cut near the 5’ or 3’ end of the 13 kb construct sequence to linearize the plasmid.
Table 4-2. The sequences of gRNA designed to cut the Sry plasmid. The sequences of all 10 gRNAs tested for cutting ability of the plasmid.

<table>
<thead>
<tr>
<th>gRNA name</th>
<th>Sequence</th>
</tr>
</thead>
<tbody>
<tr>
<td>gRNA HA1_1</td>
<td>5’- TTGGGCTTCGAGTTAGCACCA-3’</td>
</tr>
<tr>
<td>gRNA HA1_2</td>
<td>5’- ATTGCGTCTGTGCGACAAGC-3’</td>
</tr>
<tr>
<td>gRNA HA1_3</td>
<td>5’- TGGCACAAGCCGGTGAGACG-3’</td>
</tr>
<tr>
<td>gRNA HA1_NA</td>
<td>5’- TGGCACAAGCCGGTGAGACG-3’</td>
</tr>
<tr>
<td>gRNA HA1_NB</td>
<td>5’- TTACCCAGGGGACAATCTTA-3’</td>
</tr>
<tr>
<td>gRNA HA2_4</td>
<td>5’- AGTGGCTTTGCCTAGCAATG-3’</td>
</tr>
<tr>
<td>gRNA HA2_5</td>
<td>5’- TCTAATCACCTAACTCTTAAG-3’</td>
</tr>
<tr>
<td>gRNA HA2_6</td>
<td>5’- TCTCAGACACAGCTGTACT-3’</td>
</tr>
<tr>
<td>gRNA HA2_TA</td>
<td>5’- GAGTGGAGGCGCTTGCCACAA-3’</td>
</tr>
<tr>
<td>gRNA HA2_TB</td>
<td>5’- GACGTGCTGAGGAGACAAA-3’</td>
</tr>
</tbody>
</table>

The cutting ability of the gRNAs were assessed in vitro using a 1 kb PCR-amplified region of homologous arm 1 or 2 (Figure 4-1). The results (Figure 4-2) demonstrate that gRNA HA1_2, gRNA HA1_3, gRNA HA1_NA, and gRNA HA1_NB and gRNA HA2_5, gRNA HA2_6, gRNA HA2_TA, and gRNA HA2_TB are clearly able to result in cutting of the plasmid ex vivo. Because the cut sites for gRNA HA1_1 and gRNA HA2_4 are very near the ends of the PCR amplicon, it is not possible to assess their efficiency with this assay. The gRNAs were then tested for the cutting ability of the intact 15kb circular plasmid, but effectively visualizing the changes in the supercoiled versus linear plasmid prove difficult (see Appendix B).
In vitro validation of the plasmid-targeted gRNAs using PCR amplicons. The cutting efficiency of each gRNA was tested using a 1 kb amplicon of homologous arm 1 (left side of gel) or a 1 kb amplicon of homologous arm 2 (right side of gel). The lanes labeled “PCR” were the 1 kb amplicons alone. The presence of more than one band indicates cutting. The positive control included a gRNA, target sequence, and Cas9 provided with the Guide-it sgRNA Screening System kit.

In vivo approaches for generation of transgenic mouse

An alternative method to the CRISPR/Cas9 egg or zygote microinjection strategy is to exploit the traditional approaches of generating transgenic mice that utilizes embryonic stem cells (ESCs). Briefly, the genomic manipulations are carried out in ESCs under cell culture conditions. Once the desired genetic changes are integrated and validated, the genetically manipulated ESCs can then microinjected into an established blastocyst and implanted into a pseudopregnant dam. The resulting chimeras are then mated to identify germline carriers of the modification. To apply this strategy to our model, we developed t<sup>w<sup>2</sup>-carrying ESCs and induced pluripotent stem cells (iPSCs).
Mouse Embryonic Fibroblasts (MEFs)

Several cell lines were generated from $t^{w2}$ mice. Primary MEF lines were generated from 12-14 day old embryos. Mixed genotype and sex (pooled, Figure 4-3) as well as separate lines were generated, with male and female $+/+$, $t^{w2}/+$, and $t^{w2}/t^{w2}$ lines.

![Confluent plate of MEF cells](image)

**Figure 4-3. Confluent plate of MEF cells.** These cells were generated from a mixed embryo population (male, female, wildtype, and $t^{w2}$), 50x magnification.

Induced pluripotent stem cells (iPSCs)

Two iPSC lines were generated, one from a mixed MEF population and one from a male $t^{w2}/+$ MEF line. The MEF cells were transduced with the Sendai virus and the reprogramming factors KOS, Myc, and Klf4 and then observed for the formation of colonies (Figures 4-4, 4-5, and 4-6).
Figure 4-4. iPS cells one week (day 7) after transduction. A), B), and C) represent different areas of the plate where stem cell-like colonies are starting to form.
Figure 4-5. iPS cells on day 30 after transduction. Colonies are still spread out across plate, but starting to become bigger. A) and B) two different colonies on the same plate, 50x magnification.

Figure 4-6. Day 40 iPSC colonies. Colonies have reached their maximum size and need to be passaged. A) and B) two colonies from the same plate, 50x magnification.

Since the first iPSC line developed was of a mixed population of MEF cells, sub-cloning was employed to get a homogeneous population. The ultimate goal was to have a male
The $t^{w2/+}$ iPSC line. However, this process is tedious and the majority of sub-cloned colonies were later determined to be female. Several MEF lines were then established by keeping each embryo separate instead of pooled by litter, with the genotype and sex being later determined by PCR. A male $t^{w2/+}$ MEF line was then used for iPSC generation (Figure 4-7), eliminating the need for downstream sub-cloning and separation by genotyping and sex.

Figure 4-7. Day 20 iPSC cells of second round. A), B), and C) colonies on the same plate at 50x magnification.
There are advantages and disadvantages to generating either iPSCs or ESCs. One advantage of generating iPSC is that live mice are not needed, only a MEF culture. The process of inducing pluripotency is also less technically demanding than generating ES cells, the reprogramming factors are added and then the cells are observed until colony formation occurs, where the colonies are rounded with distinct borders (Figure 4-8). However, one disadvantage of generating iPSC cultures in this manner is the time it takes for the Sendai virus to clear the cells and for the MEFs to become fully reprogrammed. Both ESC and iPSC-based methods were therefore used in parallel here.

Figure 4-8. Growth of iPS cells across passages. A) a passage 3 plate, B) a passage 4 plate, C) a passage 5, and D) a passage 5 right before being passed again. The amount of stem cell like colonies are increasing and the amount of MEFs are decreasing with every passage.
Embryonic Stem Cells (ESC)

Primary ESC lines were generated from E3.5 blastocysts collected from a 129S1/SvImJ female mated to a t^{wo}+/+ male. The blastocysts were collected and allowed to grow on a plate until the inner cell mass (ICM) expanded (Figure 4-9).

Figure 4-9. The progression of a blastocyst culture. A) Immediately following collection. B) a 24 hour blastocyst is in the process of hatching from the zona pelucida. C) a blastocyst that has completely hatched, 48 hours. D) the cells attached to the plate, 72 hours. The inner cell mass (ICM), which gives rise to the embryonic stem cells (ESCs) is in the middle and the flat cells surrounding it are trophoblast cells. E) the 96 hour and F) the 120 hour progression of the cell growing and the ICM increasing in size. All panels are at 50x magnification.

After the ICM increased in size, it was moved to a new plate for stem cell colonies to form (Figure 4-10). The difficulty is only moving the ICM cells and not the more differentiated cells, such as the trophoblast cells that surround the ICM. Once the ES colonies formed, they were moved to a new plate and expanded (Figure 4-11).
Figure 4-10. Progression of an ES cell colony. A) the colony formation 4 days after it was removed from a gelatin plate to a MEF feeder layer, 50x, B) 5 days 50x, C) 6 days, 100x, D) 7 days, 50x, and E) 8 days, 100x magnification. The visible ES colonies were passed for the first time on day 8.
Figure 4-11. Expansion of stem cell colonies. A) and B) represent two separate ESC cultures on day 5 of passage 1, 50x magnification.

**FUTURE STEPS**

Once a stable line of either iPSCs or ESCs are generated, the Sry construct can be inserted into the t-haplotype sequence in the cells through electroporation and the CRISPR gene editing capabilities. These transgenic stem cells can then be used to generate the t/Sry mouse. Once the mouse has been generated, either through microinjection or stem cells, there will need to be studies on the fitness of the males and the transmission ratio of the t-haplotype containing the Sry gene. This will be important to understand if the transgenic model will be able to successfully spread through an island population with the purpose of causing a population eradication of the invasive species. Modeling the various scenarios will be important for this step (61). There are also different transgenic models that can be explored, such as killing one of the sexes or making one sex infertile (60). Creating a synthetic gene
drive using CRISPR would make the genetic eradication model applicable to other species besides *Mus musculus* and enable unique sequences to be targeted to avoid the unintended spread of the gene drive past the island. Resistance to the gene drives is also a consideration in the transgenic models (88).

Beyond invasive species eradication, another use of gene drives would be for disease resistance, similar to what is being done in mosquitos (28, 29) This same idea can be applied to mammal disease vectors, such as preventing *Peromyscus* sp. from transmitting Lyme disease (89)

Public perception is another concern for the success of this new model. The public is generally accepting of genetic modification in medicine applications, but more resistant to genetic modification for food or for eradication purposes. Another concern would be to ensure bio-containment of the transgenic species on the target island to prevent unintended spread of the gene drive. These concerns need to be addressed before seeking government approval for the use of this transgenic species for eradication purposes.


12. South Farallon Islands Invasive House Mouse Eradication Project; Farallon National Wildlife Refuge, California; Revised Draft Environmental Impact Statement. Lanham: Federal Information & News Dispatch, Inc; 2013 October 25,


23. Vanderplank F. Experiments in the Hybridization of Tse-tse Flies (Glossina diptera) and the possibility of a New Method of Control. Transactions of the Royal Entomological Society. 1947;98:1-18.


and increase their feasibility on islands. Biological Conservation. 2015 May;185:47-58.


APPENDICES
Appendix A

13 kb Sry construct sequence

5'-
TACAGTGTTGTATATAAAATACAAATAATATCTTTTAAAATCTTTAAATAATAT
ATAAAATATATATACTTTAATACACGATTTTTTTTTGTCTTTAGAAAAGACACACTGTGTGT
GATTTAATGTTCTCTGTCTCCCTGTGTCTAACGTGCAGCCAAAGATCTCTGTGTCT
CCCAACTGGAAGTGTGTTTTATTTTGTGATGTTAAATATTTGGATATGAAATTTGGGAT
GGGAAGAAAGGAAGGACTATGCTTTAAACGATATTGCTTGAGCCAGCACAGCTG
GCACAAGCCGGTTGAGAGGAGGAGAATCTCGAACAGTCGCCCAAGACACACTGTG
CTGTTGGGGTGATGGCATTCTCTACGCTCTTCCTGTGTTTGTTGATAAACGAGGCTAG
CTTTCTGTCTCTAGGCTTTACGCTTCACGCTTTAGGTTGAACTCTATGAGACTGAC
GTCCACAGTTCTCTATTTGCTCATTAGGCAGACACAGGCCAAACCAGCCTTCCACT
ATACAGACAGACAGCATAAGCATTTCCTTATTTGCTATACAGATTTTTTATAT
TAGAATGAGAAAGGATAGAGGATGATGTTGCAAACAAAGGAGAATCTCAA
GGGTGATTAGCTTGGTGACAAAGCTATAACAGTAAACATACATGTGTGCTAGTC
CTGTTAAGCTTCTGTTTACATTACGCAATGCACTTTGTGTTGAGAGATGACATATGTGTG
CTGTGGAACGATAGTGGACCTGTGGGTGAGATGAGAGGAAGTCTCTGTGTG
CTTCTCCAGGTGTTCTTTTGGTCATGGTGTGTTTTACACAGATAGACGGCTG
GAAAGGATGTTGTGATCAGGTTGACTGCAACCTCCTCAGGGAGATCCAAACATC
ACTTTGGTGTGAATATACACTATGACAGATACTGAAAACAGAGACGTTT
ATTTGATTACAATTTCATCATTACGACTGTCGTGTCGCTATAGACTCCAAACAGG
CAGGAACCTGGAAGGACTATGCTTGAGCCAGACAGAACAGGCAGCTC
GGCCACAGTTCTCTACGCTCATTAGAGCTTTGTGTTGATGAGAGATGACATATGTGT
GCTGTGGAACGATAGTGGACCTGTGGGTGAGATGAGAGGAAGTCTCTGTGTG
CTTCTCCAGGTGTTCTTTTGGTCATGGTGTGTTTTACACAGATAGACGGCTG
GAAAGGATGTTGTGATCAGGTTGACTGCAACCTCCTCAGGGAGATCCAAACATC
ACTTTGGTGTGAATATACACTATGACAGATACTGAAAACAGAGACGTTT
ATTTGATTACAATTTCATCATTACGACTGTCGTGTCGCTATAGACTCCAAACAGG
CAGGAACCTGGAAGGACTATGCTTGAGCCAGACAGAACAGGCAGCTC
GGCCACAGTTCTCTACGCTCATTAGAGCTTTGTGTTGATGAGAGATGACATATGTGT
GCTGTGGAACGATAGTGGACCTGTGGGTGAGATGAGAGGAAGTCTCTGTGTG
CTTCTCCAGGTGTTCTTTTGGTCATGGTGTGTTTTACACAGATAGACGGCTG
GAAAGGATGTTGTGATCAGGTTGACTGCAACCTCCTCAGGGAGATCCAAACATC
ACTTTGGTGTGAATATACACTATGACAGATACTGAAAACAGAGACGTTT
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CAGGAACCTGGAAGGACTATGCTTGAGCCAGACAGAACAGGCAGCTC
GGCCACAGTTCTCTACGCTCATTAGAGCTTTGTGTTGATGAGAGATGACATATGTGT
GCTGTGGAACGATAGTGGACCTGTGGGTGAGATGAGAGGAAGTCTCTGTGTG
CTTCTCCAGGTGTTCTTTTGGTCATGGTGTGTTTTACACAGATAGACGGCTG
GAAAGGATGTTGTGATCAGGTTGACTGCAACCTCCTCAGGGAGATCCAAACATC
ACTTTGGTGTGAATATACACTATGACAGATACTGAAAACAGAGACGTTT
ATTTGATTACAATTTCATCATTACGACTGTCGTGTCGCTATAGACTCCAAACAGG
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CTTCTCCAGGTGTTCTTTTGGTCATGGTGTGTTTTACACAGATAGACGGCTG
GAAAGGATGTTGTGATCAGGTTGACTGCAACCTCCTCAGGGAGATCCAAACATC
ACTTTGGTGTGAATATACACTATGACAGATACTGAAAACAGAGACGTTT
ATTTGATTACAATTTCATCATTACGACTGTCGTGTCGCTATAGACTCCAAACAGG
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GGTGCAAGCCTGGGCTGCTGCTGTCTGTCTGTCACAGACCTCCAGCTCAAAAGGGCCC
TTGTGGCAAGGCCCTCCAACCTCTCATCTCCTCTTCTGTCTCCCTTTGTCCTCCTCAGC
ACGTCACCTGATATGTCACATACCCCTCCTGCCACCAGAAGTGACTAGGCTTCT
CATTTCGAGGTCTGTGCGCTGGCAGCGGCAGTGACAGTGCTGTGCTGTGTGCC
CTTGTCCTGCCGCTGCTGCTGCTGCTGCTGCTGAGATGCTCTCTCAGAGCTTCTCAGA
CAAGCGTGACTTGGGCTAAGTATAAATTGTGGCCATTGCAACTGTCTGCAATCCTC
TTAGAGTTAGGTGATTGAGATTGTGTGGGAGGAGAGTGCGCTTGCCCTAGCAAT
GAGGGAAGGTGCTAAGTCTCACAACAGTGCTGGTCACAGTGCTCTGGAAGGCCCTTA
CTC-3’
Appendix B

Figure A. gRNA validation in the circular plasmid. Samples run on a 0.75% agarose gel. A) Gel results after 60 minutes and B) gel results after 90 minutes.