AMAZING PAPERS:
Teaching the Applications of CRISPR/Cas9: Using the African Turquoise Killifish as a Novel Model of Aging and Age-Related Diseases

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The development of genome editing technologies, including the novel CRISPR/Cas9 technique, has advanced scientific research concerning the contribution of genetics to disease through the creation of new model organisms. The subject of this review is a 2015 study done by Harel et al. from the journal *Cell*. This study is a prime example of using CRISPR/Cas9 to create a new model organism to accurately model the effects of aging and age-related diseases on a short-lived vertebrate. This study found that the African turquoise killifish is a reliable model to study the physiological process of aging due to its compressed lifespan. In addition, it provides a genotype-to-phenotype platform to study genes related to the hallmarks of aging and age-related diseases. This paper demonstrates this by showing that killifish deficient in the protein subunit of telomerase display telomerase-related pathologies faster than other established vertebrate models. From a teaching perspective, this paper could be used as a resource for educators to teach students about new technologies emerging in the field of neuroscience and the importance of model organisms. Specifically, for upper-level undergraduate students, this paper could serve as a real-world example of how scientific techniques such as CRISPR/Cas9 could be used to answer scientific questions. Further, it shows how these techniques could bring forward new model organisms better suited to answer the scientific questions being asked. Learning these techniques and being open minded to new approaches will be advantageous to students' future careers in science.

Key words: genome editing; CRISPR/Cas9; model organism; African turquoise killifish; age-related diseases; aging

An active field in neuroscience today is the study of aging and its relationship to neurological diseases. Aging, measured in part by the reduction of size in chromosomal telomeres, is associated with a myriad of chronic diseases such as hypertension and dementia (Rizvi et al., 2014). Scientists are interested in how telomere reduction and other genetic factors contribute to differences in aging across individuals and whether genetic markers can contribute to early detection and treatment. The subfield of genetic research itself has exploded in popularity among neuroscientists in recent years due to the development of the CRISPR/Cas9 system of genome editing, a process which allows scientists to program the prokaryotic Cas9 protein to make extremely localized cuts in a cell's DNA, allowing for the deletion and insertion of specific genes. Using this method, scientists can more accurately model human genetic pathologies within animal models (Cong et al., 2013; Platt et al., 2014). With such powerful tools available, it is important that neuroscience students learn how they can utilize such techniques to better understand and form more specific research questions. In this paper, we review the work of Harel et al. (2015), who identified a novel model organism, the African turquoise killifish, as an efficient platform for genetic research concerning age-related disease, and we discuss how their work can be used in an undergraduate teaching environment.

RESEARCH ARTICLE

Using the novel genome editing technology CRISPR/Cas9 to manipulate the genome of the African turquoise killifish, Harel et al. (2015) produced an optimal platform to study aging and age-related diseases. The killifish has a lifespan shorter than any other vertebrate model used in scientific research. This presents a promising tool for carrying out high throughput, longitudinal studies on aging which have proven difficult with previous long-lived, vertebrate models. The killifish's compressed lifespan of 4-6 months, which is 6-10 times shorter than other vertebrate animal models, such as mice (3-4 years) and zebrafish (5 years). This allows scientists to examine phenotypic traits of aging more efficiently. In addition, the killifish presents many phenotypes similar to those in humans, making them a viable model for studying human aging and disease. For example, they show cognitive degradation, a decline in fertility, sarcopenia, and neurodegeneration. The killifish also has important physical similarities to humans. These include organs and biological systems conserved across vertebrate species, as well as similar telomere lengths, which are an important measure of aging. These similarities, their short life cycle, and the fact that they are cheap and easy to maintain makes the killifish an ideal model for studying aging and age-related diseases.

The use of the killifish in scientific research has previously been limited due to its un-sequenced genome and the lack of tools to manipulate the genome. The innovative CRISPR/Cas9 technology has advanced scientific research and genome editing techniques. The most influential application of CRISPR/Cas9 has been the creation of genetically modified cell and animal models for numerous human diseases. It has also shown great

promise in future therapeutic applications. Since its development, CRISPR/Cas9 has been widely used on animal models such as yeast, flies, worms, zebrafish, and mice. The current study provides the first platform for examining aging using the killifish alongside CRISPR/Cas9 manipulation. Using this methodology, genes involved in the known hallmarks of aging can be genetically altered for proof of principle studies. In addition, CRISPR/Cas9 can be used to explore new genes of interest involved in the biological process of aging, including pathological aging which results in disease.

First, researchers generated a wide range of genomic data sets since the killifish is an emerging animal model. After generating the data set, they were able to narrow it down to 13 genes that are known to play a part in the hallmarks of aging. They were able to build genetic models using the sequenced killifish genome. The data sets that were generated were useful to target genes in the killifish, as well to serve as a resource for comparative genomics, aging, and longevity studies.

Next, Harel et al. (2015) designed a CRISPR/Cas9 genome editing strategy that allowed them to test the effects of aging on different genes. First, they tested their strategy by studying *TERT*, a gene that encodes the enzyme telomerase, to model telomere attrition. Telomerase elongates telomeres after replication and is important in preventing telomere shortening, which researchers use as a biomarker of biological age. Mutations in *TERT* in humans can result in diseases that result in tissue homeostasis failure. This in turn causes dyskeratosis congenita, which is characterized by symptoms of premature aging. Therefore, modeling mutations in *TERT* provides valuable information as to how these mutations can lead to pathogenic aging phenotypes. The researchers found that telomerase components are well conserved between the killifish and humans, allowing for the successful application of CRISPR/Cas9 to the animal model.

Further, killifish have advantages for modeling *TERT* mutations over other vertebrate models such as mice, since they need to breed for 4-6 generations to manifest a phenotype. This is because their telomeres are longer than humans' and take more generations to shorten enough for phenotypes to manifest. Harel et al. (2015) were able to use CRISPR/Cas9 to produce a stable line of genetically modified *TERT* within two months, showing that rapid genome editing of the killifish is possible. The modified killifish with a deficiency in the protein component of telomerase were shown to be outwardly normal but exhibited loss of telomerase function. Thus, demonstrating that the killifish is a viable animal model for manipulating *TERT* to study aging.

Harel et al. (2015) confirmed that the killifish is a reliable model to study human disease, as it was found that TERT-deficient fish show signs of genetic anticipation, in which symptoms of a genetic disorder increase in severity, or present earlier in the next generation. This is consistent with human disease, thus showing the proof of concept for genome editing in a short-lived vertebrate to test the effects of human genes on aging and disease. To further examine the viability of the killifish model, they tested if they could replicate a specific mutation that causes disease in humans. Since many human diseases are caused by non-synonymous mutations (a mutation that changes the amino acid sequence of an encoded protein), they used a known non-synonymous mutation in humans that causes dyskeratosis congenita and were able to successfully replicate it in the killifish. This demonstrated the feasibility of using the killifish as a model organism for human genetic diseases.

Finally, the researchers tested the success of their platform through proof of principle studies on genes that are common hallmarks of aging. Some genes have been proven to affect lifespan in both invertebrates and vertebrates, but many have not. It is important to use a vertebrate animal model to study these genes, as they do not have orthologs in invertebrates or yeast. The researchers made gene models and predicted protein sequences for 13 genes that were associated with aging. They were able to successfully manipulate 11 genes in the experimental population of killifish. They then studied how these manipulations manifested in and affected the development of subsequent generations. Of the original 11 genes manipulated, 5 exhibited germline transmission. The platform that this study has created will be extremely important in the scientific community, as it is a valuable tool to study aging and age-related diseases in vertebrates.

TEACHING VALUE

Harel et al. (2015) shows students the process of applying CRISPR/Cas9 to a novel model organism. Importantly, the paper covers every step of the CRISPR/Cas9 process. Students can follow along with the process of identifying target genes, creating modified RNA sequences which act on those genes, and recording the phenotypic variations which results from this genetic manipulation. This is an organic way for students to learn the preparatory work that must be done before genetically modifying novel organisms. Furthermore, the paper provides students with a real-world example of the kinds of research questions CRISPR/Cas9 can be used to answer. Given that CRISPR/Cas9 is an important neuroscientific method in use today, it is important that students study resources such as this which provide a comprehensive overview of the methodology in a relevant laboratory context.

While Harel et al. (2015) is a valuable example of the successful application of CRISPR/Cas9 technology, educators should also use it to demonstrate the continued relevance of animal models in neuroscience research. Animal research has long been the focus of ethical debate both within the neuroscientific community and in the public sphere. Recently, the conversation has focused on whether animal research is still useful/ethical given the advances made in stem cell and in-vitro research models (Hunter, 2008). Cerebral organoids, for example, allow scientists to explore brain development in-vitro using stem cells harvested from an adult human patient; they allow for a personalized neurological model which mirrors the patient's unique genetic makeup. Furthermore, scientists have already used cerebral organoids to explore the genetic causes of diseases such as microcephaly which do not

manifest in mouse models (Lancaster et al., 2013). With such exact recreations of human neuroanatomy available for manipulation, students may falsely believe that animal models are becoming less relevant to neuroscience research. Like all research methods, however, in-vitro methods have their limits, namely that cultured cells and organoids cannot be used to explore diseases involving adult neurodevelopment. Harel et al. (2015)'s killifish model, with their rapid development, short generation times, and receptibility to CRISPR/Cas9 genetic modification, uniquely address this problem. This example suggests to students that animal models remain a valid counterpart to advanced, in-vitro methods.

With their killifish model, Harel et al. (2015) also demonstrate that scientists continue to find and use animal models uniquely tailored to answering specific questions. As they suggest, established vertebrate models such as mice and zebrafish are unsuitable for studying late-age onset diseases due to their long lifespans. In addition, quickly generating invertebrate models such as yeast do not have the complexity to faithfully capture the various aspects of human age-related diseases. These established animal models, like in-vitro models, are not suited for exploring aging, a primary risk factor for most neurodegenerative diseases. Thus, the Killifish could have an immense impact on uncovering new therapies for these diseases.

The killifish model will become an important model organism for studying the neuroscience of aging, making Harel et al. (2015) an important paper for students to study. Due to its advantages, the model has already been used to study Parkinson's Disease (PD; Matsui et al., 2019). Previous animal models used in neurodegenerative disease research have been unsuccessful in fully recapitulating the human phenotypes seen in this disease state. Modeling the age-dependent neurodegeneration of neurons using the killifish is useful in uncovering the pathological mechanisms of these diseases. Specifically, this model is useful in modelling the idiopathic forms most seen in humans. One of the main problems with extrapolating results from current neurodegenerative disease models to the clinic is the discrepancy of the pathological timeline of disease. In humans, most neurodegenerative diseases present symptoms and lead to death in old age. This has been difficult to replicate in model organisms which manifest symptoms at an early age. This model provides a significant advantage in studying complex neurodegenerative diseases.

In conclusion, the authors looked outside of the boundaries of established animal models to find the killifish, a model which suited the requirements of their research. It is important for neuroscience students to learn that animal models are not only still relevant, but that scientists continue to discover new organisms tailored to answering questions that current models cannot address. Their own research questions may one day require novel animal models; they should know that, somewhere, a yet unused organism may fit their requirements. Harel et al. (2015) demonstrates that animal models are not a static, declining methodology and that enterprising neuroscientists can utilize novel organisms well-suited for specialized fields of research.

AUDIENCE

The Harel et al. (2015) article is relevant across different disciplines and educational levels in the field of neuroscience, from introductory courses to upper-level undergraduate and specialized postgraduate courses. In an introductory level course, this article would help to highlight the continued importance of animal models and encourage students to think critically about what they consider to be ethically appropriate when using live animals in experiments. In addition, this paper would allow students to discuss the field of neuro-ethics, which is currently growing due to the development of new technologies such as CRISPR/Cas9. The opportunity for students to apply critical thinking skills when discussing topics such as genome editing with CRISPR/Cas9 and the use of animal models would be ideal for a beginner-level course. Alongside the chance for students to explore the ethical considerations of this article, educators could also use this article as a model example for an experiment that uses genome editing with CRISPR/Cas 9. This article could serve as a basis for exam questions about ethical considerations/risks of an experiment of this nature, serve as a hypothetical scenario for in-class discussions about the necessity of animal experimentation, and serve as an example for project proposals using genome editing techniques.

In specialized postgraduate courses, this article could serve as required reading for students interested in using genome editing in their future research. The further development of courses that focus on teaching students the techniques required to perform the kind of research detailed in the article could prove to be important to neuroscience programs at different universities. The incorporation of such courses will allow for students to get involved in more varied types of research during their educational careers. In addition to this, it can give students a chance to learn with a more hands-on approach when using animal models and techniques such as CRISPR/Cas9, leading to a broader understanding of the approach and methodology. Learning how to utilize these techniques early in a student's career is advantageous and will lead to success in their future professional research environments.

Overall, the paper by Harel et al. (2015) is a useful resource for scientists, but it can also serve as a teaching tool for neuroscience students. The incorporation of this article in early and late-career stages in neuroscience programs will benefit young researchers and aid in progressing the field of neuroscience research.

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