Pic 1Transgenic Mice

SINGLE-SEX LITTERS

Filip Colovic, Noah Hunziker and Michael Windsor

Content

1. Preface

Increasing temperatures due to climate-change has affected various animals in different ways. Birds of paradise for example have started to decrease in size, due to their bodies not needing to retain heat.[7] Sea Turtles are other animals threatened, not only by the advance of climate change but by the fishing industry.[9] In various Asian countries their carapaces are used for medicinal purposes. The sea-turtles lay and bury their eggs on sandy beaches, where the sex of the offspring is determined by ambient temperature. With the rising mean temperatures more and more turtle hatchlings end up being female, further threatening the survival of one the oldest species, one that is already close to extinction.[8] The fact that there was a study, occupying itself with the breeding of mice through gene-manipulation, was extremely interesting. We had already asked ourselves, whether the technique could be used on animals close to extinction, in order to breed sexes, which are lacking in the wild.
Could this technique be used on reptiles, like the endangered sea turtles?
Could this technique find application in other areas and not just in farming test mice?

2. Introduction

-What is the context of the chosen topic?

Currently, in many areas there is a need for a single sex litter. This need can be found in agriculture, where a dairy farmer only has use for female bovine, as only a cow produces milk. A beef farmer mainly requires male bovine, as the increased muscle mass leads to leaner meat.[1] Likewise, one can find the same need in laboratories, that study sex specific diseases, like breast- or prostate cancer and so the mice which get tested must be of one specific sex.[5]
The surplus of the undesired sex results in them being culled, which is a waste of resources but at same time an ethical burden.[4] The chosen topic deals with this issue, trying to alleviate it.

-What is the scientific history?

The concept of gene editing was first conceived in the 1970s when they successfully created transgenic mice for the first time using transgenesis. Transgenesis was a powerful tool to analyse a multitude of disease, however it was incapable of inserting itself into genomes, limiting itself. In the 1980s, they soon began dabbling with embryonic stem (ES) cell therapy which could direct embryonic stem cells to transform into desirable cells. Unfortunately, the success rate was appallingly low, less than 10% success rate, and brought abortion and human rights controversy along with it. The progression in gene editing technology became mostly stagnant for the next twenty plus years, until 2005, when artificial restriction enzymes were first synthesised, which allowed for genomes to be cut and allowed for gene insertion. Zinc finger nuclease (2005) and later transcription nucleases (TALENs) (2010) were now at the peak of gene editing technology, until it was found out, that Crispr found in E. coli (1987) could be used in gene editing.[3]

-Where and why is the technique used?

This technique currently finds application in laboratories only, although it has potential for use in fields outside of research, such as agriculture and pest control. The reason for the use in laboratories, is to increase animal well-fare. Studying male or female reproduction requires lab-rats of the required sex. For agricultural farming, certain sexes are preferred over the other, resulting in culling.[1] With this technique, unnecessary execution is preventable. In pest-control the induction of an only male population results in fewer females laying eggs and ultimately the reduction of the number within the colony.

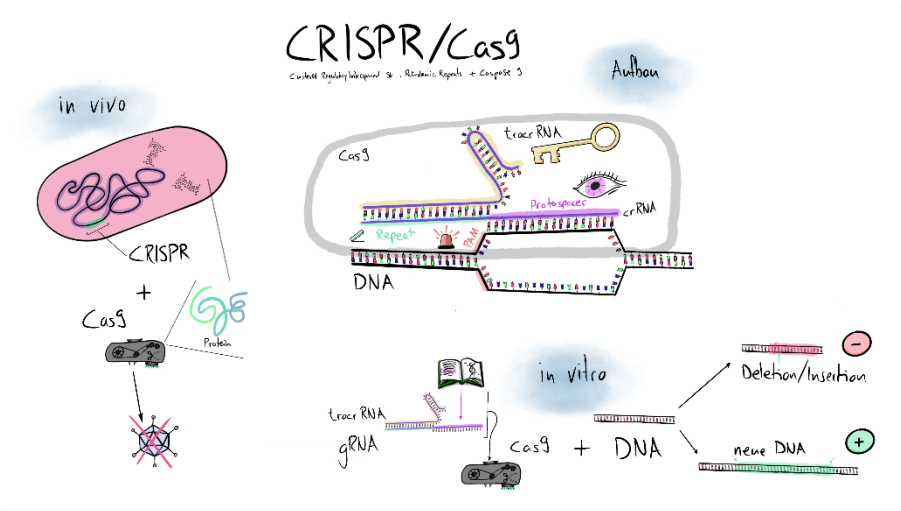
-Are there alternative treatments to CRISPR/Cas9?

Alternative treatments include a sorting of X- and Y-bearing sperm. This technique is used to a certain degree in cattle, although a reduced fertility rate is associated with this method. On top of the fertility reduction, sperm sexing, as it is called, is not feasible in other species.
A second alternative includes embryo selection via lethality of male or female embryos. This method is widely applicable, in order to control the offspring's sex ratios. This selection can be achieved by a synthetic lethal strategy, in which a dormant suicide gene carried on the paternal X or Y chromosome is inherited in a sex-specific manner. This suicide gene is activated by a second trigger gene contributed by the mother. Embryos inheriting both genes die, while those carrying only one of the genes survive.[3]

3. Description of engineering technique

-Explanation of the applied technique.

In order to drastically reduce culling, researcher have used CRISPR/Cas9 to genetically alter the offspring. The method consists of a two-part system, that inactivates embryos shortly after their fertilization, thus allowing only the desired sex to develop.
CRIPSR/Cas9 consists of two parts. The guide RNA carries the Cas9 to a specific location on the genome, allowing the Cas9 enzyme to then alter the region.
In this instance the researchers targeted the Top1 gene, which is responsible for the DNAs replication as well as its repair. The scientist placed one part on the mother's X-chromosome and another on the fathers X- or Y-chromosome, respectively depending on the desired sex. In order for the mutation to take effect, the offspring must inherit two altered chromosomes, which results in the triggering of the gene. This leads to the embryo not developing past 16 or 32 cells. To produce a litter consisting of only males, the fathers as well as the mothers X-chromosomes were altered in order to eliminate any X, X-embryos. In order to produce only females, the researchers altered the father’s Y-chromosome and the mothers X-chromosome, leading to the elimination of all X, Y-embryos.[2,5]



Picture 2: CRISPR/Cas9 system

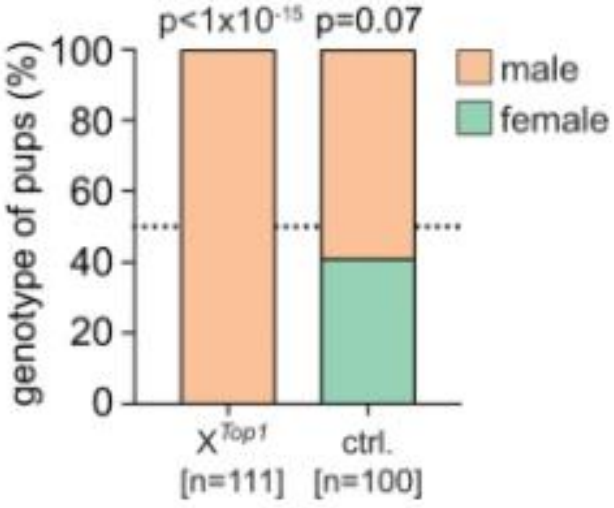


Diagram 1: Graph displaying the result of the X,X-Chromosomes having Top1 targeted
p-value shows deviation of 1:1, n-value stands for the number of offspring

4. Documentation of research institutions visited

Q: What I do not fully understand is the respective role of the sgRNA2 and 3 guides. From what I read the two different guides guide the Cas9 enzyme to different locations within the gene.

A: We simply wanted to test several guides to see which ones worked best. In CRISPR/Cas9work, the binding of guide RNA to the chromosome is affected by chromatin structure, e.g., the precise location of nucleosomes along the DNA and what histone modifications they have. Although you can try and predict which guides will work well, it's always best to just try a few to be sure.

Q: Do you then cut out base sequences from the Trop1 DNA, thus inhibiting it, or add base pairs to create a nonsense? And do the different guides just guide the Cas9 enzyme to different locations in the Top1 gene, or do they fulfill other functions?

A: The guide DNA simply guides the Cas9 to the Top1 DNA and makes it cut the gene. Different guides will create cuts in different parts of the gene. Once the DNA has been cut, the cell will repair it – but if it repairs it perfectly, then the guide RNA will still be able to bind and so the Cas9 will cut the DNA again. This goes on and on - potentially several cycles of cutting and re-joining – until the cell makes a mistake during the repair and either adds or removes a few bases. Once that happens, the guide RNA cannot bind anymore and Cas9 will stop cutting the DNA. The extra bases added or removed during the process then alter the Top1 gene, usually creating a frameshift that destroys the gene function. So, effectively, the gRNA tells Cas9, "keep cutting this piece of DNA until the gene it encodes is no longer functional"

Q: What do you use as the vector?

A: We used several different vectors for different parts of the process, each described in the relevant parts of the materials and methods.

Q: What method do you use to clone the guides into the vector?

A: Usually, restriction enzyme digestion and directional cloning, but increasingly these days you can ask a company to synthesise the construct you want directly, which we did in some cases.

Q: So, the mutation is a Trop1 gene with a premature stop-codon, rendering it useless. By passing on the mutated gene, the offspring will not develop passed 16 to 32 cells. Did I understand this correctly?

A: If an embryo inherits both the Cas9 gene (from the father) AND the gRNA gene (from the mother) these will then act together in the embryo to cut and inactivate the Top1 gene. Once Top1 is inactivated, the cells in the embryo can no longer divide, and so the embryo will not develop.

Q: It was not too long ago that I read an article about the population decrease of sea turtles. This is due to the rising ambient temperatures, which cause many of the hatchlings to be female. As long as reptiles have the Trop1 gene or a gene analogous to that of the Trop1, one could use your technique to produce the lacking males. Is this technique even applicable to reptiles?
A: These three questions belong together, and there are a few factors to consider.
Firstly, this technique mostly doesn't give you any *extra* animals, it just means that the sex you don't want stop developing early in development, rather than having to cull them after birth. If you could breed sea turtles in captivity, then you can already produce whichever sex you want and use these in repopulation efforts.

Secondly, this technique only works for animals that have sex chromosomes. The reason it works is because the Cas9 gene is passed on by the father on either the X or the Y chromosome, meaning that you can kill off either the daughters (which inherit the father's X) or the sons (which inherit the father's Y). For sea turtles, sex is controlled by temperature, and not by sex chromosomes. For reptiles in general, it will depend on whether it's one that has temperature dependent sex determination, or sex chromosomes.

Thirdly (and see below) the barrier for any use of this type of technique to modify wild populations – as distinct from laboratory animals or domesticated livestock - is extremely high. I don't see it being used for anything except possibly control of pest species that cause significant death and destruction.

Q: If yes, have you had any thoughts about its application?

A: Yes – any application in species other than mouse will require significant work to be done in cell lines before any transgenic animals could be made. There may well be significant applications in the livestock industry, i.e., animals bred by humans in controlled conditions such as chickens and dairy animals. Related techniques are also being considered for management of pest insect species if you can induce them to be all males, then the lack of females means fewer eggs are laid, and so the population decreases.

Q: Do you perhaps have any photographs of the laboratory and procedures that I could share with my colleagues?

A: Unfortunately, I don't have photos I can share because the animal work was done by my colleagues at the Francis Crick Institute.[5]

5. Discussion

-What progress was made with the application of the chosen technique?
After the first series of experiments were complete after successfully creating a single-sex litter which only yielded males, the researchers decide to create sex chromosome-linked Cas9 transgenes. The Cas9 transgene is linked onto an eGFP (enhanced green fluorescent protein) via a sequence. When targeting the X-chromosome, Crispr/Cas9 was used to help assimilate a pCAG (mammalian expression vector) promoter-driven Cas9-eDFP into a specified genetic marker (Hprt locus). With Y-chromosomes, the Cas9-eGFP undergoes homologous recombination with the previous chromosome to help integrate itself into the specified genetic marker (Uty locus) in the Y-Chromosome. When this experiment was conducted four different times, it was revealed that the mean litters' mean sizes were 66%, 72%, 61% and 61%, meaning that the lethality rate was lower than 50%, which was the expected result.

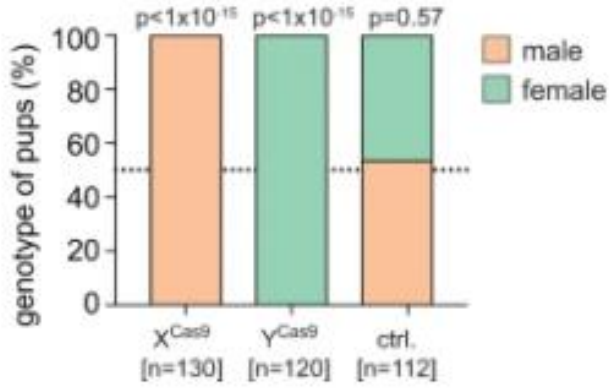


Diagram 2: Graph displaying the result of the X,X & X,Y-Chromosomes having Cas9 transgenes being sex chromosome-linked
p-value shows deviation of 1:1, n-value stands for the number of offspring

-What are future research steps?

The Crispr/Cas9 technique is a very versatile tool, which can be used in almost any given situation with regards to gene engineering. With the creation of single-sex litters being possible in mice populations, the prospect of undergoing this process with other animals opens a multitude of further research possibilities. Mammals which produce an overabundance of eggs and pre-implantation embryos, like pigs, would be the most logical candidates to further conduct this research on. With an excess of embryos provided by these, researchers will have a higher yield of the desired genotype from the offspring. Another possible direction in which this research will steer towards, would be single-sex litters in domesticated fowls. With the avian group's genders having their homo-/heterogametes reversed in contrast to mammals, this technique can only be applied to the embryos in females and not the sperm in males. In agriculture, pests are often the biggest factor when it comes to a loss of profit in the industry, whether it be the ravaging of crops or the requirement to invest in pest repellent products. With the implementation of this research technique on these pests, which are most often arthropods, you can deny further generations being spawned stemming from these individuals, since none from the opposite gender will be able to occur.

-Discussion of ethical aspects: Strengths (pros)

From an ethical perspective, the Crispr/Cas9 technique boast a drastically better success in fulfilling its function, in comparison to other outdated methods, with its success rate being nigh 100%. This eliminates any undesirable or spontaneous mutations that could occur. With that being said, single-sex litters also have an ethical appeal to them. If this technique were to be implemented in the dairy industry, the embryos would die off before they can fully mature to the point where they could perceive pain, making their culling painless, in contrast to calves being slaughtered in slaughterhouses. Once again, these genetically augmented organisms would not have any spontaneous mutations occur, rendering them essentially as the same as other members of their species.

-Weaknesses(cons)

On the other hand, this technique does have potential flaws. Firstly, Crispr/Cas9 is only a recently developed technique (less than 10 years), meaning that scientist have yet to fully unearth everything regarding these molecules, which could prove disastrous if a sudden threat related to Crispr/Cas9 would occur. There is also the concern, that humans would be subjected to Crispr/Cas9. Humans however are impervious to gene editing of this fashion, since we regularly encounter the bacteria which has this molecule incorporated within its metabolism.[11]

-Opportunities

CRISPR/Cas9 has great potential in many fields. In medicine, it can be used to cure hereditary, physiological, and pathological diseases. This would benefit doctors, as well as the victims of these diseases.[6] With the single-sex litters, it would usher in new opportunities in the global supply chain. With the meat and dairy industry, it would help reduce the production cost and boost overall productivity, which would benefit large-scale farms, big corporations, and consumers. As previously mentioned, it could also be used in agriculture. On the one hand, it can

help eliminate pest by genetically altering them to produce single-sex litters, reducing pest population which in turn increases crop yield. With Crispr/Cas9, it can also be used to genetically modify plants more efficiently, to have to be more robust, fertile, and productive. Once again, large corporations, stores, and the consumers would benefit from the overall increase in crop production.[10]

-Threats

The list of possible threats is held to an extreme minimum, with the only considerable threat, being the escape of the genetically altered animals and the passing on of the altered chromosome with the disrupted Trop1 gene. However, since the offspring is of one sex, it would not take long until the group of escaped animals dies off, and as a result their altered gene, too. From a financial standpoint, large corporations, small family farms, which are already dying out, would suffer further from the competition gap widening further with more GMO crops and single-sex litter livestock. This would also affect the environment, since if this method were to be abused because of humanity's voracity, the amount of greenhouse gas emissions would increase proportional to the livestock (especially cows).

6. Summary

-Short summary of the whole paper

The CRISPR/Cas9 technique is a very important discovery for the future. Considering the recent publication of the study, not a lot of experience has been gathered. In order to truly optimize the technique, further research must be conducted, as well as to expand its use in other fields of application. The method should find use in areas such as agriculture, pest-control, farming of test mice and perhaps medicine. Although so far, its use has already been applied to breed sex specific mouse-litters, as well as sex specific phenotypes. The resulting benefit lies in the fact, that there is no undesired sex breed and that as a result, no culling takes place, drastically improving animal welfare.

7. Table of figures

Table of figures:

8. Bibliography

(1) Barthel, T. (kein Datum). Animal Gender and the Taste of Meat. (s. f. association, Hrsg.) Snake River Farm, Becker, Minnesota, United States. Abgerufen am 10. 02 2022 von https://www.sfa-mn.org/animal-gender-and-the-taste-of-meat/

(2) CleanNA. (kein Datum). What is CRISPR-Cas9? (CleanNA, Hrsg.) Waddinxveen, The netherlands. Abgerufen am 09. 02 2022 von https://www.cleanna.com/crispr-cas9/

(3) Cribbs, A., & Perera, S. (19. 12 2017). Science and Bioethics of CRISPR-Cas9 Gene Editing: An Analysis Towards Separating Facts and Fiction. (PMC, Hrsg.) Von https://www.ncbi.nlm.nih.gov/pmc/articles/PMC5733851/ abgerufen

(4) Dictionary, C. (kein Datum). Definition Culling. (C. Dictionary, Hrsg.) Abgerufen am 01. 02 2022 von https://dictionary.cambridge.org/dictionary/english/culling

(5) Douglas, C., Maciulyte, V., Zohre, J., Snell, D., Mahadevaiah, S., Ojarikre, O., . . . Turner, J. (03. 12 2021). CRISPR-Cas9 effectors facilitate generation of single-sex litters and sex-specific phenotypes. (n. communications, Hrsg.) Kent, United Kingdom. Abgerufen am 10. 02 2022 von https://www.nature.com/articles/s41467-021-27227-2

(6) Hazafa, A., Mumtaz, M., & Farooq, M. (15. 12 2020). CRISPR/Cas9: A powerful genome editing technique for the treatment of cancer cells with present challenges and future directions. (N. L. Medicine, Hrsg.) PubMed.gov. Abgerufen am 09. 02 2021 von https://pubmed.ncbi.nlm.nih.gov/33031826/

(7) Lambert, J. (12. 11 2021). Climate change may be shrinking tropical birds. *Climate change may be shrinking tropical birds*. (ScienceNews, Hrsg.) Abgerufen am 09. 02 2022 von https://www.sciencenews.org/article/climate-change-shrinking-birds-tropical/amp

(8) NOAA. (01. 03 2021). What causes a sea turtle to be born male or female. United States. Abgerufen am 09. 02 2022 von https://oceanservice.noaa.gov/facts/temperature-

dependent.html#:~:text=The%20temperature%20of%20the%20developing%20eggs%20is%20what,81.86%20Fahrenheit%2C%20the%20turtle%20hatchlings%20will%20be%20male.

(9) seeTurtles. (kein Datum). Sea Turtle Threats. (seeTurtles.org, Hrsg.) 5605 SOUTHEAST RURAL STREET, PORTLAND, Oregon, United States. Abgerufen am 09. 02 2022 von https://www.seeturtles.org/sea-turtles-threats#:~:text=%206%20top%20threats%20to%20sea%20turtles%20,other%20important%20coastal%20areas%20can%20affect...%20More%20

(10) Vasiliou, S., Diamandis, E., Kirche, G., Greely, H., Baylis, F., Thompson, C., & Schmitt-Ulms, G. (01. 10 2016). Oxford academic. *CRISPR-Cas9-system: opportunities and considerations, 10*(Clinical Chemistry, Volume). Oxford, Great-Britain. Abgerufen am 07. 02 2022 von https://academic.oup.com/clinchem/article/62/10/1304/5611942

(11) Zingl, M. (31. 10 2018). *Laborpraxis*. Abgerufen am 25. 01 2022 von https://www.laborpraxis.vogel.de/anwendung-von-crispr-cas9-am-menschen-sind-wir-immun-a-771796/