A Review on the Molecular Mechanism, Superiorities, Applications, Limitations and Experimental Workflow of CRISPR/Cas-9 System, and the Future of Gene Engineering

Xiaoke Wang
Supervisor: Lingfang Tang
The Affiliated High School of Peking University

Abstract: Gene engineering has been in development since the 1970s. The appearance of CRISPR/Cas-9 system, a gene engineering technology, even brought the wave of developing to its unprecedented peak. Meanwhile, the drastic growth and maturity of CRISPR made the public, represented by popular presses, to question the integrity and righteousness of scientists to develop and apply CRISPR system, causing intense worldwide ethical battles of humanity. However, without relative knowledge background, rational and logical judgment can be hard to make. Using qualitative research techniques, this research has thoroughly reviewed previous literatures that introduced the molecular mechanism, superiorities over other technologies, applications and limitations of CRISPR/Cas-9 system in order to give comprehensible introduction for the public. Using quantitative research methods, this research investigated the attitudes came from the public and science community toward the ethical issues around the use of gene engineering tools such as CRISPR in the form of numerical comparisons. More than 50% of people stand neutral in this battle, but there were more supporters of developing gene engineering technologies in the science community than that of in the public presses, which produces a necessity for countries to call for a pause of current developments, and to assemble national leaders to have a serious discussion.

1. Introduction
Have you ever thought about designing your own babies with enhanced intelligence or strength? Gene engineering has the potential to allow us to make it happen someday in the not-distant future. However, gene engineering has been a hot, controversial topic in recent years, gaining supportive and contradictory forces’ attention from the globe. Last year, a Chinese scientist named He Jiankui announced that he has successfully edited the genomes of two human embryos using a gene engineering technology - CRISPR/Cas-9 system. These two babies, with genes modified, were given with the ability of preventing HIV infection. World-wide debates around the righteousness of editing human-embryo genomes and employing gene engineering technologies were initiated ever since. Mainstream scientists doubt the safety of the clinical use of this technology on human embryos since it is different from editing genes of adults, as the traits being modified will be inheritable, therefore influencing the gene pool globally. While other scientists supported it because of the significant, profound leap of science and ethic it leads to. Regardless the morality of such attempts, the general public are lacking related knowledge about what CRISPR is and what CRISPR can do. Only armed with knowledge, the judgments can be made rationally and logically, therefore leading us to make the right decision on the use and development of gene engineering. This research is divided into mainly two parts; to provide the necessary informative background of CRISPR, the first part of research is a thorough review of previous literature on the
molecular mechanism, superiorities, applications, limitations and methodological workflow of the CRISPR/Cas-9 system. To trigger the audiences to ponder over the gene engineering issue, the second half of this research reevaluates the use of gene engineering technologies such as CRISPR by reviewing the articles published by popular presses and the literatures written by scientists in chronological order, so to predict the future of gene engineering.

2. Applications of CRISPR

CRISPR, as a newly-emerged bioengineering technology, has tremendously great potential in many areas of study and extensive applications, which include but not limited to: medical applications, agricultural and environmental applications. This section will present how the world is a better place with CRISPR by listing out various functions derived from its genome engineer nature of CRISPR.

2.1 Medical Applications

2.1.1 Genome and base editing

CRISPR provides scientists the ability to do certain editing on a base or a particular locus on the genome with incredible precision, which could be applied on massive production of a desired protein or hormone, corrections of gene mutations and treatments to genetic diseases.

2.1.2 Genomic-scale screening

High revolutionized screening approached are able to be conducted by using the RNA interference (RNAi) pathway, whereas, interestingly, nowadays some researches have shown that CRISPR system could accomplish the particular same goal with higher adaptability. Traditionally, CRISPR/Cas9 screens have been used to study intracellular phenotypes by combining with positive, negative or marker/reporter gene selection.

2.1.3 Cell therapy

The immune cell therapy and stem cell therapy can be realized by CRISPR/Cas-9 system. For example, in a research that studies the repression and activation of bacterial gene expression, patients with cancer or autoimmune diseases were treated with CRISPR-engineered T-cells with PD-1 gene-knockout. These clinical trials of CRISPR system have been validated for approved treatment of muscle-invasive bladder cancer, castration-resistant prostate cancer, metastatic renal cancer and metastatic non-small cell lung cancer.

2.1.4 Modification of human embryos

Before one is born, using CRISPR technology, it became possible for heritable disease-carrying parents to correct the pathogenic gene mutation and prevent the progression of the disease right from the beginning. In 2017, a Chinese research group consisted of Ma et al. claimed that they have corrected the heterozygous MYBPC3 mutation in the genes of a human embryo before the embryonic implantation happened. Notably, embryonic genome editing with a base editor showed higher efficiency: targeted deep sequencing on injected embryos revealed that 17 out of 17 (100%) or 6 out of 9 (67%) embryos carried the targeted point mutations at the target site in FANCF or DNMT3B gene, respectively. Despite these achievements have raised much more attention on the ethical implications, they did support that CRISPR system is remarkably beneficial for the whole population as long as it is used with a kind initiative.

2.2 Agriculture applications

CRISPR/Cas-9 technology can be applied to the production of trans-genetic rice with higher yield production and higher quality. Recent breakthroughs are around the generation of climate-change-resistant crops and with higher nutrient values.
2.3 Environmental applications
Along with understand CRISPR/Cas-9 technologies deeper and more thorough, our understanding of microbes and its interactions with phages has improved as well. In 2018, a group of researchers comprehensively collected information about CRISPR/Cas systems and anti-CRISPR systems to construct a CRISPRminer web server, seeking to accelerate their understanding of the co-evolutionary relationship between the microbes and phages. This unprecedented work facilitated us to explore more about the unknowns with, unfortunately, limited knowledge, but the return of it is what we are really looking forward to.

3. Experimental workflow of CRISPR
The CRISPR system is capitalized in labs for various purposes but with similar core methodologies. Here I present a generalized step of workflow of employing CRISPR/Cas-9 technology in the labs: 1) Target selection for sgRNA and design of reagents. 2) Construction of reagents. 3) Delivery of sgRNA and Cas Enzyme to the required cells. 4) Design of repair template (optional). 5) Clonal isolation and expansion modified cell lines. 6) Functional testing and validation. 7) Clonal expansion of desired cells.

3.1 Method
Reviewing previous literatures, the standpoints of scientific authors on the development of CRISPR technology can be reasonably inferred from the discussion and acknowledgement sections. Similarly, the attitudes from the public, represented by public presses, can also be interpreted by analyzing the published articles. I reviewed 60 literatures investigating CRISPR that were submitted on Nature by scientific authors, and 60 articles published by popular presses in both mainland China and also other countries. Each standpoint was recorded, and all of the reviewed documents were published after 2015 and selected randomly, meaning that the numbers collected are able to represent the latest and most authoritative voices. In doing so, the total number of supporters, the neutral’s, and the opponents in these 120 documents were added up, presented in a bar graph, showing a broad statistical difference.

4. Data and data analysis

4.1 Data
As shown in the figure above, among the 60 scientific authors, there were 18 supporters and 7 opponents. Most researchers (35) stood neutral regarding the issue of developing CRISPR technologies. While in the 60 presses who represented the voice of public, there were only 7 of them who stood supportive. However, the number of denying CRISPR technology development was significantly higher than that of the scientists, which was up to 19. The rest of them (34) took stances at neutral in this specific problem.
4.2 Data analysis

One of the most probable reasons for why there were more supporters of CRISPR than the public is that, compared with the public, scientists have richer knowledge database and deeper understanding of what CRISPR is capable of bringing us in the very non-distant future. For instance, various possible clinical, pharmaceutical uses derived from developing CRISPR technology. For the same reason, the public tends to not fully understand the real power of CRISPR and not sure about its future directions. Such concerns may make them to reject CRISPR, and to desire for a cease in unregulated developments, which explains why there was a higher count of people who against CRISPR. There was a similar share of people who took neutral stands in both communities. That can be caused by the profound understanding of both the advantages and disadvantages of CRISPR, since CRISPR could either lead the world into a better, bigger picture, or a worse, uncontrolled place.

4.3 Discussions

One of the reasons so many scientists are obsessed with CRISPR is that, it not only allows them to correct the mutations that cause genetic diseases, but it also has the potential to develop many beneficial applications for the society, for example, high-yield rice production. Although ethical issues are sometimes raised along with its prevalence, we have to admit that we cannot truly keep a powerful tool like CRISPR under-developed. This research eventually aims to call for a pause in the unregulated developments of gene engineering technologies, so that the public and the science community could have a serious discussion on its future directions. Here are some of the most urgently important topics of the debate around CRISPR: 1) Safety. We need to ensure the safety of gene-editing, especially if it were to be used on human embryos. 2) Prioritization. The use of gene engineering based on technologies like CRISPR should be limited to the cases where there are no alternatives, or the abuse of power in any form could be dangerous. 3) Access. The right to have access to CRISPR should be available for everyone, but not only the wealthy. However, to ensure this could happen really depends on much more complicated issues that yet to be solved around the globe. 4) Engagement. It is incredibly important that
citizens everywhere have a voice in deciding the use of powerful technologies like CRISPR, concerning their own welfares where other might not relate, and making a careful, thorough decision. Hopefully, a compromise could be achieved.

One of the founders of CRISPR system, Jennifer Doudna, thinks that it would be a mistake for us to keep these technologies under-developed only because we are uncertain about and fear for what it might bring us. For following researchers, regardless of expertise, should give more serious thinking about the morality of using technologies like CRISPR, and also how could we prevent the possible negative consequences as a result of unregulated development of these life-changing technologies.

5. Conclusion

The CRISPR/Cas-9 technology is based on a bacterial immune system that helps bacterium to fight viral infections. It uses a programmable enzyme called Cas-9 that can be programed with little bits of RNA with particular sequences to locate and cut cleavages on the DNA strand. This double-strand break (DSB) triggers the cell to repair the cut, and, in the process, introduce a change in DNA precisely. Through analytical reviewing, I found that there were more supporters of developing gene engineering technologies in the science community than that of in public presses, and there were less opponents among the scientists than that in the presses. Most people stand neutral in this battle.

Acknowledgments

This research did not display how have the attitudes changed in the science community and the public over time, and did not review the documents in the chronological order of publish dates, which could be one of the topics of future analyzations. The interpretation of standpoints came from each document could be subjective to some levels, as I did not have access to actually communicate with all the authors. But I promise that I was being as objective as I could, and I only reviewed those articles that expressed their opinions relatively clear. I gratefully thank Dr. Tang who provided me professional instructions and advices, and also the technical access offered by Peking University Biology Lab. I give my heartfelt thanks to all the ones who have helped me during the whole research process in any form.

References

[1] Marilynn Marchione (2018). Chinese researcher claims first gene-edited babies. Retrieved From https://www.apnews.com/4997bb7aa36c45449b488e19ac83e86d
[2] Cui Zhang, Renfu Quan and Jinfu Wang. (2018). Development and Application of CRISPR/Cas9 Technologies in Genomic Editing. Human Molecular Genetics, Vol. 27, No. R2, R79–R88.
[3] Bikard, D., Jiang, W., Samai, P., Hochschild, A., Zhang, F. and Marraffini, L.A. (2013) Programmable repression and activation of bacterial gene expression using an engineered CRISPR-Cas system. Nucleic Acids Research, 41, 7429–7437.
[4] Ma, H., Marti-Gutierrez, N., Park, S.W., Wu, J., Lee, Y., Suzuki, K., Koski, A., Ji, D., Hayama, T., Ahmed, R. et al. (2017) Correction of a pathogenic gene mutation in human embryos. Nature, 548, 413–419.
[5] Fan Zhang et al. (2018). CRISPR-miner is a knowledge base for exploring CRISPR-Cas systems in microbe and phage interactions. Nature: Communications Biology. Vol.1, Article number: 180.
[6] Jennifer Doudna (2015), How CRISPR lets us edit our DNA [Video File]. Retrieved from https://www.ted.com/talks/jennifer_doudna_we_can_now_edit_our_dna_but_let_s_do_it_wisely
[7] Amitai G, Sorek R. (2016) CRISPR-Cas Adaptation: Insights into the Mechanism of Action. Nat. Rev. Microbiol. 14(2):67–76
[8] Barrangou R, Doudna JA. (2016) Applications of CRISPR Technologies in Research and Beyond. Nat. Biotechnol. 34(9):933–41
[9] Charpentier E, Marraffini LA. (2014) Harnessing CRISPR-Cas9 Immunity for Genetic Engineering. Curr. Opin. Microbiol. 19:114–19
[10] Doudna JA, Gersbach CA. (2015) Genome Editing: The End of the Beginning. *Genome Biol.* 16(1):292
[11] Esvelt KM, Mali P, Braff JL, Moosburner M, Yang SJ, Church GM. (2013) Orthogonal Cas9 Proteins for RNA-Guided Gene Regulation and Editing. *Nat. Methods* 10(11):1116–21
[12] Fu Y, Foden JA, Khayter C, Maeder ML, Reyon D, et al. (2013) High-Frequency Off-Target Mutagenesis Induced by CRISPR-Cas Nucleases in Human Cells. *Nat. Biotechnol.* 31(9):822–26
[13] Heidenreich M, Zhang F. (2016) Applications of CRISPR Cas Systems in Neuroscience. *Nat. Rev. Neurosci.* 17(1):36–44
[14] Hou Z, Zhang Y, Propson NE, Howden SE, Chu L-F, et al. (2013) Efficient Genome Engineering in Human Pluripotent Stem Cells using Cas9 from *Neisseria meningitidis*. *PNAS* 110(39):15644–49
[15] Isaac RS, Jiang F, Doudna JA, Lim WA, Narlikar GI, Almeida R. (2016) Nucleosome Breathing and Remodeling Constrain CRISPR-Cas9 Function. *eLife* 5: e13450
[16] Jinek M, Chylinski K, Fonfara I, Hauer M, Doudna JA, Charpentier E. (2012) A Programmable Dual-RNA-Guided DNA Endonuclease in Adaptive Bacterial Immunity. *Science* 337(6096):816–21
[17] Kleinstiver BP, Prew MS, Tsai SQ, Topkar VV, Nguyen NT, et al. (2015) Engineered CRISPR-Cas9 Nucleases with Altered PAM Specificities. *Nature* 523(7561):481–85
[18] Leenay RT, Maksimchuk KR, Slotkowski RA, Agrawal RN, Gomaa AA, et al. (2016) Identifying and Visualizing Functional PAM Diversity across CRISPR-Cas Systems. *Mol. Cell* 62(1):137–47
[19] Mulepati S, He’roux A, Bailey S. (2014) Structural biology. Crystal structure of a CRISPR RNA–Guided Surveillance Complex Bound to a ssDNA Target. *Science* 345(6203):1479–84
[20] Ran FA, Cong L, Yan WX, Scott DA, Gootenberg JS, et al. (2015) *In vivo* Genome Editing Using *Staphylococcus aureus* Cas9. *Nature* 520(7546):186–91
[21] Strong A, Musunuru K. (2017) Genome Editing in Cardiovascular Diseases. *Nat. Rev. Cardiol.* 14(1):11–20
[22] Tsai SQ, Joung JK. (2016) Defining and improving the genome-wide specificities of CRISPR–Cas9 nucleases. *Nat. Rev. Genet.* 17(5):300–12
[23] Vassylyev DG, Sekine S-i, Laptenko O, Lee J, Vassylyeva MN, et al. (2002) Crystal Structure of a Bacterial RNA Polymerase Holoenzyme at 2.6 Å Resolution. *Nature* 417(6890):712–19
[24] Wang H, La Russa M, Qi LS. (2016) CRISPR/Cas9 in Genome Editing and Beyond. *Annu. Rev. Biochem.* 85:227–64
[25] Xiong X, Chen M, Lim WA, Zhao D, Qi LS. (2016) CRISPR/Cas9 for Human Genome Engineering and Disease Research. *Annu. Rev. Genom. Hum. Genet.* 17(1):131–54
[26] Zhao H, Sheng G, Wang J, Wang M, Bunkoczi G, et al. (2014) Crystal Structure of the RNA-Guided Immune Surveillance Cascade Complex in *Escherichia coli*. *Nature* 515(7525):147–50