～Improvement & Application of Genome Editing ～

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1. Introduction

Images of human genome (source: McGovern Institute for Brain Research at MIT, YouTube)

"Genome editing" is a new technology which allows researchers to change specific gene sequences easily. There had been two kinds of genome editing systems developed previously, Zinc Finger Nuclease (GFN) in the 1990s and Transcription Activator-Like Effector Nuclease (TALEN) in 2009.

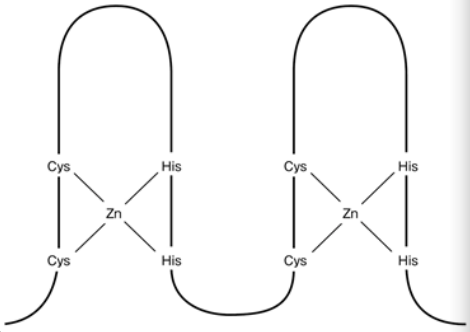


Figure1, Kobayashi, 2016

ZFN is the first genome editing technology (Figure1). It contains zinc at the centre of its substructure and nuclease like cysteine (Cys) and histidine (His) is tied to the central zinc. These combined structures play a role to cut the DNA sequence (Kobayashi, 2016, p.132). The name comes from its structure looking like finger. When this system works, a kind of protein which is designed by researchers for each use recognises and combines to the target DNA sequence. Then the nuclease put with the protein cuts the DNA (pers. comm. Dr Hotta).

TALEN, like GFN also recognises and combines the target with a kind of protein and nuclease put with the protein also cuts the DNA sequences (figure 2). This system selects one target with two arms. Therefore miss editing happens rarely in TALEN (pers. comm. Dr Hotta). However these two systems have two problems. First, they are difficult to use because designing the protein needs high-level processing skills. Second, these two cannot be used for general-purpose as the structures have to be designed each time (Kobayashi, 2016, p.15).

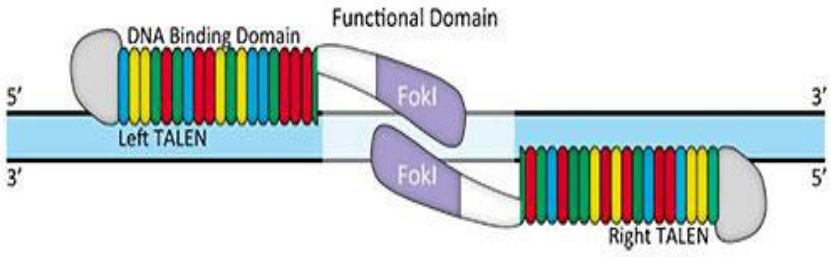


Figure2 by Kosumo・Bio Inc.

In 2012, the third genome editing system, CRISPR/cas9, was developed. It solves the previous two problems. It gained attention in the scientific world because it allows researchers to change genes faster and more easily than the previous gene editing systems, GFN and TALEN (University of Cambridge, 2016). It is because CRISPR/Cas9 is composed of RNA and nuclease (Kobayashi, 2016, p.121). This novel technology raises the question, what can people do with CRISPR in the future. I will focus on CRISPR's development and application for medical therapy, which is expected to contribute to treatment for serious diseases.

2. Literature Review

There has been a lot of research on genome editing. Many books and articles have been published and there is increasing attention on the subject, for example, the possibility to apply for medical treatment, agriculture, and a natural resource.

Genetic recombination is a way of recombining genes by damaging them from outside of the cells and expecting mutations to be caused (Kobayashi, 2016, p.105). This was commonly used to change genomes once, but it succeeds rarely and the outcome of which gene sequence is changed is uncertain (NHK Publisher, 2016, p.18). Researchers worked to create a new way to change genomes easily, fast and reliably. As noted previously, in the 1990s, the first technology for genome editing, Zinc Finger Nuclease (ZFN) was developed. In 2009, the second genome editing technology TALEN was developed (Kobayashi, 2016, p.18). These two technologies have a problem in that they are difficult to use because they utilize protein. In addition, these two are not adapted to use on different species of animals. For example, a sacksful TALEN genome edit made for mice cannot be used for other animals. Finally, in 2012, the third genome editing system, CRISPR/Cas9, which uses guide RNA and nuclease, was created. This system overcomes the previous technical problems and allows researchers to change genes like editing sentences (Kobayashi, 2016, p.14). The user only has to design the guide RNA to match the target order of DNA, and put it with Cas9, a kind of enzyme acting like scissors for DNA (Kobayashi, 2016, p.120), into the cells then the two materials edit the genome, as Figure3 shows. Cas9 exists in nature such as inside some bacteria. Also, it can work with any guide RNA sequences. Therefore CRISPR/Cas9 system can be used for several species without altering every time.

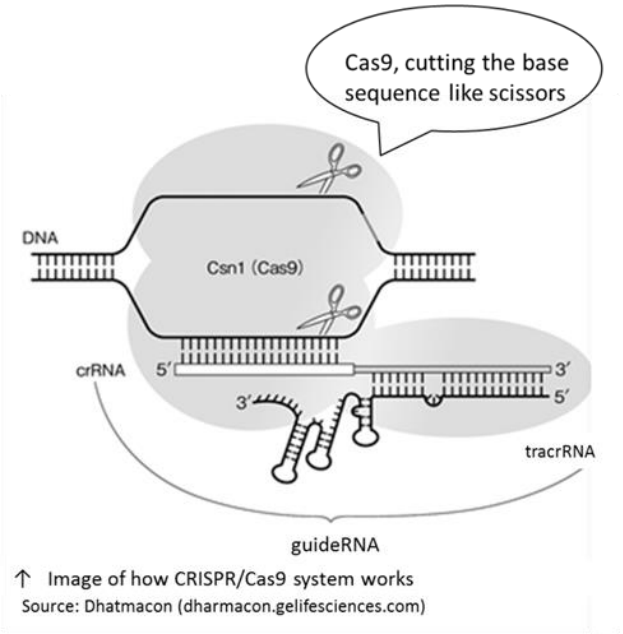


Figure3,
Dhatmacon,(dharmacon.gelifesciences.com)

There are, however, still some problems with the technology, safety and ethical concerns with CRISPR. This system is not 100% accurate and therefore, sometimes, unexpected mutations happen, called “off-target effects”. The rate of these has been decreasing thanks to the efforts of scientists, but concerns still remain, especially with regards to medical applications for humans (NHK Publisher, 2016. P106). There is discussion among both ordinary people and researchers about whether genome-edited food is safe to eat. This will be important when attempts to edit the genome of foods, such as creating fish with more muscle and drug-resistant crops, progress. Ethically, it could allow people to make designer babies in the future. The border between medical therapy and designer babies is a source of debate. Discussion on this subject is badly needed.

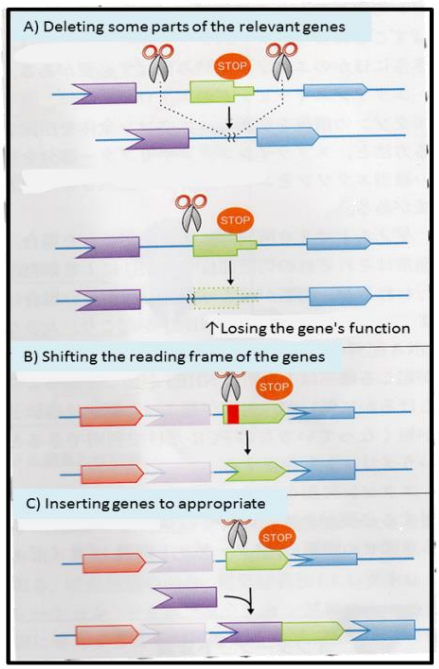


Figure4: Development of three types of treatments for Muscular Dystrophy (Xu, Hotta, 2016)

Currently, several researchers in gene editing are making progress

in medical areas. They are trying to treat serious diseases like AIDS, Muscular Dystrophy and even cancer with gene editing. A clinical experiment on the treatment of AIDS is underway in America (NHK Publisher, 2016, P.95). In the Center for iPS cell Research and Application, Kyoto University (CiRA), some research teams are trying to treat the symptoms of Muscular Dystrophy by injecting genome-editing chemicals into the patients' muscle (pers. comm. Prof. Hotta and Dr Takenaka). They think there are three ways to achieve this goal, deleting some parts of the relative genes, shifting the reading frame of the relative genes, and inserting genes to appropriate parts of the relevant genes (Figure4). The first one is thought of the most practical (pers. comm. Dr Hotta). Now there are several approaches to cancer research using genome editing, for instance, recognising the cause of cancer and stopping its progress with genome editing.

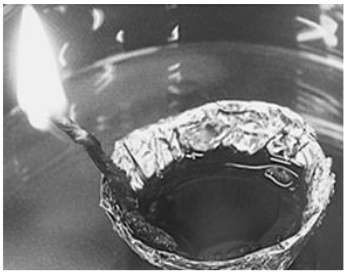


Figure5 (NHK Publisher, 2016):
Barning fuel made from genome-edited algae

Also, genome editing is used in agriculture, for example, allowing bigger plants to be made, vegetables without poisons to be produced, and animals and fish to be bred with more muscle. An example of a unique application of genome editing is from Prof. Harayama in Chuo University who is trying to make algae which can be a fuel, like oil (NHK Publisher, 2016, P.91). The potential is immeasurable.

3. Methodogy

The studies on gene editing above raised a number of questions and interesting ideas. To answer these, two researchers at CiRA and six researchers at the University of Cambridge were visited and interviewed about the development and application of genome editing.

Four main research questions were made, below, alongside follow-on questions.

The main questions are:

1) Which method is the best, most efficient, or safest to apply gene editing to the medical field, by medicine, cell therapy or genetic therapy?
2) Do many parts of the genome whose roles are not known affect the outcome and probability of off-target results? Can we continue to use genome editing technologies even in the situation where gene function is unknown?
3) Why is there no success in genome editing experiments on human fertilized eggs (a research team in China failed in such experiments in 2014)? Some research teams have already succeeded in experiments on some kinds of ape. So are human eggs quite different from other apes' eggs?
4) How can the ethical problems of gene editing or transplantation be overcome? What do you think about concerns over the safety of gene edited food?

Through the preliminary research, it is possible to make predictions, below, and discuss them during the interview.

The predictions are:

1) That combining gene editing and stem cells can be the best way to apply this technology to the medical field.

2) The core site of serious diseases can be treated with genome edited cells by cell injection or other similar methods in the future.

The people interviewed were:

・Dr Nana Takenaka, a researcher of Sakurai lab at CiRA

・Junior Associate Professor Akitsu Hotta, a Principal Investigator at CiRA

・Prof Ludovic Vallier, a professor of department of surgery at Cambridge

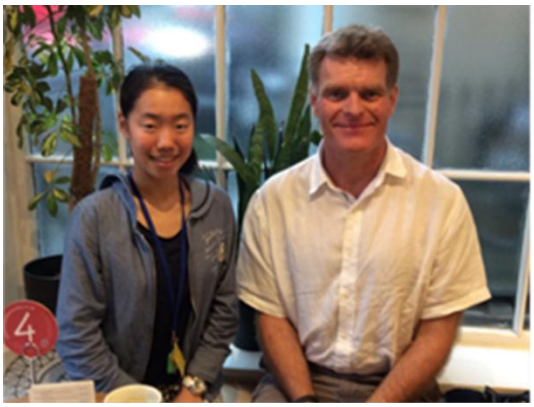
Picture1

・Dr Shota Nakanoh, a researcher of department of surgery at Cambridge
・Dr Kim Jee Goh, a researcher of department of surgery at Cambridge

Picture2

・Prof Roger Barker, a professor of Brain Repair Centre of Cambridge

Picture3



・Prof Allan Bradley, a professor of Cambridge Stem Cell Institute

Picuture4

・Dr Stefano Pluchino, a doctor of department of neuroscience at Cambridge

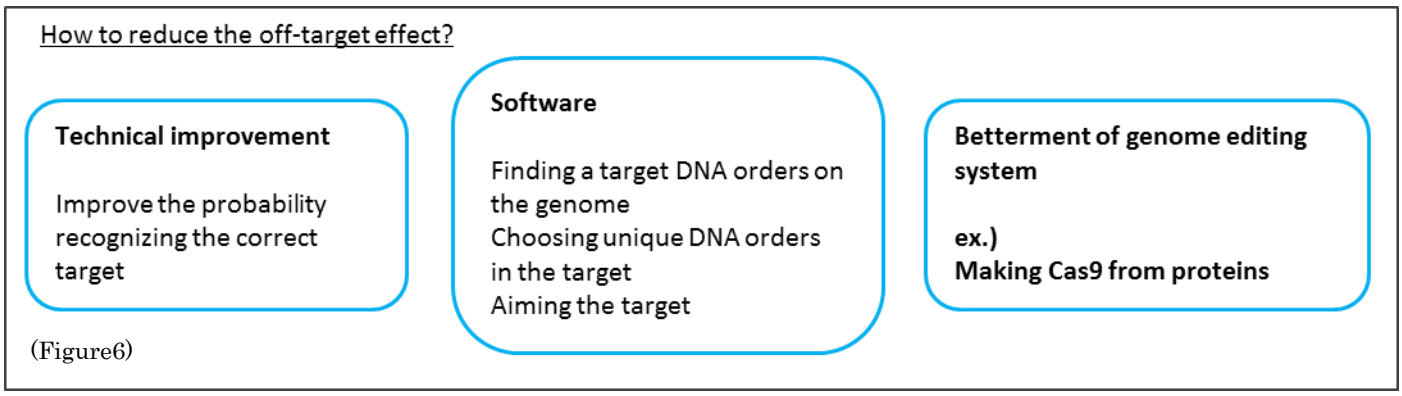
4. Result

From the interviews, the following points were raised.

A. Re: the research questions

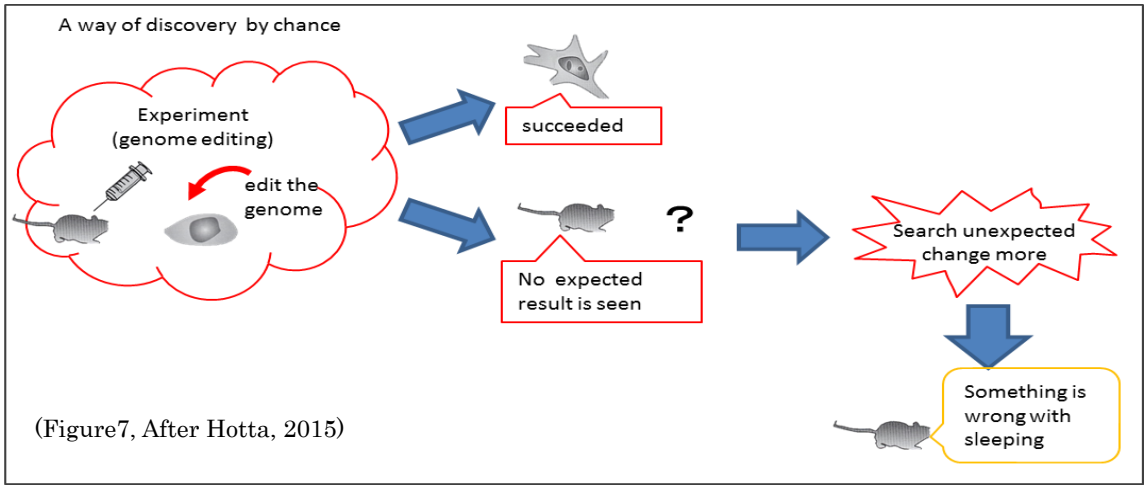
1) Which method is the best, most efficient, or safest to apply gene editing to the medical field, by medicine, cell therapy or genetic therapy?
A.→Cell therapy in vitro (outside of body).
Why?→The genome-edited cells can be screened before they are placed into a patients' body. Therefore the risk of off-target or other errors are reduced (pers. comm. Prof. Barker).

2) Do many parts of the genome whose roles are not known affect the outcome and probability of off-target results? Can we continue to use genome editing technologies even in the situation where gene function is unknown?
A.→Yes (both of the questions above).
Additional information →It is really difficult to reduce off-target effects and it is a fact that unclear parts of the genome affect off-target results very much. Now researchers use software developed to design the guide RNA. To reduce off-target effects, targeting unique DNA sequences is a good method because the CRISPR system sometimes cuts a different sequence which has a similar DNA order. Their software helps researchers to find unique sequences in the genome. Using such software can reduce off-target effects, but they can still happen. This is why researchers have to be careful. A co-worker of Dr Nakanoh is trying to structure the Cas9 of a protein to reduce off-target effects. Since Cas9 is an enzyme found in natural creatures, it contains nucleic acid (pers. comm. Dr Nakanoh). Therefore, very rarely it is taken into the DNA sequences that researchers try to edit. It is said to be one of the causes of off-target effect. This development can contribute to reducing the off-target effect.



(Figure6)

Sometimes there is difficulty in recognizing what happened inside the cells after genome editing because the change might happen somewhere unknown or unexpected (pers. comm. Dr Hotta). For example, a research team at Tsukuba University found a gene related to sleep by chance. Originally, they tried to recognize another gene's function, but they failed. This success happened thanks to their careful observation (pers. comm. Dr Hotta).



(Figure7, After Hotta, 2015)

It is important to reveal such parts of a gene sequences' role by genome editing technology and it is very valuable in other biotechnology applications. For this reason, the combination of genome editing and mysterious genes is not always negative.

Dr Takenaka spoke of her experience of genome editing. She has edited 24 cells' genomes in a part of her study, but she succeeded perfectly on only one cell. The failures had either the wrong genes edited or no result. This failure is not only an off-target result but also left an unedited genome. This rate of success can seem to be low, but such results are common knowledge among researchers in this field.

3) Why is there no success in genome editing experiments on human fertilized eggs? Are human eggs quite different from other apes' eggs?

A.→Researchers think there were two problems in the study which failed to edit the genome of fertilized human eggs: sample size and egg quality (pers. comm. all of the interviewed researchers). It is no more difficult to handle human cells than any other animal cells, consequently humans are not different from other animals regarding genome editing systems (pers. comm. Dr Hotta). For sample size, 85 eggs were used, but they could not edit the genome as they wanted on any eggs (Kobayashi, 2016, p.21). According to researchers, such a success rate when genome editing can happen for any animals' fertilized eggs (pers. comm. Dr Hotta, Prof. Bradley). Since the object was a human cell, the media said the success rate was too low. Of course it is currently not good enough to apply to human medical therapy, but no researchers I interviewed think the technique is fully developed. This news is not relevant long-term as scientists will learn more about this technology and the mystery of the genome. For the problems with egg quality, the research team used fertilized eggs that did not grow into an embryo and contained multiple sperm each (Kobayashi, 2016, p.20). They explained that, 'It was because the aim was only to measure the probability of genome editing on human fertilized eggs' (Kobayashi, 2016, P.24). Researchers think these kinds of eggs might be weak or lower quality in some ways than others, so this is one of the reasons for the failure.

4) How can the ethical problems of gene editing or transplantation be overcome? What do you think about concerns over the safety of gene edited food?

A.→The answers to this question varied depending on the person, but all thought that they have to overcome such ethical problems and safety concerns to develop medical therapy with genome editing.

The answers:

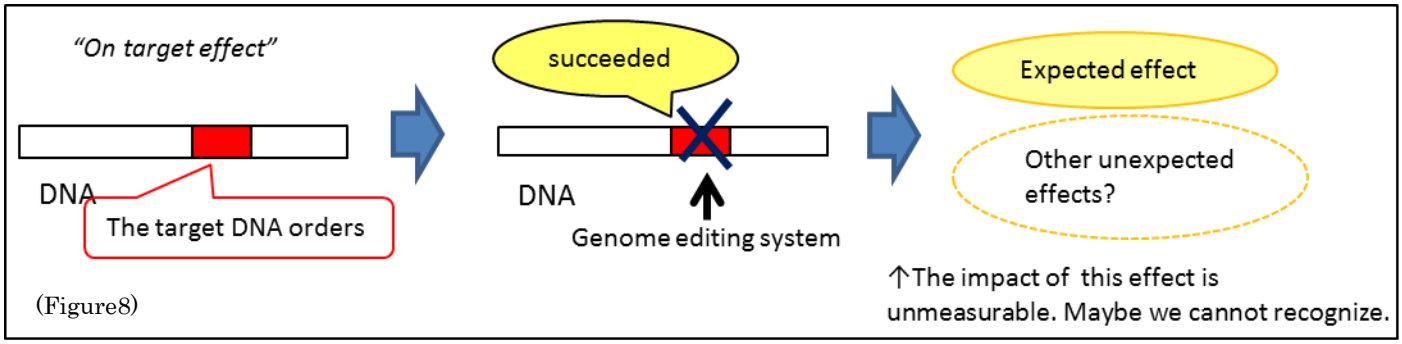
・Dr Takenaka said, 'as a researcher, I do not think genome-edited food is dangerous to eat, but I am not sure

if I would allow my child to eat them as a mother. Many of my friends who are mothers and researchers here have the same opinion.'

・Junior Associate Professor Hotta said gene-edited food is safe because there are many mutations with sudden genome changes in nature. Also, to continue explaining the risks and benefits of genome editing, people can get a general understanding of genome editing, he thinks. The ethical department at CiRA plays this role. This kind of department is rare in Japan.

・Prof. Ludovic mentioned that this new technology is a great tool but we have to improve it first. We should not hurry more than is needed and forget that only a little is known of the genome.

・Prof. Bradley said that stem cells are good enough to overcome the ethical problems except in the case of the ES cell which is made by killing an embryo. In addition, we have to limit the use of genome editing for humans to prevent the development of dangerous techniques with unexpected consequences. He also thinks genome edited food is safe. Prof. Bradley also said that the "*on-target effect*" is much more dangerous than off-target results because what is happening inside the cells may be unknown as only the surface consequences are visible. If a genome can be edited at will, the safety is unclear.



(Figure8)

・Dr Pluchino tried to overcome the ethical problems on his study by using direct inducible cells (not inducible pluripotent stem cells) which are made from human skin cells and turning them into human nerve cells. Recently, this kind of system has been developed by researchers.

B. Predictions

1) Can combining genome editing and stem cells be the best way to apply this technology to the medical field?

A.→These two technologies have great potential so many researchers have been trying to improve biotechnology with this combination. However Prof. Barker cautioned not to confuse these two because originally they were completely different tools. Each of them has a lot of potential singularly and there are still many problems that can be solved by each.

2) Can the core site of serious diseases be attacked with genome edited cells by cell injection or other similar methods in the future?

A.→Dr Hotta, who studies genome editing and iPS cells said such therapy would be great, but it has difficulty delivering factors or medicine to treat the target on purpose. On the other hand, Dr Pluchino, who studies cell injection, thinks such a direct way can be developed using cell injection as a vector against illnesses attacking in the body.

C. Others

During the interview, I asked some specific questions about the researchers' field of study. Their answers follow below:

1) Prof. Vallier and his team developed the enhanced CRISPR system, which allows researchers to switch multiple specific genes' expression on and off whenever they like while the cell is growing. It makes scientists able to investigate the role of whole families of related genes by reducing the activity of the genes to focus on specific gene's functions. However the preparation for this system takes from two to three weeks each time, which may be too long to be viable.

Consequently I asked: Is this length of preparation too long or not a problem?

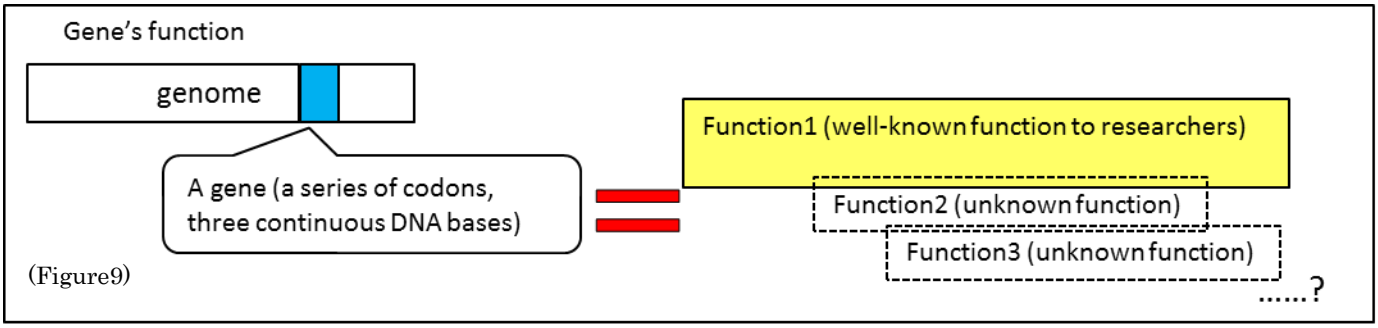
A.→It is a little long for researchers but not so long as it becomes a problem. It is because stem cells originally take 5 to 6 months to grow into differentiated types. The researchers who usually handle cells feel that experiments with stem cells are like growing babies since they need a great deal of care and time. Of course they feel it is hard to do that and shorter time with fewer steps is better, but they do not require these advances urgently.

2) Prof. Barker seemed to focus on how to assess the treatment, so I asked "what do you pay attention in order to assess the treatments?"

A.→ He told me that when he assesses the efficacy of a new treatment in his research, he wants to see how the factor works in the brain by a scan. However it is difficult. It is challenge for scientists in the field of brain repair. Moreover it is better to observe the long-term results on brain repair. So it needs a long time to collect results.

3) Prof. Bradley has discovered new roles for the many genes by knockout mice, mice whose genome has had specific genes edited by researchers to observe the function of the genes, and provided open access to the data. I asked, 'do you think you can completely reveal all roles for each gene?'

A.→ Eventually. However, now they can look at the surface of the gene's function though the genes work more deeply in our bodies. We should understand more about the genome.



(Figure9)

4) Prof. Pluchino studies cell injection technology. I asked, can cell injection be used as a tool for cell transplant?

A.→ Yes, it can be used to transplant clinically. The proportion of stable cells in the target is small, but there is long-term research on cell transplantation by cell injection.

5. Discussion

In applying the results and the interviews to the research questions, it seems that:

A. Re: the Research Questions

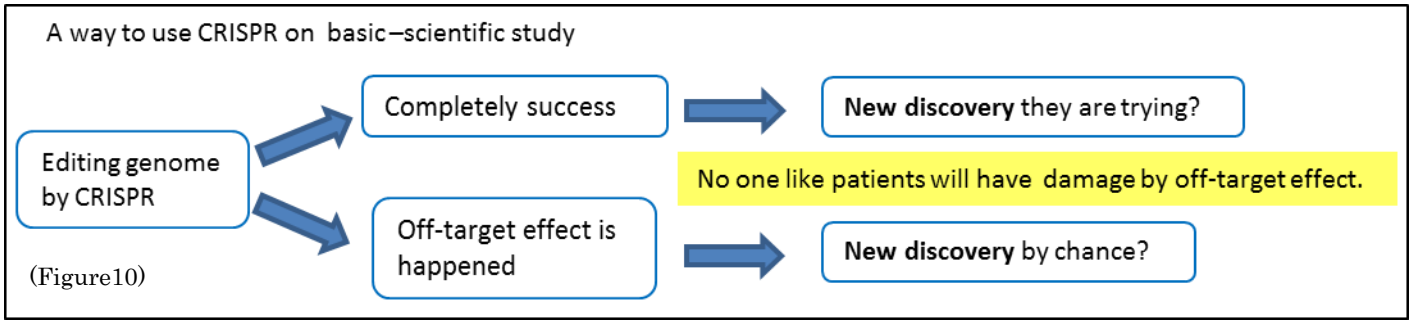
1) The best way to apply genome editing for medial application is therapy *in vitro*.

I had believed that cell therapy *in vitro* is the safest approach but the ideal is something *in vivo* (inside of the body) because it seemed more efficient. However, in fact, researchers do not think the benefit of *in-vivo* therapy is so much great because the theory of cell transplanting is established. Therefore cell therapy *in vitro* is the best way to apply genome editing for medical treatments, I think.

2) The unclear parts of the genome can affect the result of genome editing experiments.

There is concern about some effects of unclear parts of the genome, researchers agree this is a problem. This is one of the biggest issues that researchers face every day. Even if more and more studies and clinical experiments make progress, it is impossible to solve this problem completely. Effort to reduce concern is badly needed, but perfect treatment is most likely unrealizable. Thinking in terms of "risk and benefit" is essential for researchers and patients (pers. comm. Prof. Bradley). In terms of the effort needed, it includes advancement of the software to design guide RNA and attempts to structure the Cas9 protein.

In fact, there are several researchers trying to discover gene functions by genome editing and sometimes it is useful in recognizing the causes of serious diseases like cancer. This kind of usage of genome editing most likely has fewer concerns about off-target effects than studies of medical treatment for humans because such failures might induce new discoveries. So I believe that these kinds of studies should progress more in the near future. It can contribute to a better understanding of the genome and vitality.



(Figure10)

3) The reason why the attempt of Chinese research team could not succeed in editing human fertilized eggs' genomes is that there must have been a problem with the egg quality and the sample size.

In this case, my prediction was right because I was wondering whether the fertilized eggs in the experiment, which have multiple sperms in each of them and never grow into embryos, can exist after the editing of the genome with a small sample size of only 85 eggs. However, I also shocked by the practical sense among researchers of this field, where to have the probability of a perfect success in genome editing is quite low. It is natural to have no success in 85 samples. I am sure that few people think this is high enough to apply to humans. This is one of the reasons why most of the researchers do not hurry to apply the techniques to humans for medical treatment. I strongly believe the media should tell ordinary people about

this kind of information.

4) There are multiple ways to overcome the ethical issues of genome editing.

I remembered three important aspects from the answers to this question. First, ethical and safety problems of research are serious issues for researchers and they have difficulty in making progress in studies with explaining complex information to people and trying to improve their experiments. In fact, there has already held an international conference by researchers to discuss such issues (NHK Publisher, 2016, p.123). It shows their high-level of ethical commitment. Second, I was sure of the importance of the "iPS cell" because many researchers use it for their research. Several researchers said that iPS cells have the possibility to overcome ethical problems with ES cells, which are made by killing embryos, because iPS cells are made from differentiated cells and no embryos die. It is true that we should discover the potential of each technology, genome editing and pluripotent cells, but the combination of these may contribute to the field of medical science greatly. Third, scientific studies are related to each other in many ways. For example, Dr Pluchino uses a new system to turn skin cells into neuron cells for his experiments to overcome the ethical problems and shorten the preparation time. Wide fields of studies are developing which influence each other.

B. Re: the Predictions

1) Combining gene editing and stem cells can be the best way to apply this technology to the medical field.

Although there are many articles which combine pluripotent stem cells and genome editing (Kobayashi, 2016, p.5), the two technologies are quite different and should be studied separately. However, some researchers actually pay attention to combining these two, and the media also focus on that. Therefore, both kinds of research should progress. In the future, I hope for some great discoveries in each field, the separated areas of research of genome editing and stem cells can be put together and may make an amazing method.

On the other hand, I feel that there might be the need for some novel system to apply to genome editing. There are still limitations where genome editing can be used by itself. Advancement of new technology seems to be needed.

2) The core site of serious diseases can be attacked with genome edited cells by cell injection or other similar methods in the future.

I was happy to get a positive response to my prediction. I could find a new possibility to connect separated fields. Dr Pluchino said that cell injection works as just a vector. I believe there needs to be vectors working *in vivo*, because there are many technologies for controlling and observing cells inside animals on prepared slides, or grown *in vitro*. However, there is often difficulty taking them *in vivo* and this skill is necessary for use in a clinical environment. I expect this kind of skill will be developed in the future.

C. Others

1) The length for preparation of enhanced CRISPR system is a little long for researchers but not so long as it becomes a problem.

I was convinced by the statement of the researchers, 'experiments with stem cells are like growing a baby'. I got a strong impression that they seemed to enjoy taking care of the cells even though it is hard. Anyway I was surprised at the length of time research using stem cells took. I hope it will be shortened in the future. This experience reminds me of the care needed involving research with creatures.

2) Prof. Barker wants to see how the factor works in the brain using scans to assess any new treatments.

Brain repair techniques have garnered a lot of attention in the scientific world. However, since the brain is difficult to observe, sometimes the data is unclear. This should be solved as quickly as possible. On the other hand, they collect long term results to look at the efficacy of the treatment. I wonder if the difference in mental situations of the patients might affect the result a little. Many fields like psychology study the environment and of course brain repair seems related to this field (pers. comm. Prof. Barker). I think they should be combined to consider the result.

3) Prof. Bradley believes that he can eventually completely discover all roles for each gene.

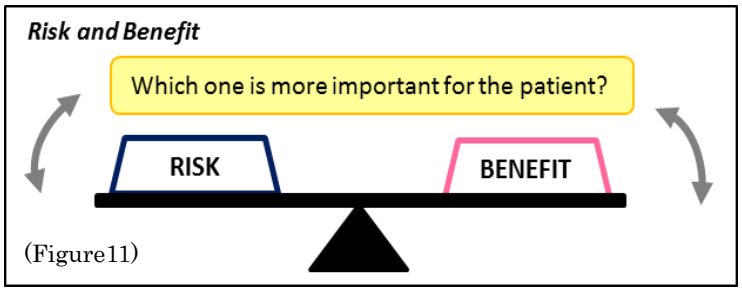
It would be amazing if this can come true in the future. It will contribute to a wide range of study. However, he cautions that now they only recognize the surface of a gene's functions and the deep mechanism is unclear. This should be emphasized greatly. If someone loses this awareness, they might make serious mistakes in their observation of experiments. I hope there will be an understanding of deep functions of the genes. To achieve this can lead new discoveries.

4) Dr. Pluchino said that cell injection can be used to help transplants in clinical medicine.

From this answer, I recognized a new possibility of treatments with transplantation because there are many people who hesitate to receive direct transplantation into the body, but they have less hesitation about injection into their body. If cell injection technology will become established, the mental stress of such patients might be decreased. It means that combination of cell injection and genome editing or stem cells can contribute to improvement of medical treatment with transplantation as I expected.

Then I got a new prediction about the efficacy of this transplantation. In fact, it is the same as therapy with medicine that injected cells to treat illness diffuses widely inside a patient's body, and when researchers tried to treat serious illness by medicines to edit genome, the attempts was failed because the medicines could not stay in specific points for a long time. So I am afraid that cell injection does not work so efficiently as normal transplantation because of the same reason as the attempts with medicines to edit genome. This is my new prediction.

D. Overview of the Research



(Figure11)

Through this research the techniques, ethics, safety and the difference between media stories and the researchers' opinions all proved to be important. As for the result above, CRISPR or other genome editing is an amazing tool for researchers, but still not perfect. The concerns and risks outweigh the benefits for many researchers if the usage of genome editing was expanded. Though the media cover genome

editing as a dream technology, like all new technologies, it needs much study and improvement. Kobayashi said, "The UK is historically not afraid of the taboo, like designer babies, so they are making progress on research of genome editing now." Actually this statement is inaccurate. The researchers I met in the UK understand the "*risk and benefit*" of genome editing very much. Therefore they know we have to study more about this new technology. This fact makes for a large volume and high speed of study, even using human cells, in the UK. It is one such example that shows the UK is a reaching country in science. For the ethical problems, ordinary people think it a complex topic, so researchers are seriously worried. The safety of genome edited food is controversial, moreover even researchers do not know where the limit of genome editing lies with regards to ethical concerns. It made a strong impression that one researcher has different opinions as a professional and a mother.

There is a long way for a new technology to develop to help people. This experience showed me the difficulty in studying. Researchers' views on the ethical problems are also very interesting because these are not covered often in the literature.

For the predictions, I was surprised that the answer was, "possibly." Most of the time, researchers had already considered my ideas, but they answered the questions seriously. Science seems much closer to ordinary people than before.

6. Conclusion

All the people interviewed at Cambridge were so kind and I provided a wonderful experience with many great answers. I was surprised at the attitude of the researchers, welcoming a high school student like me. I was impressed with the fact that there were many persons using cells on their study, even though it was in the middle of summer vacation season I visited there. I learned the difficulty of research they face every day. They have to try not only to improve their research, but also to make people understand. I also found that research is more developed than ordinary people think because there are many unpublished topics which are under way. I became even more interested in scientific research.

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