Regeneration Biology
Rajesh Ramachandran
Department of Biological Sciences
IISER Mohali
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W11L53_Stem cells for tissue engineering-Use of CRISPR-Cas9 for genome targeting

Hello everyone, welcome back to another class on regenerative biology. In today's class, we will learn about how stem cells are tweaked or modified using CRISPR-Cas9 technology to alter their genome. Many of you would have heard about CRISPR-Cas9 technology. I think in the year 2021, two women scientists received a Nobel Prize for CRISPR-Cas9 technology, and I have also written an article in Resonance about it. Those who are interested can read that article on the CRISPR-Cas9 technique. Let us have an overview of the CRISPR-Cas9 technology.

Induced pluripotent stem cells can differentiate into specific cell types or tissues of the eye or any other organ or tissue in humans. You can make them whatever you want. And they have several application prospects in retinal cell transplantation, corneal cell transplantation, and lens regeneration. But what is important to understand is that if a given cell type is not functioning in a tissue or organ, such as the retina, it could be because of a genetic mutation.

Maybe in that given cell or maybe in its progenitor itself, that is why gene editing comes into the picture: gene editing technology of clustered regularly interspaced short palindromic repeats, which is the short form of CRISPR, and also the CRISPR-associated protein 9, known as Cas9. Which is basically a nucleus, and in short, they are known as CRISPR-Cas9, which can effectively introduce causative mutations of inherited ocular diseases into human induced pluripotent stem cells. Means you can do it in both ways. Say you have a normal stem cell and you want to study the effect of mutation on a given cell type. So what you have to do is have the stem cell, introduce this mutation, and then differentiate into a given cell type and study its physiology, that cellular physiology.

Other way around, you have a patient who has a mutation in a given gene; hence their organ is suffering. Collect the cell; of course, that is a mutated gene-bearing cell, but you correct it using CRISPR-Cas9 technology and now differentiate it back into a normal cell that is now lacking this defect. So in both ways, it's possible. Then they can be further differentiated into specific somatic cells, maintaining a genetic disease background, to, just like I told you, introduce a mutation and study it, which can mimic the occurrence of inherited ocular diseases in vitro. And if it's an animal model, you can study it in vivo as

well.

The cell model may help scientists study the mechanism of human disease development, and you can establish an in vitro screening platform to find therapeutic drugs and correct genetic mutations in the human genome for cell therapy. So you can study; oh, there is a defect. I have hundreds of drugs and see how the cells are going to behave. Can one of these drugs be? One approach to fixing this trouble is to consider a given mutation in a patient and determine if I can fix it to obtain a normally functioning cell from this mutated cell using CRISPR-Cas9 technology. Combining stem cells and gene editing technology revolutionizes regenerative medicine in ophthalmology, and gene therapy for inherited ocular diseases is also made possible.

Why CRISPR-Cas9? One can always ask, is there another approach? Of course, there are different approaches. And CRISPR-Cas9 is so attractive because it is very easy. It is a time saver. It's a big time-saver. The advent of CRISPR-Cas9 is possibly the biggest advance in biological science since the generation of iPSCs themselves.

If you ask someone, ask what the most beneficial thing that has come ever since the discovery of induced pluripotent stem cells is. One must say it is the CRISPR-Cas9 technology that can fix any trouble. So stem cells are a big achievement, but if there is trouble or if you want to create trouble, CRISPR-Cas9 becomes the most attractive choice. And it has opened the doors to what was only dreamed of: accessible, genetically engineered, patient-specific stem cells on demand. Now you can make whatever mutation or change you want in the genome of an organism in no time.

The latest advancement of CRISPR-Cas9 has enabled genome and epigenome editing, as well as gene repression and activation. So far, we know that CRISPR-Cas9 basically causes a cut, a nick in the DNA, and removes it, or after making a nick, you can fix it by recombination. Now, that is not it. You can use Cas9 as a molecule, not as a nicking enzyme; rather, it can act as an activator of transcription or a repressor of transcription. Say a person has cancer because a gene is activated.

You can use the appropriate CRISPR technology to suppress that gene. so that there will not be any more of that gene product being formed. So that is one of the other lines of growth that have happened in the CRISPR-Cas9 approach. So the CRISPR-Cas9 approach, the function is very simple. The mechanism of action of CRISPR-Cas9 involves the gRNA, which is called guide RNA.

Interacts with Cas9 to recognize the 20-nucleotide target sequence located upstream of the invariant 3-base pair region known as NGG. NGG, where N stands for any nucleotide

and GG. That is the sequence that will be recognized by Cas9 and is known as the PAM sequence. Photospacer adjacent motif, which is what it is called, is a sequence that causes a double-strand break in a specific genomic region; subsequently, DNA repair systems correct the gap in the sequence either by non-homologous end joining (NHEJ) or the HDR pathway. You have to have a donor cassette present for recombination, which occurs from a donor DNA cassette and gets integrated.

So you can see here this is a DNA and you have got a guide RNA that has bound to one of the loci which has Cas9 bound to it, and it is going to create a cut in the NGG region, creating a double-strand break. And the double standard breaks, once it has occurred, the system will try to repair. Repair can be NHEJ, where a random sequence can be inserted. Because of this, if the cut happened inside a gene, it is most likely that the gene has lost its function. Either it will create a stop codon, or it will create a frameshift.

Less likely that it will restore the normalcy of the genes. Another option is precise DNA replacement. So whenever there is DNA damage, the system or the cell looks for a complementary sequence that can integrate by homologous end joining. That means it will recombine. As you can see here, this is the HDR sequence that recombines, and you end up getting an insertion.

And now the whole region has been fixed up, and when you think about it, like I mentioned, you can disrupt and create a precise mutation; you can have that precise mutation in this HDR region, create a nick, and then insert that HDR cassette with a precise mutation. One option, or the other option, is if you simply want to disrupt a gene and then want to know what its effect is, you can go for the NHEJ method. So either way, it is a very effective and powerful application of the CRISPR-Cas9 technique. hiPSC. Which means human-induced pluripotent stem cells.

Okay, so that is hiPSC. means. Since the initial reports of Yamanaka and colleagues on generating iPSCs in 2007, human iPSCs have been successfully generated by ectopic expression of the four transcription factors KLF, OCT4, SOX2, and c-MYC. So subsequently, several improvements in the method of iPSC generation were reported, including the generation of human iPSCs without the overexpression of the oncogenic gene MYC. Because although MYC is one of the amenable factors, MYC always brings in some extra baggage that can make the cells, the iPSCs, more tumorigenic.

So reprogramming methods with mRNA, protein, small molecules, and microRNAs, all of which do not require genomic integration of the reprogramming factors, were also devised without too much complication. You can simply make a stem cell without manipulating the genome. You can work with various other molecules such as drugs,

proteins, or microRNAs, etc. The derivation of HIPSCs and the culture of HIPSCs are in completely defined xeno-free conditions. Xeno means strange materials.

Xeno-free conditions have also paved the way to employ these cells in basic and translational applications. The technological advances have allowed for the development of HIPSC-based models for numerous diseases. However, we should understand that the full realization of the potential of these cells in research and clinical endeavors definitely requires the ability to modify their genome at precise locations. That is what CRISPR-Cas9 offers. CRISPR-Cas9 is used to generate HIPSC reporter lines to study cell fate.

One approach we will study is. The ability to insert reporter genes into specific genomic loci, such as GFP, RFP, or any other reporter molecule, allows you to study the expression of a given gene by simply integrating a GFP downstream of that gene so that the gene is not affected, but you will be able to see it in vitro. Or in a live condition, if it is an animal that can be put under the microscope, you can see the GFP expression. To target specific genomic loci in HIPACs offers the chance to faithfully reproduce gene expression patterns with minimal effect on the adjacent genes. That is the beauty of a CRISPR-based integration of an expression reporter line or an expression cassette.

Several studies reported using CRISPR-Cas9 technology to insert fluorescent protein into loci associated with the undifferentiated state of HIPC. For example, let us think of a scenario. I have a cell. I took the fibroblast and I am trying to make it a stem cell. Stem cells have to have some markers, but who will come and tell me it has become a stem cell? So my goal is to identify when a stem cell marker starts to express.

So if that stem cell marker is now tagged with GFP, whenever I convert a fibroblast into a stem cell, I will be able to recognize it by GFP expression, and I can handpick those cells to make a new colony or new passage. For example, the green fluorescent protein was inserted downstream of the last exon of the Oct4 gene. Generation of HIPSCs reporter line could not only recapitulate Oct4 expression to monitor pluripotency, but it also did not impact the ability of the targeted cells to differentiate into other cell types. means this GFP integration did not affect the functionality of Oct4 and the functionality of the stem cells themselves. That means it's a completely harmless approach.

Most importantly, the generation of tissue-specific reporter iPSC lines provides a better understanding of the developmental stage of these cells, where a strong GFP indicates that Oct4 expression is really strong, while a weak GFP means that Oct4 expression is really weak. So sometimes strong expression may be harmful, and sometimes weak expression may be useless. So you will be able to catch what is ideally expressed, just like putting salt in a dish. Too much salt is a problem, too little salt is a problem. So that

is also possible by monitoring these reporter lines.

We will see a few examples. Wu et al. generated a knock-in mouse iPSC line with early myogenic lineage specification of a gene called PAX7 using the CRISPR-Cas9-Nicase pairs. A GFP reported with the selection markers was incorporated before the stop codon of the PAX7 gene in the last exon. That means the PAX7 gene is intact; you put an extra GFP into it. A similar example of a reporter line was generated in the human iPSC line where an early myogenic specification gene, MYF-5, was generated by CRISPR double-nicked knock-in of GFP.

So, these putting a reporter line is normally referred to as knock-in lines just before the stop codon. This allowed for the prospective identification and purification of myogenic progenitors from human iPSC cells. So strategies such as these could improve our ability to track the survival, engraftment, and localization of human iPSC-derived cell populations in real time in vivo. That means within the organism itself, which is very important for its clinical translation.

Without seeing, believing is seeing. Like without seeing, you don't know which cell is gone, where it is, and what it is doing. So using CRISPR-Cas9 in producing human iPSC-based disease models. Let us see some examples. Compared with conventional stem cell manipulation, such as the HDR system for gene modification, CRISPR-Cas9 mediated gene editing has dramatically improved gene editing feasibility by reducing the time required to recover the edited clones from years to months. Earlier, it used to take four years, five years, like that.

Now you can do it in a matter of a few months. It is so easy that you can make a modification in the genome using the CRISPR-Cas9 approach. Several patient-derived iPSC lines have been easily engineered via CRISPR-Cas9 to repair the disease-causing mutations, which I already told you. And these were then differentiated and transplanted into the appropriate organ in an animal model, leading to functional rescue.

Let us see a few more examples. Several other researchers have also reported the CRISPR Cas9 system, which was successfully used to induce large gene deletions to remove the mutation hotspot in human Duchenne muscular dystrophy. Using the patient-derived IPSCs. So in a simplistic sense, Duchenne muscular dystrophy is a male-specific gene. Females can be carriers, and the victims normally don't live beyond 30 years. Some exceptions may exist because their muscles will be very weak, their breathing will be affected, and their lungs will not function.

So, these are all very serious issues. But the problematic region itself can be deleted

because Duchenne muscular dystrophy, the dystrophin gene, is a large gene. If I'm not wrong, it's around 17 KB of RNA itself, 17,000 bases. So this approach may potentially treat 60% of the DMD population. So if there is a problematic area, delete and fix it so that no more mutations remain in that protein.

Hence, the organism survives perfectly. And more importantly, the ipse correction and the restoration of function have also been applied for the most common chronic lung disease known as cystic fibrosis. We would have heard about it because it affects the chloride balance in the lung epithelial cells. This disease is caused by a cystic fibrosis transmembrane conductance regulator, also known as CFTR. and the mutation in this CFTR gene is the main cause of cystic fibrosis. Patient-derived iPSCs were used to model the correction of the CFTR mutation by transfection with a Cas9 and sgRNA plasmid.

You put it together, and the HDR can recombine and fix the mutation. These cells were subsequently differentiated into mature airway epithelial cells with recovery of CFTR expression. So you can make them into cells of the airway epithelium, and you can transfer them. And then it was transplanted, leading to the improved chloride current in response to the stimulation and the disease amelioration. So the complexity of the disease is controlled, and the patient survives.

So if you look further into the application of CRISPR Cas9 systems in human iPSCs, this picture taken from these publications shows that human and patient-derived iPSCs are generated, and now you are culturing them after editing. So first, you culture these cells. Edit using the CRISPR Cas9 approach, and you grow the edited cells, which means the mutation is now removed, and you can allow them to differentiate directly into tissues of your interest. If you have a reporter line, it is much easier because, if it is a stem cell-specific reporter line, then Once it is differentiated, it will go off. What if you have put a reporter in a differentiation-specific line? Then, when the stem cell differentiates into that cell type, the reporter comes up.

Either way, you can do it. So treatment can also be done if you have a culture; you can treat it with certain drugs and see that when this cell behaves properly, several genetic diseases can be corrected with this approach, and mutations can be reversed back to normalcy, allowing you to implement or implant it into the patient. Surgical transplantation of the affected area can also be done effectively using these cells. So novel CRISPR-based genome engineering tools can also be applied in the IPSCs. Novel tools. As I mentioned at the beginning of the talk, it’s not just, you know, cutting the genome, cutting the genome.

You can edit it. That is a very powerful technique, but you don't want to edit it because you want to activate some genes. So you cannot say a gene is turned off in a given cell or organism. So it has nothing to do with any mutation. Gene is perfectly fine, but it is not turning on, or a gene is turned on. Strongly and because of that, there is some complexity, so you don't want to turn that gene off or create a mutation in that gene; you want to reduce the expressivity of that gene, so there are two scenarios: turning it on to increase its level or turning it off to decrease its level.

So CRISPR Cas9 applications now go far beyond DNA editing. Targeted mutations were made to the Cas9 protein on its catalytic side. Catalytic side means it will cause cutting. And the fusion of Cas9 to diverse effector domains has widened the range of genomic alterations possible in iPSCs and other cell types. It is now possible to precisely downregulate or activate genes without introducing changes to the DNA sequence or using novel CRISPR tools. DNA integrity preservation is required for clinical applications of iPSCs, and CRISPR gene regulation can potentially be applied to generate genetically engineered, safer, and genomically stable cells.

So by using this kind of activation-repression approach, you can say you did not modify the genome at all. You just made it a little more active. You just made it a little low. Just like, you know, you are tweaking the system.

Let us see an approach. CRISPR interference, known as CRISPR-I, and CRISPR activation, known as CRISPR-A, are two distinct processes. They are the gene expression modulation systems that enable the expression or activation of genes. Either you can do repression, interference, activation, or upregulation. Both systems are based on a mutated Cas9 protein called nucleus deactivated Cas9, also known as dCas9. Mutations in the RUVC and HNH domains abolish their DNA cutting abilities while maintaining unaltered DNA binding domains.

It will still bind to the DNA, but it does not cut the DNA. D-Cas9 recognizes the target sequence in the same manner as the wild-type Cas9. No change in that. However, disrupting the nuclear activity of Cas9 transforms this protein into a generic RNA-guided DNA-binding protein. It just binds to the DNA. The fusion of dCas9 with effectors converts dCas9 into a programmable transcription factor that can be engineered to target and recruit other transcription factors to any DNA region specified by the gRNA.

Because you can target any region. There is no region that is immune to d-Cas9. The CRISPR-Cas9 approach creates the possibility to modulate gene expression in a very specific and versatile manner. So this is the picture that shows the actions of deactivated Cas9 and associated proteins used to repress and induce gene expression. So you can see

two examples: the left-hand side is CRISPR-I, and the right-hand side is CRISPR-A. The interference activation concept is the same in that you have the DNA and the guide RNA comes and recognizes the PAM sequence, Cas9 binds, but this Cas9 is deactivated Cas9, or dCas9; hence, it will not cut the DNA.

Now, this dCas9 is fused with an interfering protein called KRAB, which has been introduced to prevent gene expression. The gene expression that is normally turned on is now turned off. Another option is that you can bring in something called DNMT3, DNA methyltransferase. And Cas9 fused with the DNA methyltransferase will methylate that DNA so that gene expression can be turned off. Another option for activation is that Cas9 is fused with VP64, which is an activator protein.

The other gene, Cas9, is now fused with Tet1, a 10-11 translocase, which will demethylate the DNA. So both will turn on the genes. So this is what can be easily achieved using the CRISPR approach. So editing the epigenome, the complexity of mammalian gene expression regulation opens many avenues for manipulation. On the other hand, some iPSC applications could benefit more from stable gene regulation, which can be achieved either by genome knockouts or, more recently, by sequence-preserving epigenome editing.

So these are all the approaches we can take. DNA methylation in CpG islands is known as a repressive mechanism of gene expression. We all know that methylation of CpG islands will not allow gene expression to happen. The association of dCas9 with the catalytic domain of DNMT3 can promote de novo methylation. That means normal is not methylated, but you can bring methylation to these loci of genes in mouse embryonic stem cells. When dCas9 DNMT3 was targeted against an active housekeeping gene, such as GAPDH, there was transcription inhibition, demonstrating the feasibility of this strategy for use in stem cell models.

Means any gene you can turn it off as per your will. Similar to adding methyl groups to induce gene silencing, removing repressive marks has been performed to activate gene expression by CRISPR-A. This strategy has been used for the promoter region of the BNDF gene where dCas9 is fused with the catalytic domain of TET1. Like I told you, TET1 is a 10-11 translocase that causes the demethylation of the CpG island. This led to the activation of gene expression in mouse ESCs.

So, once you remove the methyl group, you can turn the gene on. TET1 catalyzed the conversion of 5-methylcytosine into 5-hydroxymethylcytosine, playing a key role in active DNA demethylation. Moreover, the dCas9-Tet1 was used against a transcriptionally inactive gene. Dazl, it's an off gene, turned off gene. Transcription was

activated, demonstrating the predictability of the system in modulating gene expression without altering the DNA sequence and with minimal off-target effects. So the authors, the researchers have also shown that this system worked in vivo by demethylation of an imprinted gene.

Imprinted, we will not go into the details. You can read about imprinting. It's a very important step in fixing the paternal and maternal copies of the gene. So these findings are remarkable for opening the possibility of activation of the endogenous genes in a controllable and inducible manner. So we should understand from this study that even imprinted genes, which I will explain very quickly, because you will understand the depth of it. Sometimes it is referred to as human insulin-like growth factor. You have copies of genes from both your father and mother, but the insulin-like growth factor receptor will work only from your mother.

Father has the gene, but it will not work at all. Permanently it is turned off. The same way as insulin-like growth factor. It will work only from the paternal copy. Maternal copy is fine.

It is there, but it will not express itself. This is called imprinting. So sometimes what happens is that if the paternal copy has a mutation, the maternal copy is very much there, but it has a mutation, and now you are suffering. Now the maternal copy can be activated and you will get an extra life. So this is the application. So you can reverse the imprinting. So, to conclude, the crosstalk between stem cells and CRISPR technology is improving day by day.

While human iPSCs provide an infinite supply of cells for the manipulation of phenotypes and differentiation into tissues for disease modeling and possible therapies, CRISPR-Cas9 enables precise, simple, and cost-effective solutions for the genetic manipulation of stem cells to induce specific genetic alterations, unleashing its potential in both basic and applied sciences. Since CRISPR technology is only a few years old, it is reasonable to expect that more refined versions of Cas9 family proteins will provide even more fine-tuned approaches to genome engineering with fewer off-target effects, which will, in turn, facilitate the generation of edited patient-derived stem cells suited for personalized medical needs. So we will study more and more about CRISPR-Cas9 and how stem cells are used in the coming classes. Thank you.