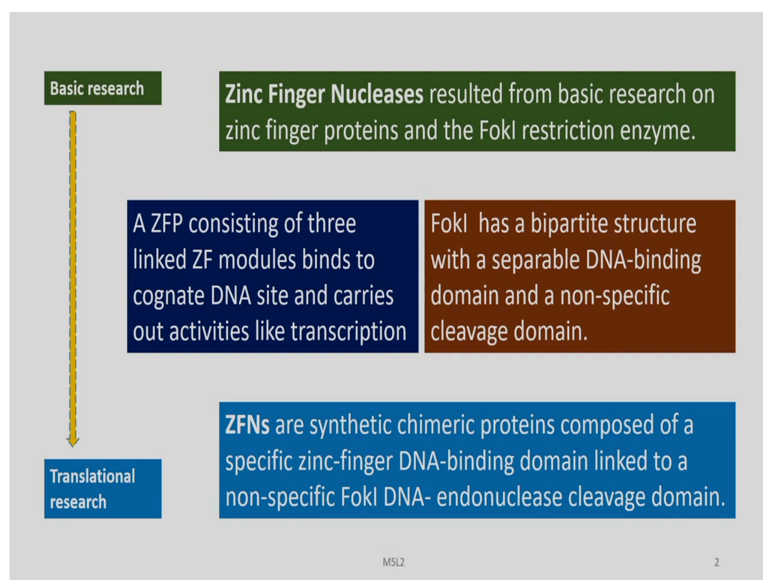
Genome Editing and Engineering
Prof. Utpal Bora
Department of Bioscience and Bioengineering
Indian Institute of Technology, Guwahati

Module - 05
Zinc Finger Nuclease (ZFN) Technology
Lecture - 11
Design of Zinc Finger Nucleases for genome editing

Welcome to my course on Genome Editing and Engineering and as part of module 5 on Zinc Finger Nuclease Technology, we are going to discuss today how we design Zinc Finger Nucleases for Genome Editing. So, this lecture, we will study about the design of the Zinc Finger motifs to bind to DNA targets of our interest.

(Refer Slide Time: 01:07)



We already have studied about the zinc finger domains in the earlier lecture, where nature offers us many zinc finger motifs and which bind to diverse DNA sequences.

And today, we have the capability to modify those zinc finger domains and make them specific to our own needs. So, these were all started with basic research on zinc fingers as well as the Fok1 endonuclease that we studied in the last lectures. And we know now that a ZFP or a zinc finger protein domain consists of three linked zf modules and they bind to cognate DNA site and carries out activities like transcription and Fok1 has a bipartite structure with a separable DNA binding domain and a nonspecific cleavage domain.

Now, put these ZFP and Fok1 knowledge together; a artificial enzyme can be constructed, which we call as zinc finger nucleases and they are basically synthetic chimeric proteins which are composed of a specific zinc finger DNA binding domain linked to a nonspecific Fok1 DNA endonuclease cleavage domain. However, overall these ZFNs bind to specific DNA sequences and cleave as per our requirements or desire.

This is one of the glaring examples of the findings of basic research being taken to the translational stage and this has revolutionized the genome engineering technology in a big way.

(Refer Slide Time: 03:12)

So, you have come to know about Srinivasan Chandrasegaran's work in the last class. And here he defined the functional domains of the Fok1 nuclease and you can see this paper which was being published in PNAS and this is the most important findings amongst others in this paper.

The modular structure of the enzyme suggests that, it may be feasible to construct chimeric endonucleases of different sequences by linking other DNA binding proteins. So, this is something very very remarkable because you have a catalytic domain which is nonspecific.

(Refer Slide Time: 04:18)

So, if we attached it that to other kind of DNA binding motifs so we can engineer other nucleases with diverse specificities. Another work which is considered as a landmark work is the work by Pavletich and Pabo in 1971, they found out from the crystal structure of the three-finger Zif268 bound to DNA that each finger used amino acid positions in minus 1, 3 and 6 of it’s alpha helix to contact a 3 base pair subsite of the DNA.

(Refer Slide Time: 04:55)

So, this knowledge we are going to use extensively in the design of artificial zinc fingers. With all these findings, this seemingly simple mode of recognition of zinc fingers inspired

many scientists to engineer zinc finger nucleases and create custom DNA binding proteins, which can stimulate gene targeting at specific genomic loci in insect, plant and human cells.

Using a linear array of several engineered zinc finger domains, many molecules are generated soon. This seemingly simple mode of recognition inspired scientist to engineer zinc finger nucleases to create custom DNA binding proteins, which can stimulate gene targeting at specific genomic loci in insect, plant and human cells.

Using linear arrays of several engineered zinc finger domains can be generated and connecting the individual DNA binding modules with a highly conserved linker sequence also can be carried out. And it become possible to create polydactyl ZFPs that recognize long sequence of DNA with high specificity.

(Refer Slide Time: 06:08)

Numerous platforms for constructing artificial zinc finger arrays using "modular assembly" became available. However it turned out to be a tedious and complex process in majority of cases as standardized reagents and protocols that permit rapid, cross-platform "mixing-and-matching" of the various zinc finger modules were not easily available.

Other problems included influence of adjacent modules in a polydactyl zinc finger which can have cooperative effects that affect activity of the entire DNA-binding array.

Wright and his associates* have compiled a comprehensive, publicly available archive of plasmids encoding more than 140 well-characterized zinc finger modules together with complementary web-based software (termed ZiFiT) for identifying potential zinc finger target sites in a gene of interest.

*Nat Protoc 1, 1637–1652 (2006). https://doi.org/10.1038/nprot.2006.259

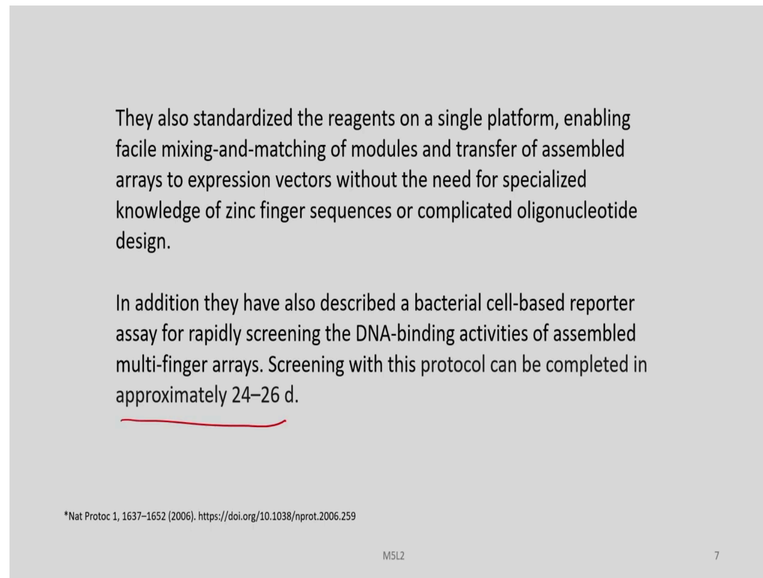
MSL2 6

For creating cells polydactyl ZFPs, a numerous platforms has been used like the module assembly platforms. However, using these modular assembly platform become turned out to be very tedious and complex in majority of cases due to the lack of reagents and protocols that permit rapid cross platform mixing and matching of the various zinc finger modules which are not easily available.

Other problems included influence of adjacent modules in a polydactyl zinc finger, which can have cooperative effects that affect activity of the entire DNA binding array. Wright and his associates have compiled a comprehensive publicly available archive of plasmids coding

more than 140 well characterized zinc finger modules together, which complementary web-based software ZiFiT for identifying potential zinc finger target sites in a gene of interest.

(Refer Slide Time: 07:19)



They also standardized the reagents on a single platform, enabling facile mixing and matching of modules and transfer of assembled arrays to express vectors without the need for specialized knowledge of zinc finger sequences or complicated oligonucleotide design. In addition, they have also described a bacterial cell based reporter assay for rapidly screening the DNA binding activities of assembled multi-finger arrays. Screening with this protocol can be completed in approximately 24 to 26 days or less than a month.

(Refer Slide Time: 07:52)

Most ZFs are 30 amino acids. However many shorter ones are also now reported

Plasmid	ZF ID	Target Seq	ZF Protein Seq
pc3XB-ZF1	ZF1	GAA	PGEKPHICHIQGCGKVYGQRSNLVRHLRWH
pc3XB-ZF2	ZF2	GAC	PGEKPHICHIQGCGKVYGDRSNLTRHLRWH
pc3XB-ZF3	ZF3	GAG	PGEKPHICHIQGCGKVYGRSDNLARHLRWH
pc3XB-ZF4	ZF4	GAT	PGEKPHICHIQGCGKVYGQSSNLARHLRWH
....	...	....	
....	....	....	
pc3XB-ZF139	ZF139	GCT	PGEKPYECNYCGKTFSVSSTLIRHQRIH
pc3XB-ZF140	ZF140	GGT	PGEKPYRCEECGKAFRWPSNLTRHKRIH
pc3XB-ZF141	ZF141	RGA	PGEKPYACHLCGKAFTQCSHLRRHEKTH

Resources

https://www.addgene.org/kits/zfc-modular-assembly/#protocols-and-resources

A spreadsheet containing information for the 141 zinc finger plasmids is available as a CSV file:

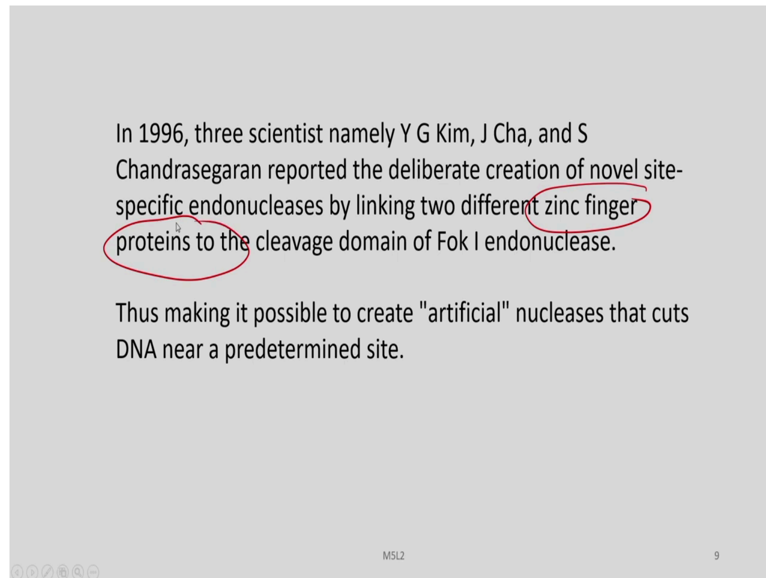
Zinc Finger Plasmid sequences 761.6 KB

M5L2 8

So, these are the various plasmids you can see, a pc3XB-ZF1 to 141, we have only listed here few for your understanding. And they have different ids ZF1 to ZF141 and all these 141 molecules have different, identify different target DNA sequences ranging from GAA to RGA. And these are the ZF protein a sequences.

Most of these ZFs are 30 amino acid long; however, many shorter ones are also now reported and you can visit this site by addgene for further information and a there is a spreadsheet containing information for all these 141 zinc finger plasmids as a CSV file, which is downloadable.

(Refer Slide Time: 08:58)



Now, let us move to the work of three scientists namely Y G Kim, J Cha and S Chandrasegaran, in 1996 they reported the deliberate creation of novel site-specific endonuclease by linking two different zinc finger proteins to the cleavage domain of Fok1 endonuclease. So, they have not only found out earlier that the binding domain and the catalytic domain of Fok1 are in two separate domains and if you divide them into two different domains, the catalytic activity is retained.

So, to this catalytic domain, they joined zinc finger proteins or zinc fingers to give create a new type of specificity. So, it became possible to create artificial nucleases that cuts DNA near a predetermined site. With all these sequence specificities at our disposal, today we can design so many different kind of ZFNs by using ZF motifs as per our DNA of interest. And fusing those modules along with a, with the nuclease domain of Fok1 and this was first pioneered by Chandrasegaran and his group and published in PNAS in 1996.

(Refer Slide Time: 10:27)

Proc. Natl. Acad. Sci. USA
Vol. 93, pp. 1156–1160, February 1996
Biochemistry

Hybrid restriction enzymes: Zinc finger fusions to *Fok* I cleavage domain

(*Flavobacterium okeanokoites*/chimeric restriction endonuclease/protein engineering/recognition and cleavage domains)

YANG-GYUN KIM, JOOYEUN CHA, AND SRINIVASAN CHANDRASEGARAN*

Department of Environmental Health Sciences, The Johns Hopkins University, School of Hygiene and Public Health, 615 North Wolfe Street, Baltimore, MD 21205-2179

Communicated by Thomas Kelly, October 3, 1995 (received for review April 13, 1995)

ABSTRACT A long-term goal in the field of restriction-modification enzymes has been to generate restriction endonucleases with novel sequence specificities by mutating or engineering existing enzymes. This will avoid the increasingly arduous task of extensive screening of bacteria and other microorganisms for new enzymes. Here, we report the deliberate creation of novel site-specific endonucleases by linking two different zinc finger proteins to the cleavage domain of *Fok* I endonuclease. Both fusion proteins are active and under optimal conditions cleave DNA in a sequence-specific manner. Thus, the modular structure of *Fok* I endonuclease and the zinc finger motifs makes it possible to create "artificial" nucleases that will cut DNA near a predetermined site. This opens the way to generate many new enzymes with tailor-made sequence specificities desirable for various applications.

M5L2 10

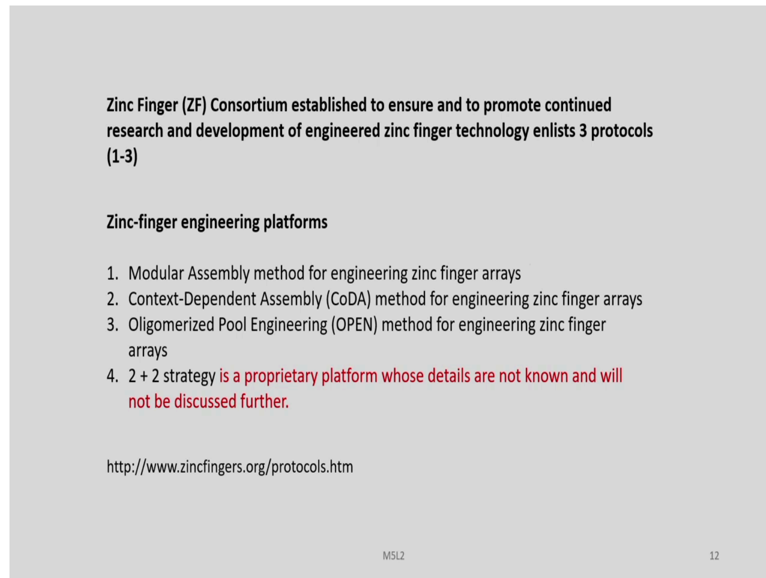
(Refer Slide Time: 10:35)

Zinc-finger engineering platforms

M5L2 11

So, what are the different zinc finger engineering platforms? So, briefly we have mentioned about the modular design of zinc finger proteins.

(Refer Slide Time: 10:47)



So, zinc finger consortium established to ensure and to promote continued research and development of engineered zinc finger technology has enlisted three protocols, which can be used for design of ZFNs.

So, there are most basically, the modular assembly method for engineering zinc finger arrays, the other is the CoDA or the context dependent assembly method for engineering zinc finger arrays and OPEN or oligomerized pool engineering method for engineering zinc finger arrays.

There are another method called the 2 plus 2 strategy this is how a proprietary platform and details are not available. And we will not discuss about this a platform in this lecture; this is just to inform that apart from these three other technologies are also available.

(Refer Slide Time: 11:51)

1. **Modular assembly:** The first generation ZFP design entailed the use of a modular assembly, in which individual zinc fingers were optimized against target triplet DNA sequences, and linked together to form three- or four-ZFPs against 9 or 12 bp sequences.

Ramirez et al., Nat Methods. 2008, 5: 374-375. 10.1038/nmeth0508-374.

M5L2 13

Let us first focus on the modular assembly. As the name suggest and we assemble the ZF modules one by one and then attach it to a nuclease enzyme. So, the first generation ZFP design entailed the use of a modular assembly in which individual zinc fingers were optimized against target triplet DNA sequences and linked together to form 3 or 4 ZFPs against 9 or 12 base pair sequences because 1 module will bind to 3 bases. So, 3 will correspond to 9 and 4 will correspond to 12 base pair sequences.

(Refer Slide Time: 12:34)

Modular assembly thus involves the joining of single zinc-finger domains of known DNA-binding specificities and Wright and his associates contributed for this platform.

While modular assembly provided examples of successfully applied ZFPs, Ramirez et al., raised concerns about "Unexpected failure rates for modular assembly of engineered zinc fingers" in 2008.
This high failure rate with this approach is likely due to ignoring the influence that neighboring zinc-fingers have on the sequence specificity of a given zinc finger.

To overcome such deficiencies selection-guided assemblies were developed.

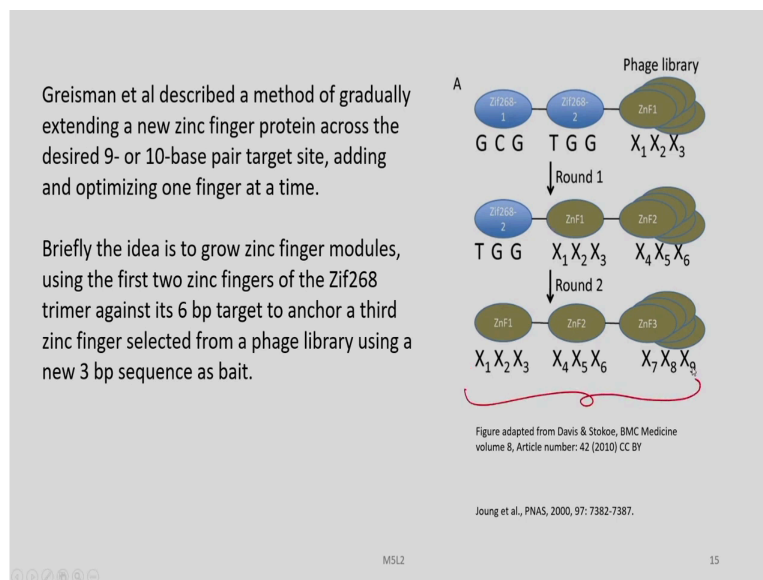
Ramirez et al., Nat Methods. 2008, 5: 374-375. 10.1038/nmeth0508-374.

M5L2 14

So, in such an approach we join a single zinc finger domains of known DNA binding specificities and through this Wright and his associates contributed hugely for the development of this technology and the platform. While modular assembly provided examples of successfully applied ZFPs, a Ramirez and his team raised concerns about unexpected failure rates for modular assembly of engineered zinc fingers in 2008.

The high failure rate with this approach is likely due to the fact that they were ignoring the influence of neighboring zinc fingers on the sequence specificity of a given zinc finger. To overcome such deficiencies selection guided assemblies were developed.

(Refer Slide Time: 13:38)



So, Greisman et al described a method of gradually extending a new zinc finger protein across the desired 9 or 10 base pair target site adding and optimizing one finger at a time. Briefly the idea is to grow zinc finger modules, using the first two zinc fingers of the Zif268 trimer against its 6 base pair target to anchor a third zinc finger selected from a phage library using a new 3 base pair sequence as a bait.

So, there are two rounds as you can see over here. So, this is the Zif268 first and the second module and the third module will be selected from this phage library; ok. And in after the first round, the second one is replaced and after the second round, the third one is also replaced. And the final construct will have a very high specificity.

(Refer Slide Time: 14:47)

This process was then repeated two additional times to replace each of the two remaining Zif268 zinc fingers (Figure A).

The resulting synthetic zinc finger trimer can display high affinity and specificity towards the desired 9 bp target sequence.

In a similar approach Joung et al., used a bacterial two hybrid system to optimize zinc finger binding to DNA sequences of interest.

A

Phage library

Zif268-1 Zif268-2 ZnF1

G C G T G G $X_1X_2X_3$

Round 1

Zif268-2 ZnF1 ZnF2

T G G $X_1X_2X_3$ $X_4X_5X_6$

Round 2

ZnF1 ZnF2 ZnF3

$X_1X_2X_3$ $X_4X_5X_6$ $X_7X_8X_9$

Figure adapted from Davis & Stokoe, BMC Medicine volume 8, Article number: 42 (2010) CC BY

Joung et al., PNAS, 2000, 97: 7382-7387.

M5L2 16

So, this process involve repetition of two additional times to replace each of the two remaining Zif268 zinc fingers. The resulting synthetic zinc finger trimer can display high affinity and specificity towards the desired 9 base pair target sequence.

In a similar approach Joung and his team used a bacterial two hybrid system to optimize zinc finger binding to DNA sequences of interest.

(Refer Slide Time: 15:20)

2. **Context-Dependent Assembly (CoDA)**

Sander et al., described the context-dependent assembly (CoDA), a publicly available platform of reagents and software that is simple to practice and has a success rate for generating active zinc-finger arrays comparable to that of selection-based methods such as OPEN.

"Context-sensitive selection" strategies attempt to identify combinations of zinc-fingers that work well together.

Using CoDA ZFNs, they rapidly altered 20 genes in zebrafish, Arabidopsis, and soybean.

The simplicity and efficacy of CoDA could enable broad adoption of ZFN technology and make possible large-scale projects focused on multi-gene pathways or genome-wide alterations.

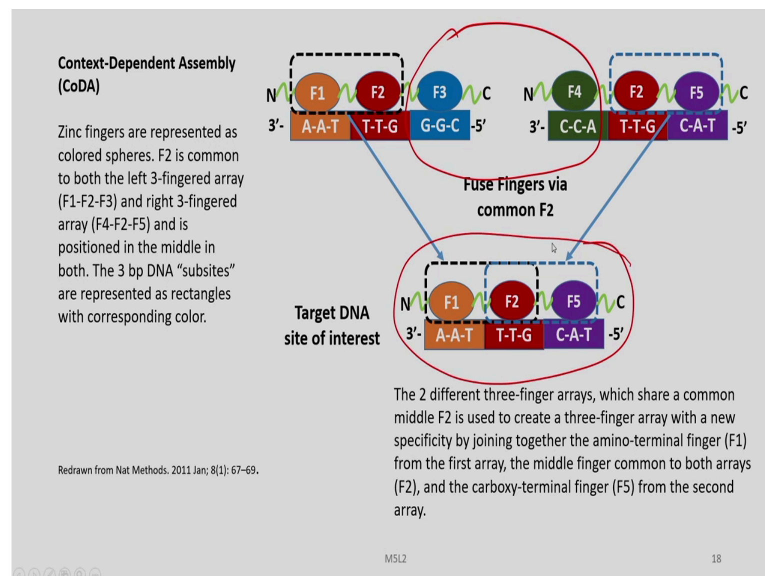
Sander et al., Nat Methods. 2011 Jan; 8(1): 67–69. doi:10.1038/nmeth.1542

M5L2 17

Next, let us move on to the little bit of discussion on the context dependent assembly CoDA. Sander and his team described this CoDA technique, a publicly available platform of reagents and software that is simple to practice and has a success rate for generating active zinc finger arrays comparable to that of selection-based methods such as OPEN.

So, context sensitive selection strategies attempt to identify combinations of zinc fingers that work well a together. Using CoDA ZFNs, they rapidly altered 20 genes in various organisms like zebrafish, Arabidopsis and soybean. The simplicity and efficacy of CoDA could enable broad adoption of ZFN technology and make possible large-scale projects focused on multi gene pathways or genome wide alterations.

(Refer Slide Time: 16:15)



So, here in these cartoon depiction or figure, you can see the zinc fingers which are represented as colored spheres, finger 1, 2, 3 with 3 different colors, binding to three different triplets. And then F4, finger F5 also binding to their respective sequences and in both these, you can see F2 is the common finger and it is binding to his respective sequence TTG.

So, here as already described F2 is common to both the left 3-fingered array F1 to F2, F3 and the right 3-fingered array F4, F2, F5 and its position in the middle in both the triplets or trimers. The three base pair DNA subsites are represented as rectangles with corresponding colors.

So, the two different 3-finger arrays which share a common middle finger F2 is used to create a 3-finger array with a new specificity by joining together the amino terminal finger F1 from the first array, the middle finger common to both arrays and the carboxyl terminal finger F5 from the second array. So, due to this fusion, this F3 and F4 are removed and these combination of F1, F2, F5 now gives a new kind of a specificity.

(Refer Slide Time: 18:21)

The modular assembly strategy, treats ZFs as completely independent units.

Cornu and associates have found that CoDA in contrast to the modular assembly strategy, yield multi-finger domains

i. that show high activity and
ii. low toxicity as ZFNs in human cells

Context-sensitive selection strategies account for potential context-dependent effects, including cooperativity of ZF binding and occasional recognition of a fourth base in the target sequence.

Molecular Therapy (2008), 16: 352-358

M5L2 19

This modular assembly strategy treats ZFs as completely independent units. Cornu and associates have found that CoDA in contrast to the modular assembly strategy yield multi finger domains, that show high activity and low toxicity as zinc finger nucleases in the human cells. These context sensitive selection strategies account for potential context dependent effects including cooperativity of zinc finger binding and occasional recognition of a fourth base in the target a sequence.

(Refer Slide Time: 18:57)

3. Oligomerized pool engineering (OPEN)

Maeder and his team described OPEN (oligomerized pool engineering), an 'open-source,' combinatorial selection-based method for engineering zinc-finger arrays for developing ZFNs.

OPEN method has been found to have a higher success rate than previously described 'modular-assembly' methods for engineering ZFNs.

OPEN selections are carried out in Escherichia coli using a bacterial two-hybrid system and do not require specialized equipment.

Maeder, M.L. et al. Mol. Cell 31, 294–301 (2008).
Nature Protocols 4: 1471–1501 (2009).

M5L2 20

Let us now move to the next platform which is the Oligomerized pool engineering or OPEN. Maeder and his team described OPEN, oligomerized pool engineering, which is an open source combinatorial selection based method for engineering zinc finger arrays for developing ZFNs. Open method has been found to have a higher success rate than previously described the modular assembly methods for engineering ZFNs. OPEN selections are carried out in e.coli, using a bacterial two-hybrid system and they do not require specialized equipment.

(Refer Slide Time: 19:43)

OPEN relies on an archive of pre-characterized zinc-finger pools that are organized based on their binding specificity to a given 3 bp sequence.

After identifying the desired genomic target sequence, the appropriate mixtures of zinc-finger pools are then randomly assembled using overlapping PCR and screened for the zinc finger assembly displaying the most potent binding activity to the desired 9 bp target (Figure B).

B Archive 1 Archive 2 Archive 3
ZnF1 ZnF2 ZnF3
$X_1 X_2 X_3$ $X_4 X_5 X_6$ X_7
PCR and assembly
ZnF1 ZnF2 ZnF3
Selection by 2-hybrid
ZnF1 ZnF2 ZnF3
$X_1 X_2 X_3$ $X_4 X_5 X_6$ $X_7 X_8 X_9$

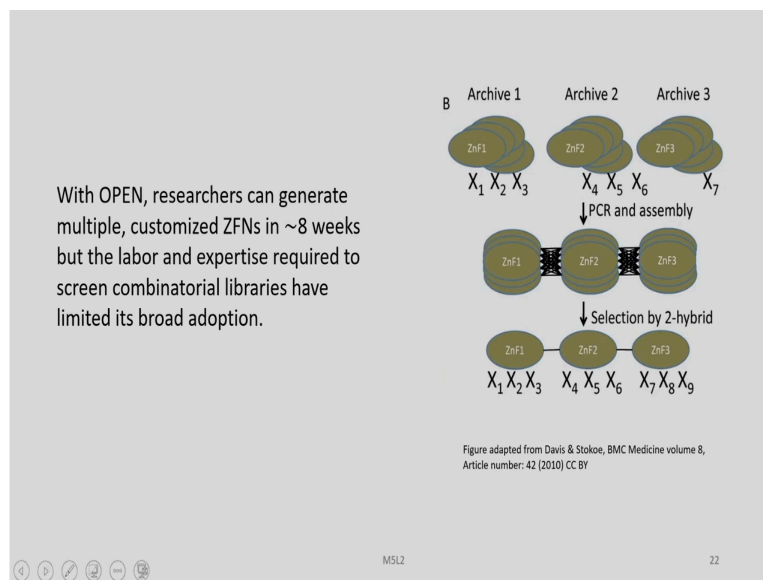
Figure adapted from Davis & Stokoe, BMC Medicine volume 8, Article number: 42 (2010) CC BY

M5L2 21

So, OPEN relies on an archive of pre-characterized zinc finger pools that are organized based on their binding specificity to a given 3 base pair sequence. After identifying the desired genomic target, the appropriate mixtures of zinc finger pools are then randomly assembled using overlapping PCR and screened for the zinc finger assembly displaying the most potent binding activity to the desired 9 base pair target.

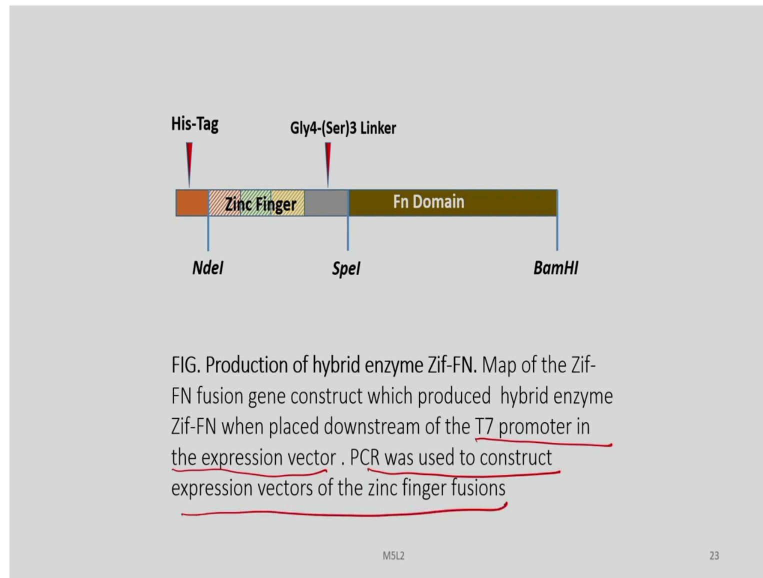
So, here we have three archives 1, 2 and 3. So, we carry out PCR and assembly of the 3 modules and we go for selection by two hybrid methods and obtain the desired 9 base pairs at the end of this process.

(Refer Slide Time: 20:41)



With OPEN, researchers can generate multiple customized zinc finger nucleases in about 8 weeks or 2 months. But the labor and expertise required to screen combinatorial libraries have limited its’ broad adoption.

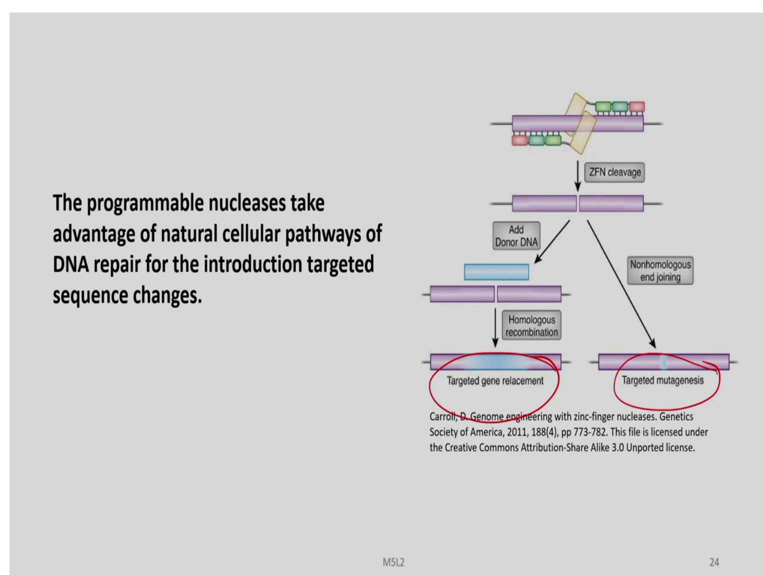
(Refer Slide Time: 21:01)



So, these figure, if you observe we have a His-Tag in one end and then we have the zinc finger triplets over here as you can see and then this is the linker which join the ZF domains to the functional domain and then these are the specific restriction sites in this construct.

So, basically this is used for the production of hybrid enzyme zinc finger nucleases. This is a map of the zinc finger FN fusion gene construct which produced hybrid enzyme ZFN, when placed downstream of T7 promoter in an expression vector. A PCR was used to construct expression vectors of the zinc finger fusions.

(Refer Slide Time: 22:09)



The programmable nucleases take advantage of natural cellular pathways of DNA repair for the introduction of targeted sequence changes. And we have discussed in the earlier lecture that the dimerization of the DNA cleavage domain is very very important and we have also discussed the involvement of the various residues or structural components in this dimerization. And once that dimerization is successful, the ZFNs will create double strength brakes, ok.

So, now it may follow two pathways as we have discussed in the beginning of our course. It can lead to non-homologous end joining and there can be some targeted mutagenesis over here. Or, it can follow the homologous recombination pathway and we may add a donor DNA over here, which will lead to the targeted genetic replacement. So, we are going to use the knowledge of a zinc fingers and Fok1 nucleases to create fusion proteins having domains of zinc fingers and the catalytic DNA cleavage activity of the Fok1 enzyme.

And then we are going to deploy them inside a cell where they will carry out double stranded DNA breakage and then we may direct the repair either in the nonhomologous end joining pathway or in the a homologous recombination pathway by other interventional steps. And thereby carry out a gene editing, which may be simple point mutations or targeted gene replacement.

(Refer Slide Time: 24:30)

Current methods to manipulate the plant and mammalian genomes have two limitations:

(1) the very low rate of homologous recombination (HR) at the targeted site and
(2) the relatively high rate of random, non-targeted integration elsewhere in the genome by non-homologous end joining (NHEJ).

In these cells, HR, occurs at a very low rate compared to, NHEJ.

For most mammalian cells, targeted recombinants via HR are overshadowed by nearly 1000-fold higher random, non-targeted integrants (NHEJ)

Cells use the universal process of HR to maintain their genomic integrity, particularly in the repair of a double-strand break (DSB), which otherwise would be lethal.

Repair of a DSB in a damaged chromosome by HR is a highly accurate form of repair which uses the homologous DNA from the undamaged chromosomal partner as a template.

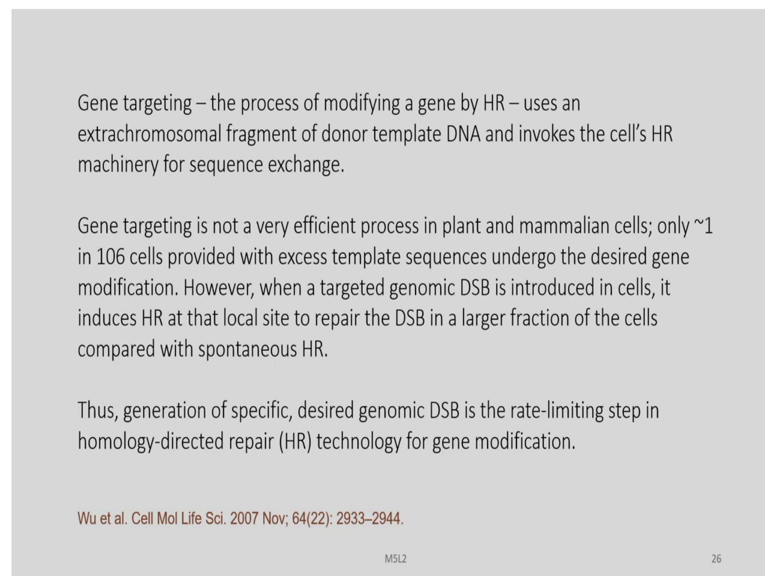
M5L2 25

The current methods to manipulate plant and mammalian genomes have two limitations, the very low rate of homologous recombination at the targeted site and the relatively high rate of

random non-targeted integration elsewhere in the genome by non-homologous end joining. In this cells, homologous recombination occurs at a very low rate compared to the NHEJ. For most mammalian cells targeted recombination by via homologous recombination are over shadowed by nearly 1000-fold higher random non-targeted integrants.

Cells use the universal process of homologous recombination to maintain their genomic integrity, particularly in the repair of double strand breaks which otherwise would be lethal and these are important facts we have emphasized in the last lectures. Repair of these DSB in a damaged chromosome by HR is a highly accurate form of repair which uses the homologous DNA from the undamaged chromosomal partner as a template.

(Refer Slide Time: 25:37)



Gene targeting is the process of modifying a gene by homologous recombination, which uses an extra chromosomal fragment of donor template DNA and invokes the cells homologous recombination machinery for sequence exchange.

Gene targeting is not a very efficient process in plant and mammalian cells, only 1 in 106 cells provide with excess template sequences undergo the desired gene modification. However, when a targeted genomic DSB is introduced in cells, it induces a homologous recombination at that local site to repair the DSB in larger fraction of the cells compared with spontaneous homologous recombination.

So, for generation of specific desired genomic DSB, which is the rate limiting step in homology directed repair technology for a gene modification, we will use zinc finger nucleases or other similar nucleases.

(Refer Slide Time: 26:41)

Molecular scissors for genome surgery. A schematic representation of a pair of 4-finger ZFNs (zinc finger nucleases) bound to their cognate sites in a gene.

Since a pair of ZFNs requires two copies of the 12-bp recognition sites in a tail-to-tail orientation, they effectively have a 24-bp recognition site, which is long enough to specify a unique genomic address in human cells.

Since the ZFN-binding sites in the gene are not identical, both ZFNs that bind these sites need to be introduced into the cell to induce a targeted genomic DSB.

The ZFN sites in the gene are separated by 5 bp.

T-G-T-A-G-C-C-G-C-T-T-T-A-A-G-G-G A-C-T C-C-G G-A-G C-C-T

A-C-A T-C-G G-C-G A-A-A T-T-C-C-C-T-G-A-G-G-C-C-T-C-G-G-A

Adapted from
Nature. 2005;435:646–651.
Cell Mol Life Sci. 2007; 64: 2933–2944

M5L2 27

So, they also called as the molecular scissors for a genome surgery. A schematic representation of a pair of 4 finger ZFNs bound to their cognate sites can be seen in this figure. There are four members over here, fingers and these are the nuclease domains.

A pair of ZFNs require two copies of the 12 base pair sequence recognition sites in a tail to tail orientation. they effectively have a 24 base pair recognition site, for these catalysis to happen, which is long enough to specify a unique genomic address in human cells. So, the off-target cleavage is highly reduced a due to this high or long recognition site involvement. So, if they are triplets, the requirement will be actually 9 plus 9 or 18 base pair recognition sites.

Since the ZFN binding sites in the genes are not identical, both ZFNs that bind these sites need to be introduced into the cell to induce a targeted genomic DSBs. So, that is another advantage of this technology; so, we need to design for both the forward and the backward or sense and the anti-sense strands and because these enzyme can act only if it is dimerized.

So, if the binding on any of the strands either the sense or the anti-sense fails, the cleavage will not happen. So, this gives additional specificity to such a constructs. The ZFN sites in the genes are separated here by around 5 base pair as shown in the schematics.

(Refer Slide Time: 28:56)

Single ZF Array Vs. Dimeric ZF Nuclease Sites.

ZF1 – ZF 2 - ZF3

T-G-T-A-G-C-C-G-C-T-T-T-A-A-G-G-G A-C-T C-C-G G-A-G C-C-T

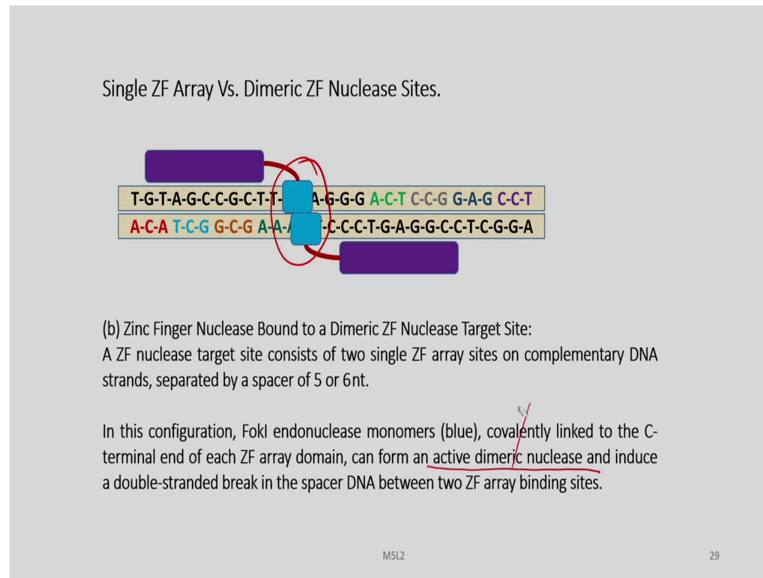
A-C-A T-C-G G-C-G A-A-A T-T-C-C-C-T-G-A-G-G-C-C-T-C-G-G-A

(a) Zinc Finger Protein Bound to a Single ZF Array Target Site:
A Single ZF Array Target Site consists of three to eight adjacent DNA triplets (9–24nt) on the same strand of DNA. Each triplet of DNA is recognized by one “finger” or “ZF module” (light blue ovals). The cartoon illustrates an array with three fingers (ZF1, ZF2, ZF3) bound to a single ZF array target site, in this case, a 9nt DNA sequence.

MSL2 28

So, single ZFN array versus dimeric ZF nuclease sites. So, here you see a single ZF array comprising of three fingers binding to one of the strands. So, ZF finger protein bound to a single ZF array target site as shown in this picture, a single ZF array target site consisting of 3 to 8 adjacent DNA triplets or 9 to 24 nuclease sites on the same strand of the DNA. Each triplet of DNA is recognized by one finger or ZF module. The cartoon illustrates an error with three fingers bound to a single ZF array target site in this case in 9 nucleotide DNA.

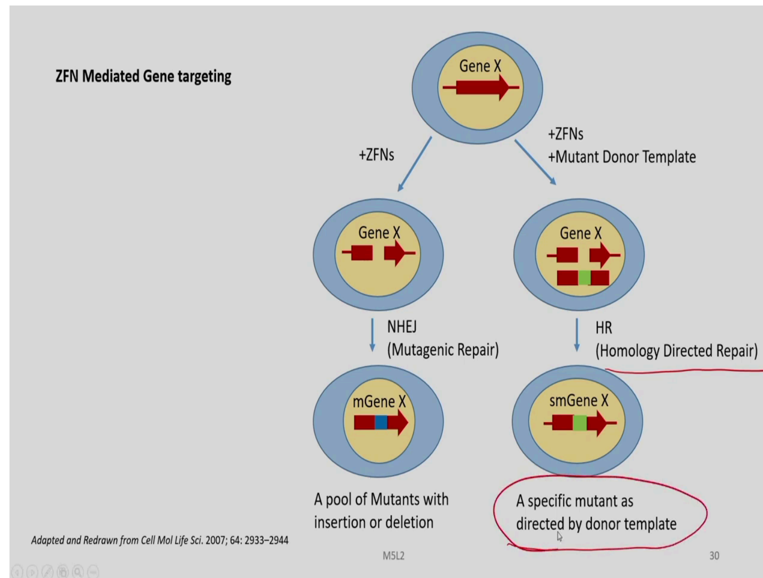
(Refer Slide Time: 29:44)



So, here is the single ZF array versus dimeric ZF nuclease sites and here you have two partners. The single, the zinc finger nuclease bound to a dimeric ZF nuclease target site is being depicted in this figure. A ZF nuclease target sites consisting of 2 ZF array sites on a complementary DNA strands, separated by a spacer of around 5 or 6 nucleotides. And in this configuration a Fok1 endonuclease a monomers, in blue, covalently linked to the C terminal end of each ZF array domain forms an active dimeric nuclease and induce a double stranded break in the spacer DNA between two ZF array binding sites.

So, these active dimeric nuclease is very very important for the cleavage of DNA. Here due to absence of its partner on the other strands, the enzyme is not active and it is not able to cleave. So, this concept is very very important to understand the mechanism by which zinc finger nucleases operate.

(Refer Slide Time: 31:10)



So, let us now study about ZFN mediated gene targeting. Let us now study about ZFN mediated gene targeting. In the earlier slide, we discussed about the requirement of dimerization of the nuclease domains to induce a double strand break.

So, we are going to use that double strand break generation by ZFNs for ZFN mediated gene targeting. So, there is a gene X as you can see in this picture and then we expose it to zinc finger nucleases, which will generate a double strand break in this gene because the zinc fingers that we have designed bind to the sense and the antisense strand and the ZFNs dimerize to generate this double strand break.

Now, due to some kind of deletion events, there may be some kind of mutation occurring here. But these pathway comes into action over here and then there is a mutagenic repair due to NHEJ, Non Homologous End Joining and we get a pool of mutants with insertion or deletion in this case. Now, similarly, if we add engineered zinc finger nucleases and we also add some mutant donor templates. So, there will be template dependent repair in this case, which is due to the homology directed repair and then we get here a specific mutant which is directed by the donor template.

(Refer Slide Time: 33:27)

ZFN-mediated gene targeting involves various steps as follows:

- Deliver ZFNs alone to induce a targeted DSB in Gene X of normal cells and stimulate NHEJ to generate a pool of mutants (mGene X), some of which will be frame-shift mutations resulting in functional deletion of Gene X, i.e. knock-outs of Gene X. Alternatively, deliver ZFNs and the mutant Gene X donor fragment into normal cells to induce a targeted DSB and stimulate HR to generate a specific Gene X mutant, i.e. knockouts or knock-ins of Gene X as the case may be. To achieve gene editing (or gene correction), deliver ZFNs and the correcting donor Gene X fragment into mutant cells.
- Monitor for gene correction (or mutagenesis) at the targeted gene loci as per the investigator-provided donor Gene X template.
- It is also critical to show that the donor DNA fragment has not integrated elsewhere within the genome of the cell by using Southern Blot.

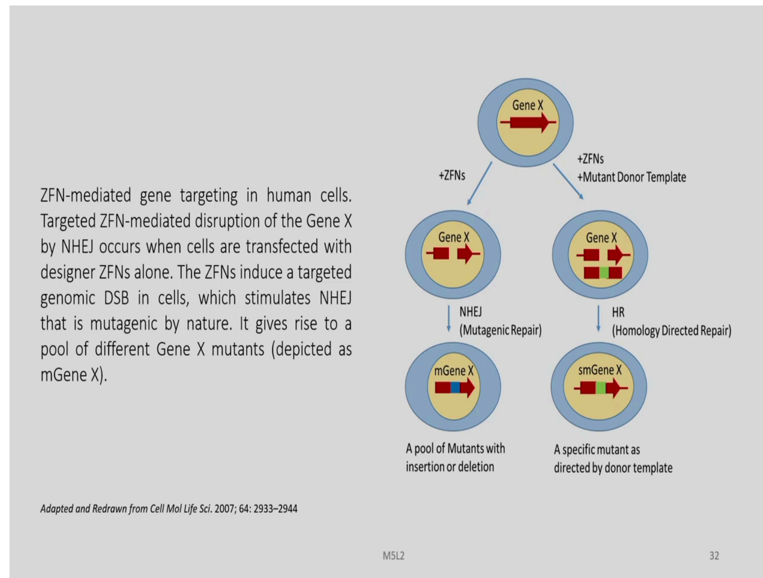
MSL2 31

So, this is the strategy by which we use the ZFN mediated gene targeting. These ZFN mediated gene targeting involves various steps as follows. Number 1, you need to deliver ZFNs alone to induce a targeted DSB in Gene X of normal cells and stimulate NHEJ to generate a pool of mutants. Some of which will be frame shift mutations resulting in functional deletion of gene X, that is knockouts of gene X.

Alternatively, we deliver ZFNs and the mutant Gene X donor fragment into normal cells to induce a targeted DSB and stimulate homologous recombination to generate a specific Gene X mutant, that is knockouts or knock-ins of Gene X as the case may be to achieve gene editing, deliver ZFNs and the correcting donor Gene X fragment into the a mutant cell.

The second step is to monitor for gene correction or mutagenesis at the targeted gene loci as per the investigator provided donor Gene X template and it is also critical to show that the donor DNA fragment has not integrated elsewhere within the genome of the cell by using southern blot. So, this has to happen exactly here and not somewhere else and that has to be confirmed.

(Refer Slide Time: 34:55)



So, it is an important step in the ZFN media mediated gene targeting. ZFN mediated gene targeting in human cells. Let us study a bit about it. Targeted ZFN mediated disruption of the Gene X by NHEJ occurs when cells are transfected with designer ZFNs alone. The ZFNs induce a targeted genomic DSB in cells which stimulates non homologous end joining that is mutagenic by nature; it gives rise to a pool of different Gene X mutants.

Let us now discuss about ZFN mediated gene targeting which involves various steps as followed. And the first step is to deliver ZFNs alone to induce a targeted DSB in Gene X of normal cells and stimulate the non-homologous end joining to generate a pool of mutants, some of which will be frame shift mutations resulting in functional deletion of Gene X that is gene knockouts of gene X.

Alternatively, deliver ZFNs and the mutant Gene X donor fragment into normal cells to induce a targeted DSB and stimulate homologous recombination to generate a specific Gene X mutant, that is knockout or knock-ins of Gene X as the case may be.

To achieve gene editing, we need to deliver the ZFNs and the correcting donor Gene X fragment into the mutant cells. Once this is done, we need to monitor for the gene correction or mutagenesis at the targeted gene loci as per the investigator provided donor X template. And it is also very important to determine that the donor DNA fragment has not integrated elsewhere within the genome of the cell by using southern blot.

(Refer Slide Time: 36:44)

ZFN-mediated gene targeting involves various steps as follows:

- Identifying ZFN target sites near the targeted locus within the Gene X of interest.
- Design and/or select ZFPs that recognize the chosen ZFN target sites.
- Convert the designed and/or selected ZFPs into ZFNs.

M5L2 32

So, identifying ZFN target sites near the targeted locus within the Gene X of interest is the first most important step in this procedure. Next, in this procedure, we go for designing and or selecting zinc finger proteins that recognize the chosen ZFN target sites. And then, we convert the designed and or selected ZFPs into ZFNs by attaching it or covalently, you know, binding it to the nuclease domain.

(Refer Slide Time: 37:27)

addgene Browse Catalog Deposit Education & Tools Help Center

Plasmid Collections / Zinc Finger Consortium

Zinc Finger Consortium Reagents

The Zinc Finger Consortium was established to promote continued research and development of engineered zinc finger technology. Consortium members Keith Joung and Daniel Voytas have deposited at Addgene various reagents for engineering and expressing zinc finger proteins for distribution to academic and nonprofit laboratories for research and educational use. Links to additional pages describing these reagents are provided below:

Zinc Finger Genome Engineering Reagents

- Zinc Finger Arrays for Zebrafish Genes - Browse pre-constructed zinc fingers targeting specific genes
- Oligomerized Pool ENgineering (OPEN) Reagents - Engineer zinc finger arrays via OPEN method
- Modular Assembly Engineering Reagents - Engineer zinc finger arrays via Modular Assembly method
- Nuclease Expression Vectors - Cloning vectors for expressing zinc finger FokI nuclease fusions

You may also like...

Keith Joung Lab plasmids

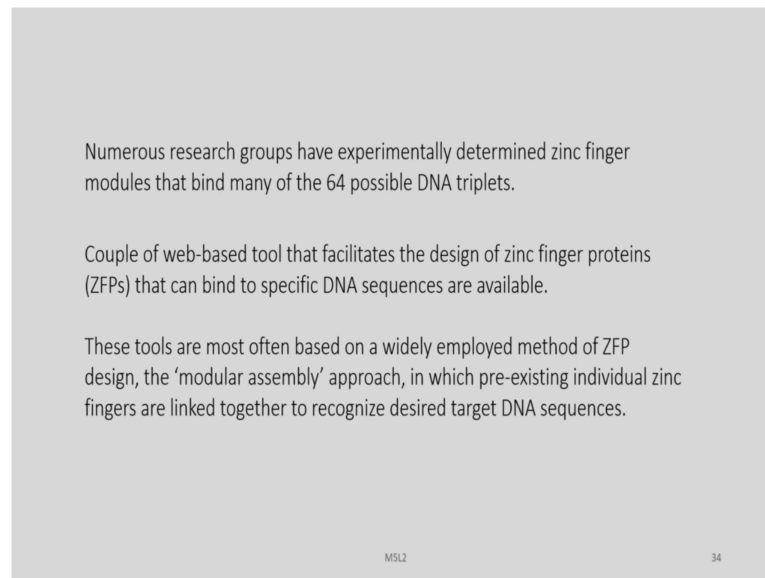
Daniel Voytas Lab plasmids

Scot Wolfe Lab plasmids

M5L2 33

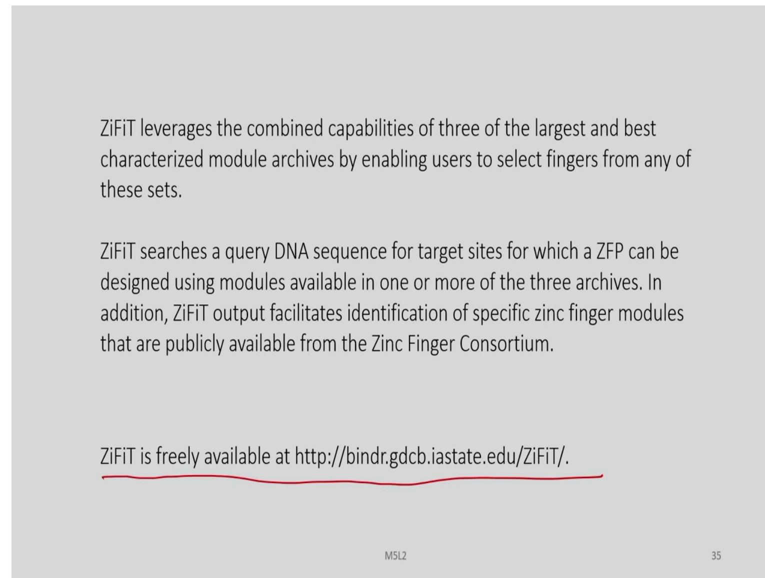
So, this is a resource site from where you can get many of the reagents required and you have these various platforms as well here, the OPEN and the a modular assembly engineering reagents and we have discussed about these a various platforms earlier.

(Refer Slide Time: 37:53)



Numerous research groups have experimentally determined zinc finger modules that bind many of the 64 possible DNA triplets. A couple of web based tools that facilitate the design of zinc finger proteins that can bind to specific DNA sequences are also available. These tools are most often based on a widely employed method of ZFP design, the modular assembly approach in which pre-existing individual zinc fingers are linked together to recognize desired target a DNA sequences.

(Refer Slide Time: 38:31)



ZiFiT leverages the combined capabilities of three of the largest and best characterized module archives by enabling users to select fingers from any of these sets. ZiFiT searches a query DNA sequence for target sites for which a ZFP can be designed using modules available in one or more of the three archives.

In addition, ZiFiT output facilitates identification of specific zinc finger modules that are publicly available from the zinc finger consortium. This is, ZiFiT is freely available from this site and you can download and you can use it or experiment with it and try to generate some zinc finger nucleases, may be just for fun.

(Refer Slide Time: 39:27)

So, this is how this website resource looks. This is a software package and which is designed to aid research in application of gene editing and expression technologies. So, these identifies potential target sites in DNA sequences for several DNA binding platforms using publicly available reagents and protocols, including for CRISPR Cas and TALEs and of course, ZFPs.

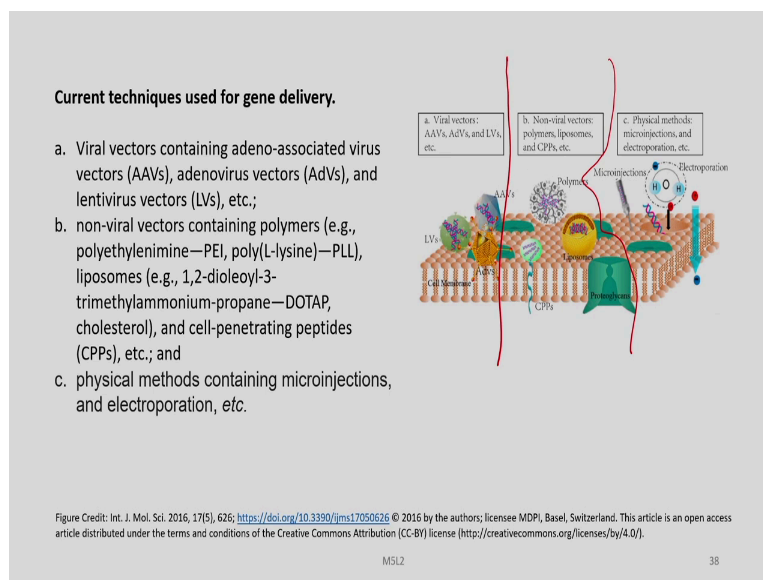
(Refer Slide Time: 40:08)

We are going to discuss about these other two technologies soon one by one. Now, how do we deliver the zinc finger nucleases inside the cell? We now have the capability to design ZF modules by various platforms and you also know about a software platforms available, which

can be used to design effective zinc finger modules. And then we can attach those two nucleases and generate ZFNs of our interest and specificity to bind to DNA targets of our choice.

Now, once we do that, we need to deliver it inside the cell so that it goes inside the cell bind to the specific DNA sequences and then generate double stranded bricks and allow the cell's DNA repair mechanisms to take over and give the desired results, whether it is a point mutation or gene knockout or gene knock in.

(Refer Slide Time: 41:19)



So, they are very different kind of delivery methods used for gene delivery. We have viral vectors like AAV, ADVs and LVs or lentivirus vectors, adeno virus vectors or adeno associated vectors. Then we have, so you can see here, the different viral vectors being used.

In this case, then we have non-viral vectors which may be a polymers, liposomes etcetera or cell penetrating peptides, CPPs. Then we have other methods, physical methods which may be micro injection, electroporation etcetera. So, these in brief, constitute the three major type of gene delivery platforms or methods.

(Refer Slide Time: 42:29)

In Vivo Delivery Systems for Zinc Finger Nucleases (ZFNs) and Their Expression Cassette

Viral Delivery

The virus life cycle involves infection and replication. In infection step, the virus enter into target cells after recognition, and release the viral genome for replication.

In replication step, the progeny virions are released outside cells after synthesizing the viral genome copies in cells. Following this fresh infection steps in nearby cells happen or circulation begin.

This is useful in using viruses vectors to encode and deliver genome editing programmable nucleases to target tissues or cells, and achieve genome editing therapy.

M5L2 39

So, in vivo delivery systems for zinc finger nucleases in their expression cassette. So, we use virus as we already told in the earlier slide. The virus life cycle involves infection and replication. In infection step, the virus enters into target cells after recognition and release the viral genome for replication.

In replication step, the progeny are released outside cells after synthesizing the viral genome copies in to cells. Following these fresh infection steps in nearby cells happen or circulation begins. This is useful in using virus vectors to encode and deliver genome editing programmable nucleases to target tissues of cells and achieve genome editing therapy.

(Refer Slide Time: 43:16)

Adenoviral Vectors (AdVs)

The first-generation adenoviral vectors (AdVs) are obtained by substituting the gene E1 (3.15 kb), or the E1 and E3 (3.1 kb), followed by the second-generation AdVs, which lack more than two early genes, including E2 and E4.

Compared to the first generation, the second-generation AdVs provide an extended genome packaging capacity.

However, it is limited by the inflammatory response of the host and the inability of replicating in vivo including a cytotoxic T-cell response.

A systemic delivery of ZFNs, against HIV infection has entered clinical trials.
The expression unit coding the right and left ZFNs is inserted into a serotype 5 AdV pseudotyped with serotype 35 fiber (AdV5/35). The AdV/ZFNs system is designed to repair the autologous CD4+ helper T cells from HIV infected patients.

M5L2 40

So, one of the earliest viral vectors used was the adeno viral vectors or the first generation adeno viral vectors, which obtained by substituting the gene E1 or E1 and E3 followed by the second generation AdVs, which lack more than two early genes, including E2 and E4. Compared to the first generation, the second generation AdVs provide an extended genome packaging capacity. However, it is limited by the inflammatory response of the host and the inability of replicating in vivo including its cytotoxic T-cell response.

A systemic delivery of ZFNs, against HIV infections has currently entered clinical trials. The expression unit coding the right and left ZFNs is inserted into a serotype 5 AdV pseudo typed with serotype 35 fiber. The AdV ZFN system is designed to repair the autologous CD4 plus and helper T-cells from a HIV infected patients.

(Refer Slide Time: 44:25)

Lentiviral vectors:
Retrovirus can spontaneously penetrate the intact nuclear membrane. For this Lentiviral vectors, are now commonly used for in vivo delivery in genome editing therapy.

As the delivery vector of ZFNs technology, lentiviral vectors which can accommodate sequences up to around 10 kilobases (kb) theoretically allow for site-specific genome modification or addition in predefined genomic sites.

However, they have been avoided because of multiple tragedies involving patient death in earlier clinical trials. Therefore, advanced types, such as Integrase-Defective Lentiviral Vectors (IDLVs) have been heavily explored in recent years.

M5L2 41

Another virus that is used is are the lentiviral vectors. The retrovirus can spontaneously penetrate the intact nuclear membrane. For this lentiviral vectors are now commonly used for in vivo delivery in genome editing therapy. As the delivery vector of ZFNs technology, lentiviral vectors which can accommodate sequences up to around 10 kilobases or theoretically allow for site-specific genome modification or addition in predefined genomic sites.

However, they have been avoided because of multiple tragedies involving patient death in earlier clinical trials. Therefore, advanced types, such as Integrase-Defective Lentiviral Vectors have been heavily explored in the recent years.

(Refer Slide Time: 45:19)

Adeno-Associated Viruses Vectors (AAVs)
Adeno-associated viruses vectors (AAVs), are the commonly used delivery vector in genome editing technologies for its potential site-specific integration ability and low immunogenic characteristics.

Eg. Icosahedral non-enveloped viruses in the *Dependovirus* genus of *Parvoviridae* family.

The major limitation of AAV vectors system is their relatively small genome size (around 4.7 kb), which restricts the genome engineering nuclease complexes they can carry up.

However, with careful design, sequences for expressing ZFNs (each sequence is around 1 kb) and an optional donor DNA template can be encapsulated well by AAV vectors.

M5L2 42

Another viral vector that is used in delivering ZFNs for genome editing is the adeno associated virus vector. The adeno associated virus vectors are the commonly used delivery vector in genome editing technologies for its potential site specific integration ability and low immunogenic characteristics unlike the others discussed earlier.

Some examples of these are icosahedral non-enveloped viruses in the dependovirus genus of parvoviridae family. However, it also has certain limitations. The measure limitation of AAV vector system is there relatively small genome size around 4.7 kb, which restricts the genome engineering nuclease complex that they can carry. However, with careful design sequences for expressing ZFNs each sequences around 1 kb and an optional donor DNA template can be encapsulated well by the AAV vectors.

(Refer Slide Time: 46:26)

Direct Delivery of ZFN Proteins

For a protein to be delivered directly overcoming the barriers in the cell membrane penetration by proteins due to their low lipophilicity, pH sensitivity and degradation by endogenous proteases are important.

Fortunately ZFN proteins can penetrate cell membranes due to the positive charge of Cys2-His2 zinc finger domains.

It has been found that the direct delivery of ZFN proteins can disrupt the CCR5 gene in both HEK-293, HDF cells, and human CD4+ T cells.

Increasing the stability of ZFN proteins is vital to achieve the direct delivery of ZFN proteins, because ZFN proteins will be completely degraded in four hours. One of possible strategies to keep ZFN stable is to modify the lysine residue needed in the degradation

M5L2 43

We can also deliver ZFNs directly. So, for a protein to be delivered directly overcoming the barriers in the cell membrane penetration by proteins due to real low lipophilicity, pH sensitivity and degradation by endogenous proteases we have to devise certain strategies.

Fortunately ZFN proteins can penetrate cell membranes due to the positive charge of Cys2-His2 zinc finger domains and it has been found that the direct delivery of ZFN proteins can disrupt the CCR5 gene in both HEK-293, HDF cells and, human CD4 plus T cells.

Increasing the stability of ZFN proteins is vital or critical to achieve the direct delivery of ZFN proteins, because ZFN proteins will be completely degraded within four hours. One of the possible strategies to keep ZFN stable is to modify the lysine residue needed in the degradation of the protein.

(Refer Slide Time: 47:49)

Table 1. Summary form of typical *in vivo* delivery systems and candidates for genome editing nucleases and their expression cassette.

Typical Delivery Systems		Assessment: Advantages	Assessment: Disadvantages	Genome Editing Nuclease	Clinical Trials: Phase	Clinical Trials: Status	Clinical Trials.Gov Identifier
AAVs		High efficiency	Low packaging capacity, cost high	ZFNs, CRISPR/Cas9	–	–	–
AdVs		Low off-target mutagenesis	Immunoreactivity, high cost	ZFNs	I	Completed	NCT01044654
					II	Completed	NCT01252641
					I	Completed	NCT00842634
HCAdVs		High packaging capacity	Cell-specific targeting is difficult to achieve	TALENs	–	–	–
CPP, e.g., TAT-TALEN proteins; CPP-Cas9 proteins		Low off-target mutagenesis	Immunoreactivity	TALENs, CRISPR/Cas9	–	–	–
Candidates for delivering plasmids of nucleases	DOTAP-cholesterol	Easy to produce, large packaging capacity	Large particle size, low targeting efficiency, toxic	–	I/II	Active	NCT01455389
	PEI	Easy to produce, large packaging capacity	Low targeting efficiency, toxic	–	II	Active	NCT00595088
	PEG-PEI-Cholesterol	Easy to produce, large packaging capacity with small particle size, low toxic	Low targeting efficiency	–	II	Active	NCT01118052

Table Credit: Int. J. Mol. Sci. 2016, 17(5), 626; https://doi.org/10.3390/ijms17050626 © 2016 by the authors; licensee MDPI, Basel, Switzerland. This article is an open access article distributed under the terms and conditions of the Creative Commons Attribution (CC-BY) license (http://creativecommons.org/licenses/by/4.0/).

M5L2

44

So, these are in brief the typical in vivo delivery systems and candidates for genome editing nucleases and their expression cassettes.

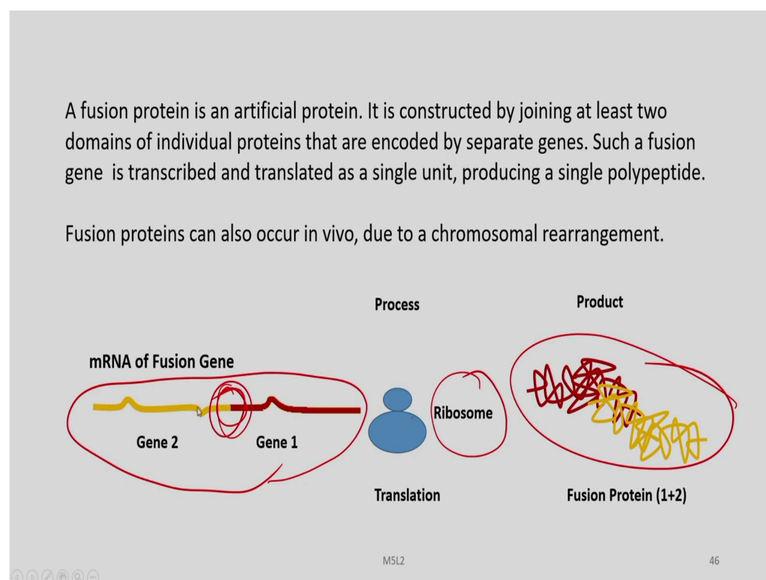
You can see here the typical delivery systems, the viral system AAV, AdV, HCAdVs, then you have here others not important for ZFN. Mostly the AAVs are used for ZFNs as well as CRISPER Cas9 and AdV is also being used over here. And this slide is important from the point of view to understand the status of the clinical trials of these particular delivery platforms. Here information is not available or they are not being put forward for clinical trials.

But here you can see that the AdVs, the clinical trials one has been completed as well as clinical trial phase 2 has also been completed. There are certain advantages of these, they are low, they show low off-target mutagenesis, but the problem is the immuno reactivity and also the cost currently.

(Refer Slide Time: 49:19)

Let us go to the next section of this lecture where we will discuss about the design strategy of ZFNs.

(Refer Slide Time: 49:33)



So, first let us have a small discussion on fusion proteins which is may be very familiar to you, particularly we use many fusion proteins by fusing the gene, green fluorescent protein to it so that we can trace the location of the targeted protein by observing the fluorescence of the green fluorescent protein. So, a fusion protein is an artificial protein, which is constructed by joining at least two domains of individual proteins that are encoded by separate genes. And

for that matter ZFN is a fusion protein in a way, it is joining the zinc finger domains with a nuclease catalytic domain of Fok1.

Such a fusion gene is transcribed and translated as a single unit producing a single polypeptide. Fusion proteins can also occur in vivo due to a chromosomal rearrangements. They are not just engineered all the time, they may happen due to natural chromosomal rearrangement due to any of the mechanisms, cellular mechanisms, inherent cellular mechanisms.

So, ok, this is the mRNA of a fusion gene, we have gene 1 here which is continuous to gene 2 and then as it is through the ribosome for the translation process, we get a product of two proteins, which are covalently bound to one another. The fusion protein product you can see this is 1 plus 2, the two domains may carry out two different continue to carry out the independent functions, which was there in the native a protein.

(Refer Slide Time: 51:36)

2A peptides are 18–22 aa-long self-cleaving peptides, which can induce ribosomal skipping during translation of a protein.

2A peptides share a core sequence motif of DxExNPGP, and are named after the virus in which they have been first described.

F2A, is the first described 2A peptide, derived from foot-and-mouth disease virus. The name "2A" comes from the gene numbering scheme of this virus.

Others like P2A is derived from Porcine teschovirus-1 2A and T2A is derived from *Thosea asigna* virus 2A.

They assist directly in synthesizing polyproteins by causing the ribosome to fail at making a peptide bond.

MSL2 47

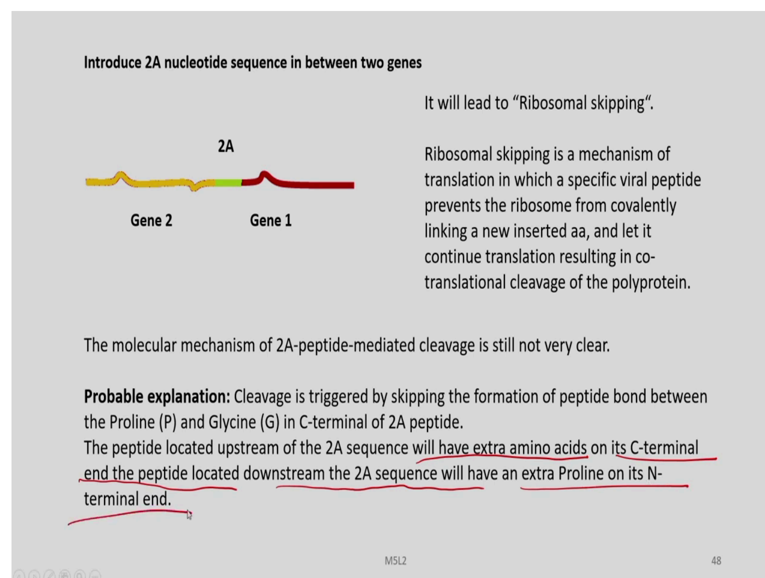
So, there is another important concept that we need to remember or learn at this moment those are the 2A peptides. So, what are these 2A peptides. So, these 2A peptides are 18 to 22 amino acid long self cleaving peptides, they induce ribosomal skipping during translation of a protein. We need to remember the term a ribosomal skipping, ok.

So, let us go back to the earlier slide, you can see mRNA becomes a protein only after translation that happens in the ribosome and we have a term here ribosomal skipping, which

is related to the word a ribosome. 2A peptides share a core sequence motif of DxExNPGP, and are named after the virus in which they have been a first described. F2A is the first described 2A peptide derived from foot and mouth disease virus, which occurs basically in bovidae or for example, in cattles.

Then name, 2A comes from the gene numbering scheme of this particular virus. So, F2A basically derived from foot and mouth disease virus. Others like P2A is derived from a Porcine teschovirus and T2A is derived from Thosea asigna virus. So, we have at least, say, three different peptides, which can induce ribosomal skipping namely, the 2A peptide known as the F2A peptide or P2A peptide or T2A peptide.

(Refer Slide Time: 53:59)



All these assist directly in synthesizing polyproteins by causing the ribosome to fail at making a peptide bond, what we mean by this we will learn very soon.

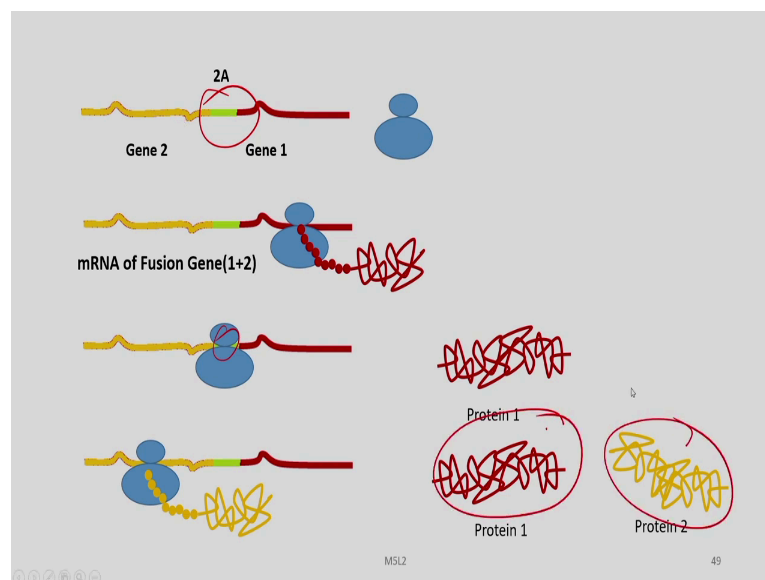
So, we have seen this construct of two genes, gene 1 and gene 2 to create a fusion protein. So, they are continuous one after the other, but in our current scheme of things, we are going to introduce these F2A peptide particularly in between these 2 genes. And by doing this, we are going to have a very different kind of an outcome. The introduction of these 2A or F2A sequence will lead to ribosomal skipping. What do you mean by ribosomal skipping?

This is a mechanism of translation in which a specific viral peptide prevents the ribosome from covalently linking a new inserted amino acid and let it continue translation resulting in

core translational cleavage of the polyprotein. However, the molecular mechanism of this 2A peptide mediated cleavage is still not very clear.

There is a probable explanation given forward by some of the scientists. The cleavage is triggered by skipping the formation of peptide bond between the proline and glycine in C-terminal of 2A peptide. The peptide located upstream of the two way sequence will have extra amino acid on its C-terminal and the peptide located downstream the 2A sequence will have an extra proline on its N-terminal end.

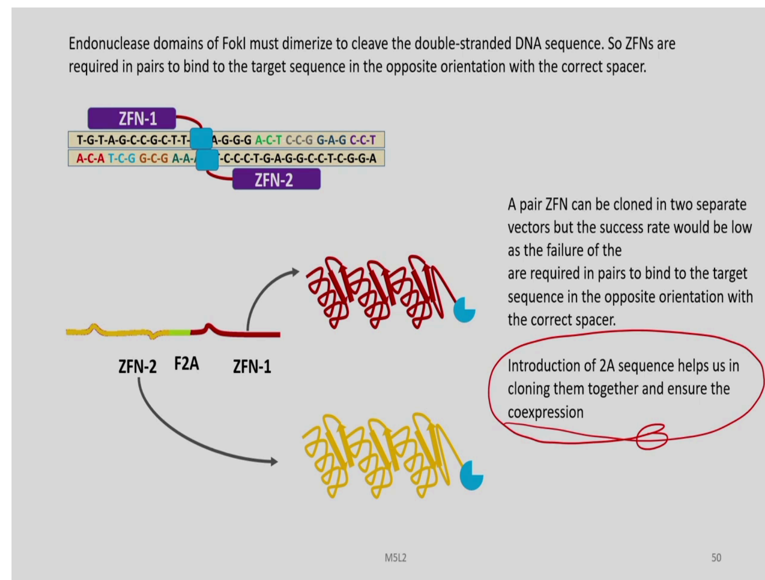
(Refer Slide Time: 55:42)



So, this is the fusion protein of gene 1 and gene 2, which has a 2A intervening separated by a 2A intervening sequence. So, when translation takes place of these mRNA, the first protein is produced, ok, and moment the ribosome come in interaction with the 2A sequence, there is a skipping or ribosomal skipping happening, as a result of which the protein 1 will be released. But the translation continues beyond 2A sequence and it goes on to produce the 2nd protein.

So, by this method of inserting 2A sequence in between the sequence of two genes mRNA sequence of two genes, we can produce two separate proteins in a single read. And if we do not have that sequence, we will have a fusion protein because there is no any 2A here. But in the case where 2A is introduced, we get two different proteins.

(Refer Slide Time: 57:11)



So, this strategy, design strategy is very very important for ZFN design. We go back to the fact that the endonuclease domains of Fok1 must form dimers to cleave the double stranded DNA sequences.

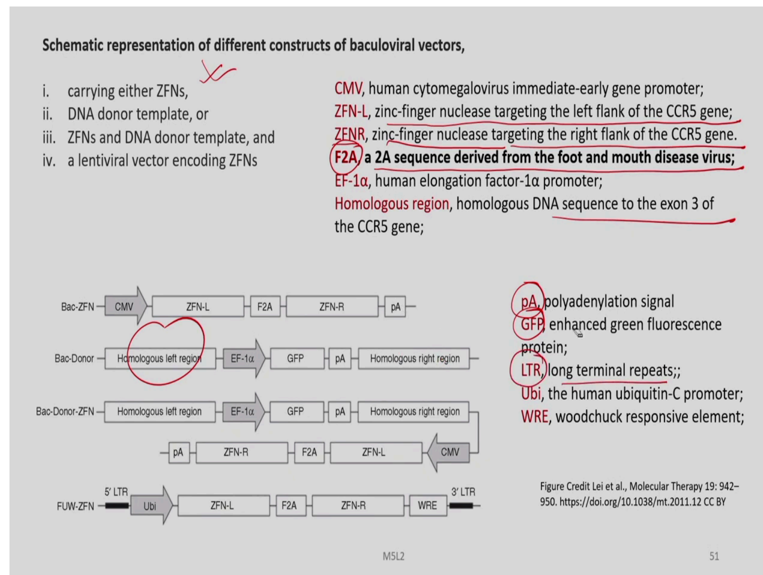
So, ZFNs are required in pairs to bind to the target sequence and we have also discussed a single ZFN molecule is not going to cleave the DNA, we need a partner which bind to the complementary strand. So, these binding of two ZFN molecules in the opposite orientation with the correct spacer is very very important for double strand breaks.

So, to provide 2 ZFN molecules inside the cell, we may clone them into a separate vectors. But the success rate would be low, because if one of the vectors do not enter or efficiency is low or for any other reason, the vectors are delivered, but one of the constructs on ZFNs are not expressed properly. The dimerization is not going to happen satisfactorily and the double strand break generated will be low.

To avoid that, we will put both the ZFNs into one single construct and separate it by a F2A sequence. We cannot a generate a fusion protein because that is not going to be helpful to us. So, this will ensure that both the ZFNs are delivered inside the single cell and they produce both the nucleases, which will bind to the specific targets and after dimerization, they will form the double strand breaks and allow the desired mutagenesis to occur.

So, the introduction of the 2A sequence helps us in cloning them together and ensure the co-expression as well as the double strand breaks.

(Refer Slide Time: 59:43)



So, here is a schematic representation of different constructs of baculovirus vectors. So, number 1, you have carrying either of the ZFN. So, you have the ZFN-L and ZFN-R and you can now understand what is this F2A sequence is all about. So, ZFN-L may be binding on one of the strands and the ZFN-R will bind on the other strand.

So, the CMV is the human cytomegalovirus intermediate early gene promoter, ZFN-L is the zinc finger nuclease targeting the left flank of the CCR5 gene, ok, in this is important gene in the pathogenesis of HIV, which we are going to discuss in the applications part. The zinc finger nuclease ZFN-R is the ZFN targeting the right flank of the CCR5 gene and this F2A is a two way sequence derived from the foot and mouth disease virus.

And these are the homologous regions of the DNA sequences and this is a polyadenylation site and in certain other constructs we use the GFP and you have the LTR, which are the long terminal a repeats. So, to sum up briefly, we can have a various constructs of baculovirus where we carry the ZFN right and ZFN left sequences, intervening sequences, with intervening F2A foot and mouth disease 2A sequences. And we know that this is being done to ensure that both the ZFN molecules are delivered together and expressed together to carry out efficient double strand breaks and the targeted mutagenesis.

Thank you for your patient hearing.