Genome Editing and Engineering
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Module - 04
Targeted genetic modification
Lecture - 9
Targeted genetic modification-II

Welcome to my course on Genome Editing and Engineering. Today we are going to discuss about Targeted genetic modification the second part, which is part of module 4.

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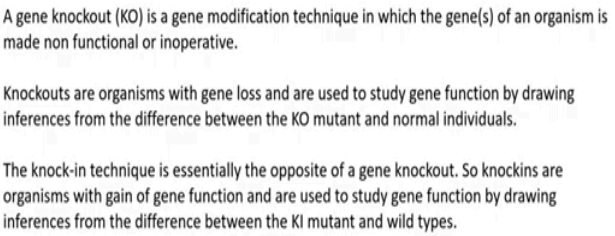

So, till now you have learnt about knockout and knock-ins and you know that knockout is a gene modification technique in which the genes of an organism is made non functional or inoperative.

So, briefly knockouts are organisms with gene loss or loss of gene function and these are used to study gene function by drawing inferences from the differences between knockout mutants and wild type or normal individuals. In contrast to these, knock-in technique is essentially the opposite of a gene knockout. So, in knock-ins organisms have gain of gene function and these are also used to study gene function by drawing inferences from differences between the knock-in mutant and wild types.

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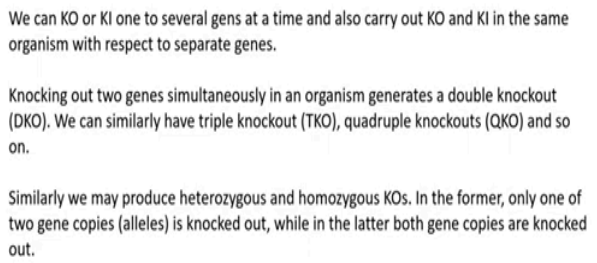

We can create knockouts or knock-ins with to one to several genes at a time and also can carry out knockout and knock-in in the same organism with respect to separate genes or we may create a knockout first and then again recreate a knock-in in the same organism with respect to the same gene.

Knocking out two genes simultaneously in an organism will generate a double knockout which we call as DKO and we can similarly have triple knockouts or TKO or quadruple knockouts and so on. And in fact, similarly we can have double knock-ins or triple knock-ins and so on and so forth. In fact, these type of techniques are used for creating humanized organisms, which we will discuss at a later point of time in this course.

Now, just focusing on one gene, we may create a heterozygous knockout or a homozygous knockout and you can easily understand, homozygous knockout both the alleles are being made in operationable. But in a heterozygous knockout, one of the wild type gene is allele is retained while the other copy is made inactive.

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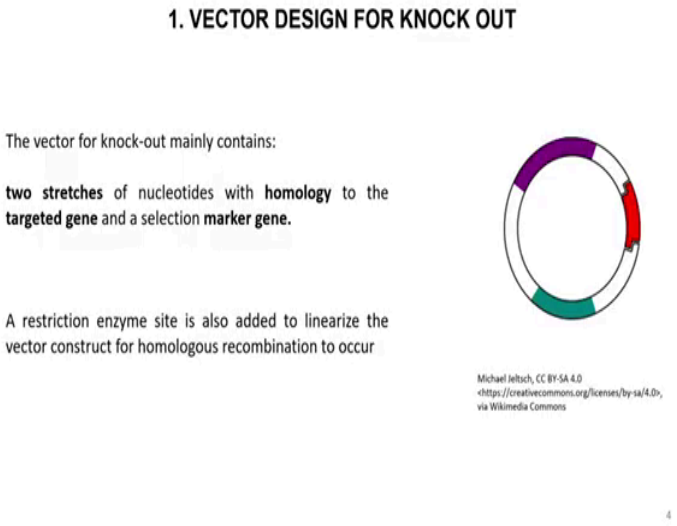

So, for carrying out knockouts, we have to take use of vectors. So, let us discuss a little bit about the design of the vector that is used for creating knockouts. In general a knock-out vector contains the following:

It will have two stretches of nucleotides with homology to the targeted gene and a selection marker. The selection marker would help us in selecting a successful knockout. Then, there is a restriction site which is used to linearize the vector construct for homologous recombination to occur because the target organism will have a DNA in a linearized form.

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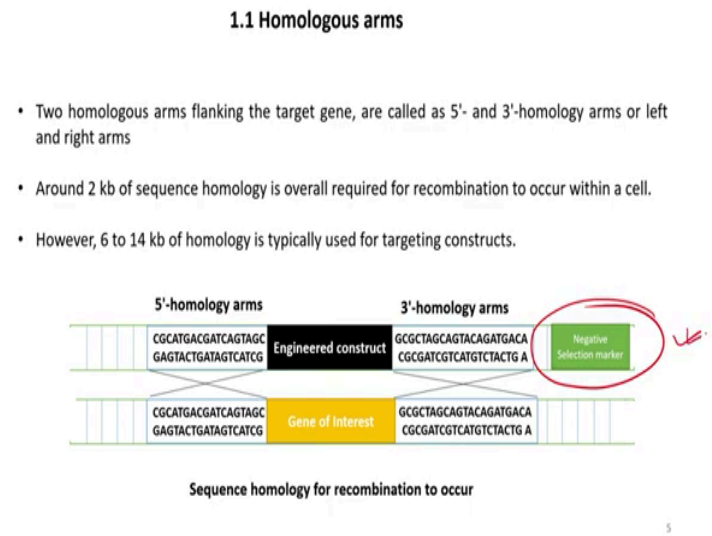

Let us focus on the two homologous stretches or the homology arms, these arms flank the target gene. So, you can see here a gene of interest and corresponding to the gene of interest we have an engineered construct and in these engineered construct you can see that there are two 3 prime and 5 prime homology arms. So, these are the flanking homology arms on the two sides of the target gene.

Then there is around 2 kb of require homology requirement for recombination to occur within a cell. However, in general 6 to 14 kb of homology is typically used for targeting constructs. In these engineered construct, you can also see in the presence of a negative selection marker or the selection marker that we discussed about in the earlier slide.

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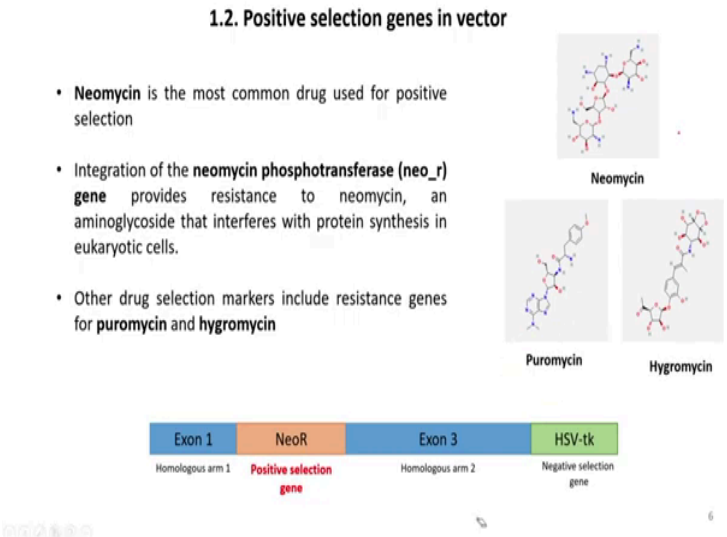

Then, let us discuss about the positive selection genes in a vector. So, basically we have various drugs, which are being used for this procedure and these drugs may be neomycin, puromycin or hygromycin.

In general neomycin is the most commonly used drug for positive selection. So, integration of the neomycin phosphotransferase gene neo_r provides resistance to these drug neomycin, an aminoglycoside that interferes with protein synthesis in eukaryotic cells and we may have similarly genes, which are offering resistance to the other two drugs puromycin or hygromycin.

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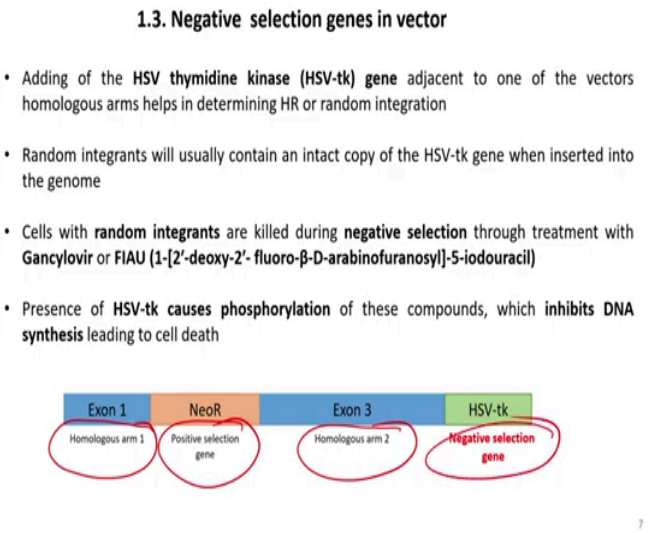

The adding of the HSV thymidine kinase gene adjacent to one of the vector homologous arms helps in determining homologous recombination or random integration. This is the role of the negative selection genes in the vector. So, the random integrants will usually contain an intact copy of the HSV-tk gene when inserted into the genome. Cells with random integrants will be killed during negative selection through treatment with Gancyclovir or FIAU.

The presence of HSV-tk causes phosphorylation of these compounds, which inhibits DNA synthesis leading to cell death. So, here you can see the construct in brief, you have a homologous arm over here, positive selection the gene, another homologous arm and a negative selection gene in these construct.

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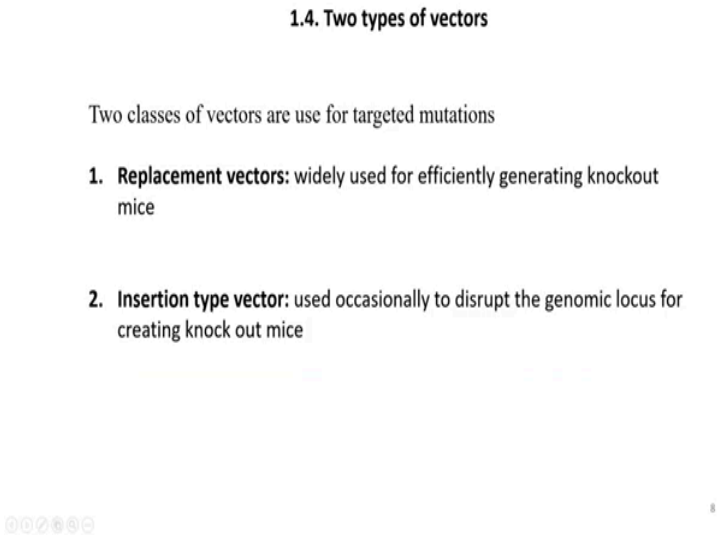

Now, we have basically two types of vectors which are used for knockouts for creating of knockouts or for the targeted mutations, they are the replacement vectors and insertion type vectors.

Replacement vectors are widely used for efficiently generating knockout mice, an insertion type vectors are used occasionally to disrupt the genomic locus for creating knockout mice. So, in the first case, the gene is totally replaced, but in the second case the gene is not replaced, but the gene is disrupted by insertion of an intervening sequences.

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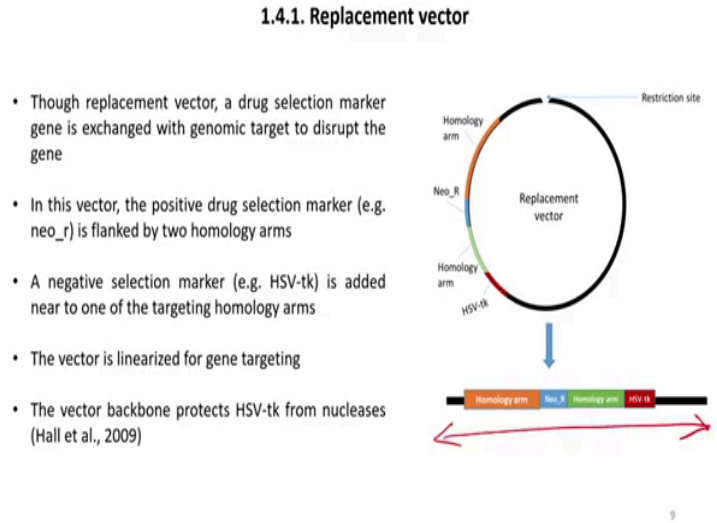

Let us now focus on the first type, the replacement vector. So, you can see here the replacement vector which has two homology arms as we have discussed earlier, then the selective markers and there is a restriction site here, when this restriction site acts on the vector it will linearize the vector as in this.

Through replacement vector, a drug selection marker gene is exchanged with genomic target to disrupt the gene. In this vector, the positive selection marker is flanked by two homology arms as already shown in the diagram and the negative selection marker is added near one of the targeting homology arms as also discussed in the earlier slide.

And the vector is linearized for targeting. The backbone protects HSV-tk from nucleases.

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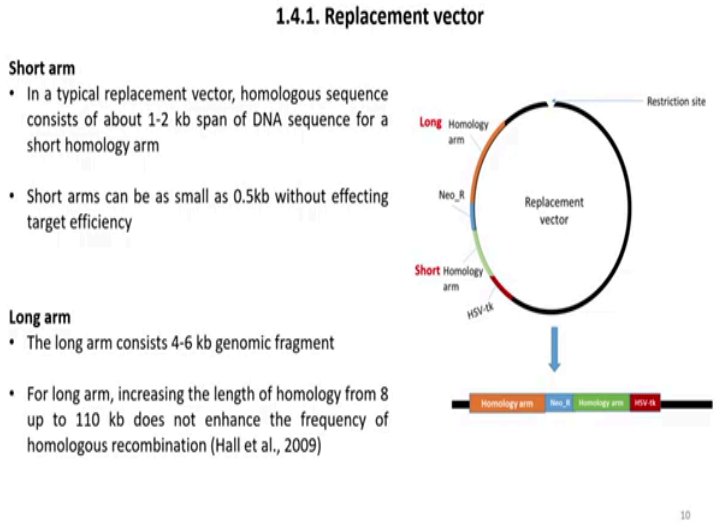

Now, there is a short arm and there is a long arm in this replacement vector. So, in the short arm of a typical replacement vector, homologous sequence consist of about 1 to 2 kb span of DNA sequence for a short homology arm and it can be as small as half kb without effecting a target efficiency.

In the long arm there is presence of around 4 to 6 kb genomic fragment. Here increasing the length of homology from 8 to 110 kb can be done, but it does not enhance the frequency of homologous recombination. So, it is better to retain an optimum land.

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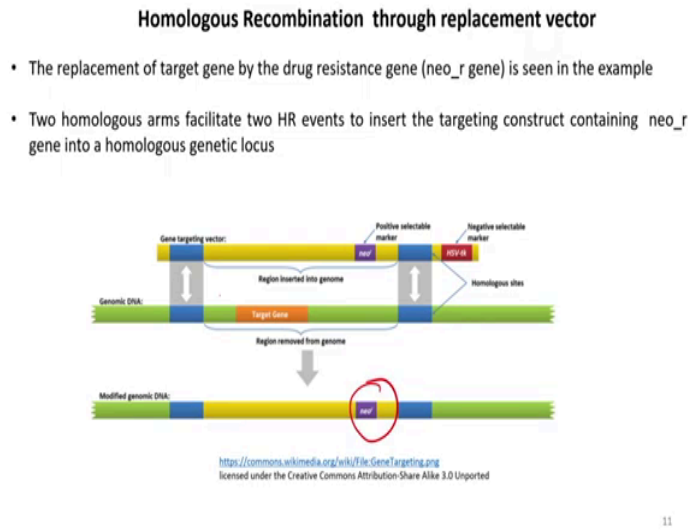

How do we do the homologous recombination through a replacement vector?

So, here you have a gene targeting vector and then you have the genomic DNA here into which you want to do the replacement of a target gene and then you have a region here in the vector, which would be inserted into the genome. The replacement of target gene by the drug resistance here can be seen in this picture.

So, the two homologous arms would facilitate two homologous recombination events to insert the targeting construct retaining neo_r gene into the homologous genetic locus. So, recombination homologous recombination has taken place in the two homologous combination arms and you can see here that these region from here to here, ok, is getting replaced and this region is removed in the final product and as a result of these the neomycin r gene will get into this genetic locus of the targeted gene.

And then we can use these for selection purposes.

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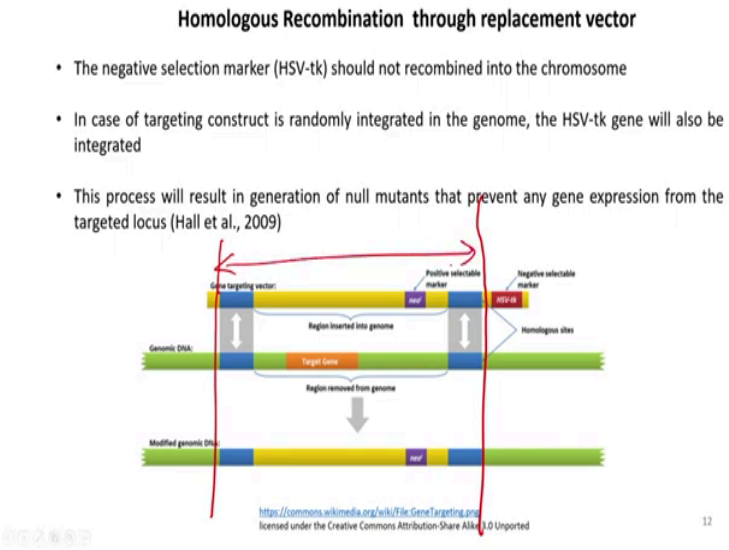

The negative selection marker HSV-tk should not be recombined into the chromosome. In case of targeting construct is randomly integrated in the genome, the HSV-tk gene will also be integrated. So, the process will result in generation of a null mutant then prevents any gene expression from the targeted locus.

So, there is a loss of function over here because these gene is being removed and as already told these HSV-tk marker is not included here, the region that is included in the targeted region range from this area to this area as you can see.

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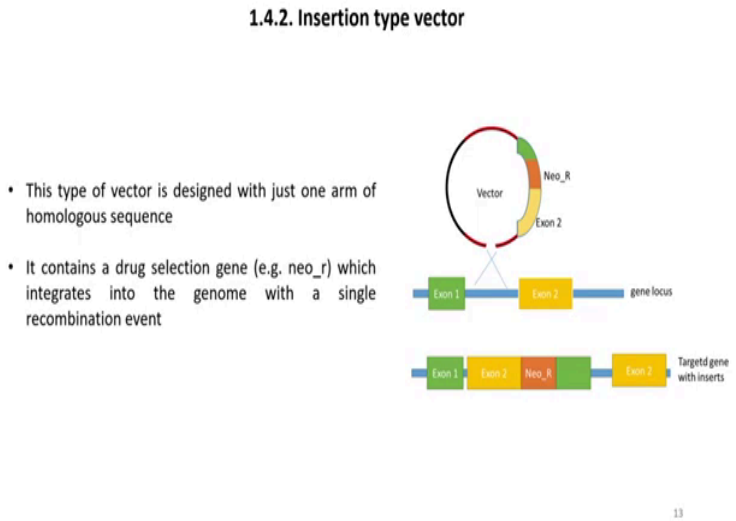

Now, let us discuss about the insertion type vectors. So, here you have 2 exons and then you have a neomycin-r gene over here.

So, this type of vector is designed with just one arm of the homologous sequence. It will contain a drug selection gene which integrates into genome with a single recombination event. So, you can see here that this is the gene locus and recombination has taken place in this particular region and as a result of which you have the insertion of exon 2 of the vector. And the neomycin-r gene flunk by this region attached to the neomycin - resistance gene and this exon 2 belongs to the targeted organisms DNA.

And then as a result of these, you can see in the targeted organism there is no any loss of any DNA sequence. Both the axons as well as the intervening region is retained. But we have here insertion of the sequence, which is there in the vector or the insertion type vector.

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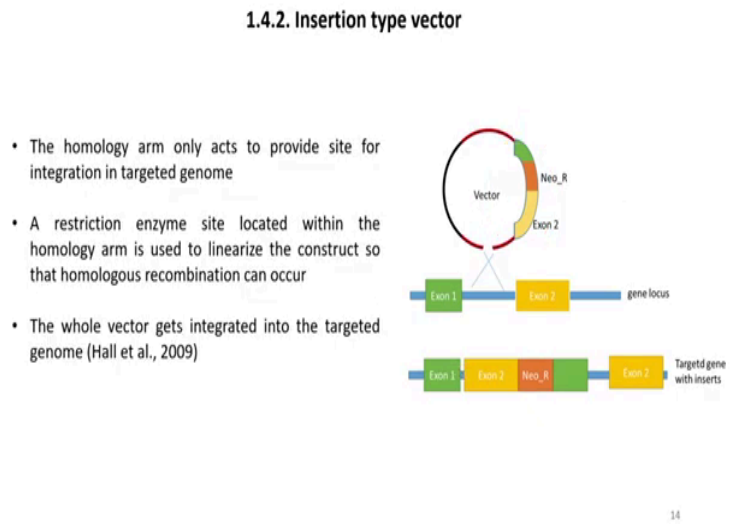

The homology arm only acts to provide site for integration in the targeted genome. A restriction enzyme site located within the homology arm is used to linearize the construct, so that homologous recombination can occur. The whole vector gets integrated into the targeted genome as already discussed.

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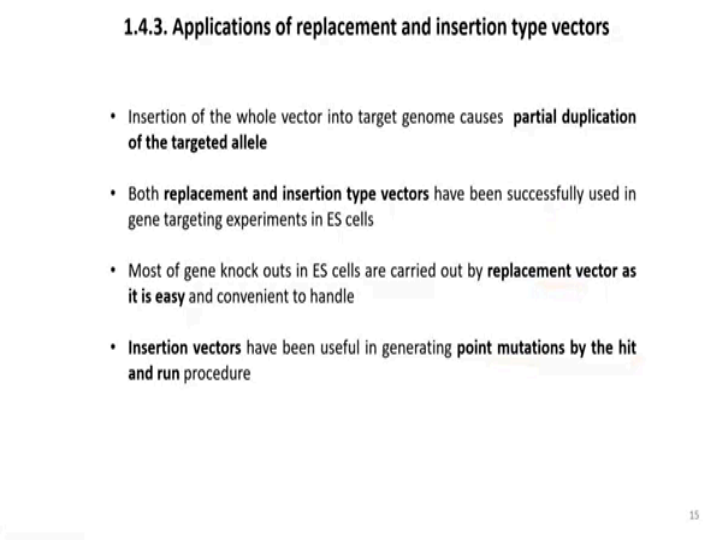

Now, we have an idea of replacement vectors and insertion type vectors, what are the applications of such vectors let us discuss one by one. Insertion of the whole vector into target genome would cause partial duplication of the targeted allele. Both replacement and insertion type vectors have been successfully used in gene targeting experiments in embryonic stem cells.

Most of the gene knockouts in embryonic stem cells are carried out by replacement vector as it is easy and convenient to handle. Insertion vectors on the other hand have been useful in generating point mutations by the hit and run procedure, which we will discuss a little later.

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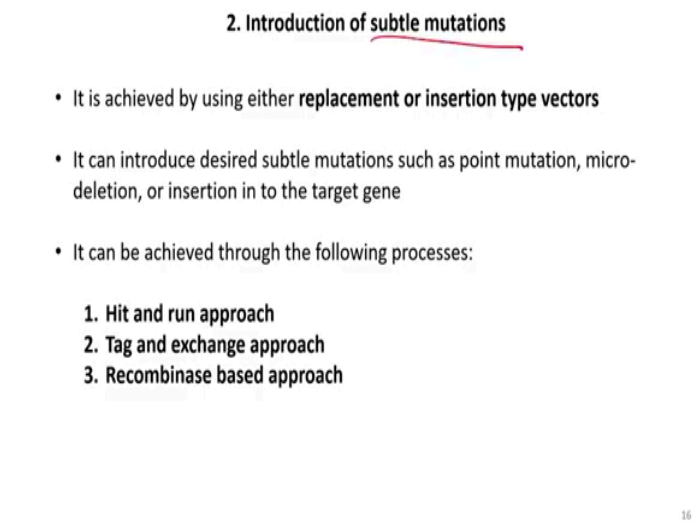

Let us now discuss about the introduction of a subtle mutations, which is achieved by either using replacement or insertion type vectors. It can introduce desired subtle mutations such as point mutation, microdeletion or insertion in to the target gene.

This can be achieved through various processes like hit and run approach, tag and exchange approach and recombinase based approach. We will study all these various approaches one by one in the following slides.

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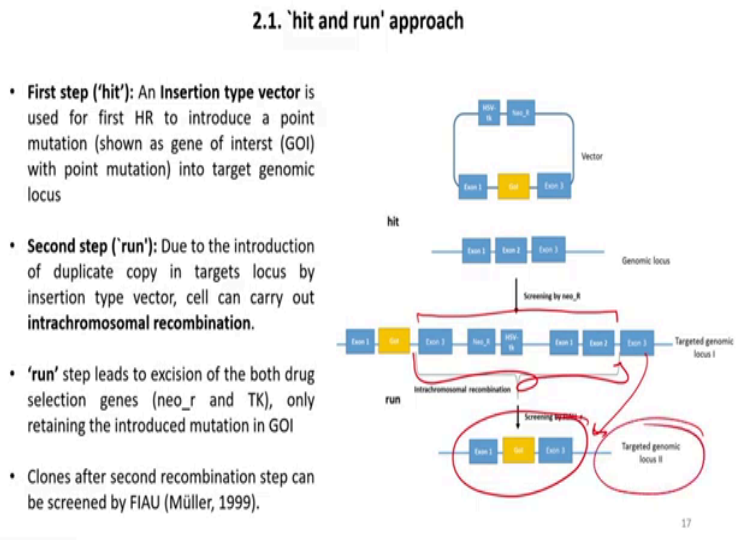

Let us first discuss about the hit and run approach. So, in the hit and run approach, the first step is to use an insertion type vector for first homologous recombination in order to introduce a point mutation. So, this is shown as gene of interest GOI here with point mutation into the target genomic locus. So, this is the vector construct over here you have two exons, 1, 3 which flanks the gene of interest and then you have the HSV-tk marker and neomycin - resistance marker.

So, the first step is the 'hit' step as already discussed and this is the genomic locus which we are targeting which has three exons 1, 2 and 3. The second step comprises the 'run' step, now what is this run step?

Due to the introduction of duplicate copy in target locus by insertion type vector, cell can carry out intrachromosomal recombination. The 'run' step would lead to excision of both drug selection genes, only retaining the introduced mutation in the in gene of interest.

So, here you can see the target genomic locus 1. So, this has the exon 1, the gene of interest and the exon 3 and neomycin - resistance and if you read it from this side you can understand the sequence up to here and then this is flanked by the three exons of the targeted genomic locus 1.

Now, in the 'run' step the GOI is only retained because of this intra chromosomal recombination from this stretch, to this stretch these are removed and these exon will become part of the targeted genomic locus 2. So, the clones after second recombination stand step can be screened by the FIAU.

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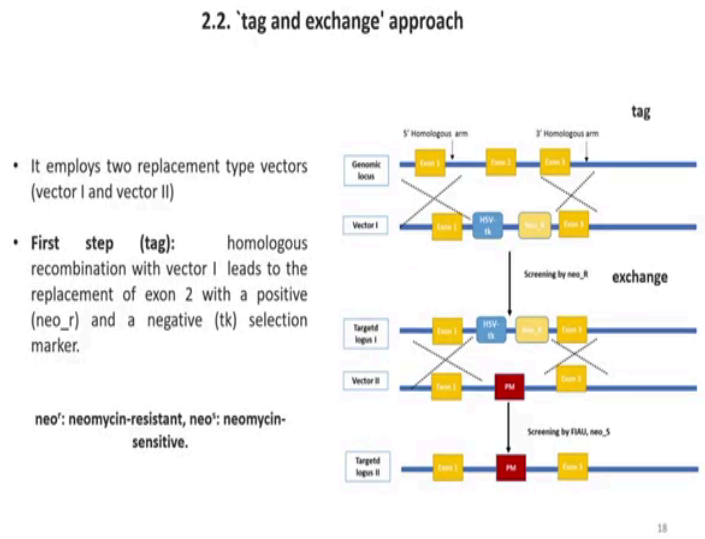

Let us discuss the second approach of which is a tag and exchange approach.

So, in the tag and exchange approach you can see here there is a vector 1 and there is another vector 2. So, let us study the first step the tag step and you have the genomic locus with three exons here, 1, 2 and 3 and there is homology arm between exon 1 and 2 and then the homology arm flanking the exon 3 region.

And then similarly, you have these HSV-tk and neo-r selection markers which lie between exon 1 and exon 3 and due to the homology of these regions there will be homologous recombination. So, in the first step homologous recombination with vector 1 will lead to the replacement of exon 2 with a positive neomycin-r and the negative tk selection marker.

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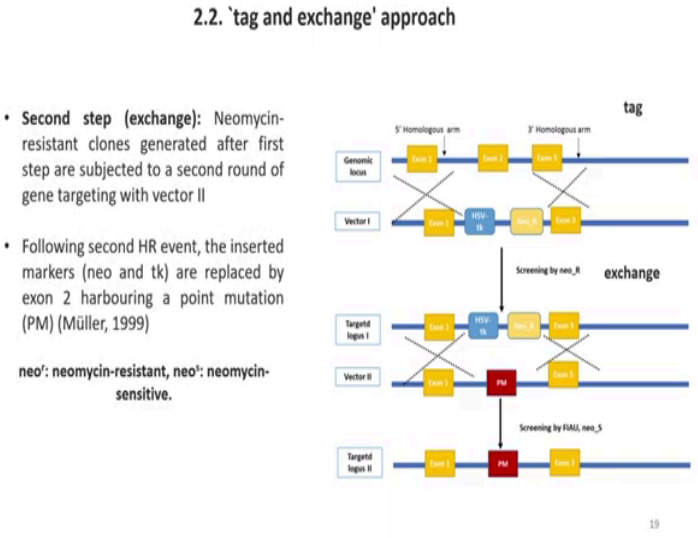

Now, in the second step which is the exchange step, the neomycin - resistant clones, which are generated after first step are subjected to a second round of gene targeting with the vector 2 and this is the vector 2 with exon 1 and exon 3 and another sequence which lie in between exon 1 and 3. And then there is homology between exon 1 on the left side as you can see and homology on the right hand side between the exons 3.

Now, due to homologous recombination effects, the inserted markers neo and HSV-tk will be replaced by harbouring a point mutation as shown in this figure.

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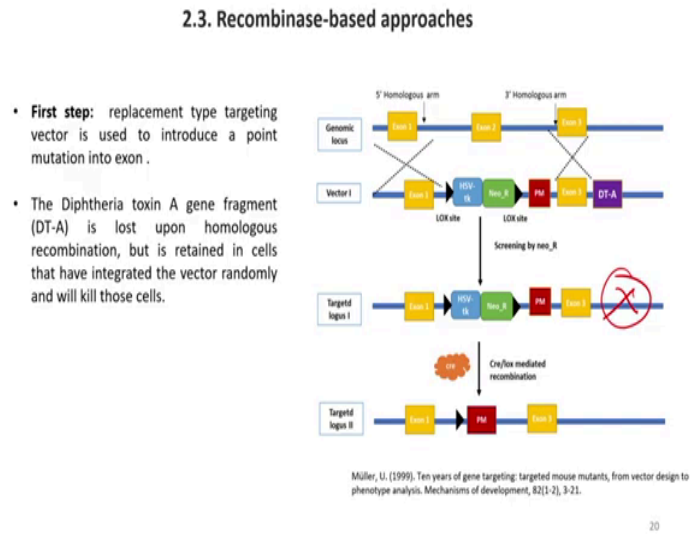

The third procedure is the recombination based approach. So, here also you can see that you have a vector, which is having two exons 1 and exons 3 and then you have these markers HSV-tk and neo_r and you have the lox sites which are adjacent to the HSV- tk and on the one side and neomycin-r on the other side. And we have discussed about this Cre - Lox mechanism in our earlier classes, you also have here one point mutation and you have a diphtheria toxin gene a here, ok; and this is the map of the genomic locus that we are targeting with 3 exons and 2 homology arms.

So, in the first step the replacement type targeting vector is used to introduce a point mutation into the exon. So, here due to this homology you have these HSV-tk and neomycin-r and the point mutation as well as the lox sites incorporated. So, this is the targeted locus 1, output of the first step.

So, diphtheria toxin gene fragment is actually lost here, but it is retained in cells that have integrated the vector randomly and therefore, these toxins will kill those cells. So, we are using these to make our selection much more efficient.

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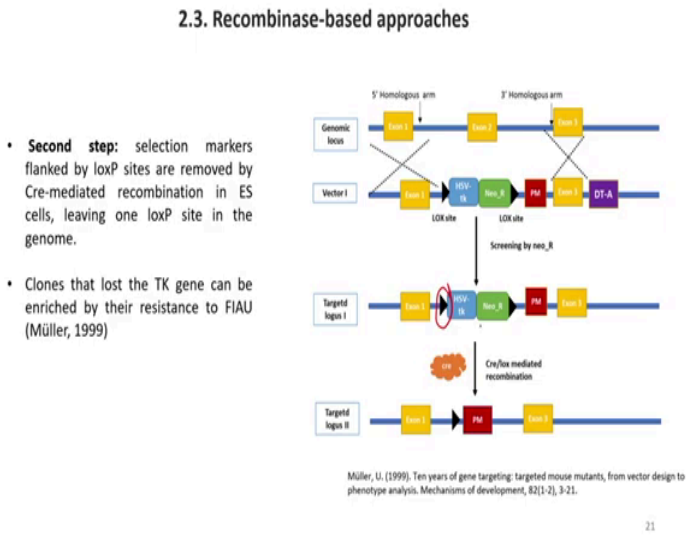

In the second step, selection markers flanked by these loxP sites are removed by these Cre-lox mediated recombination reaction and we have already learnt about the Cre-lox mediated recombination reaction.

So, the clones that lost the TK gene can be enriched by the resistance to fewer selection. So, finally, we lose these genes over here and then we only have exon 1 and exon 3 with a point mutation in between. So, this is how the recombinase or the Cre-lox recombination system is used to create a knockout.

There are certain drawbacks associated with these processes; for example, we may not have a complete knockout sometimes.

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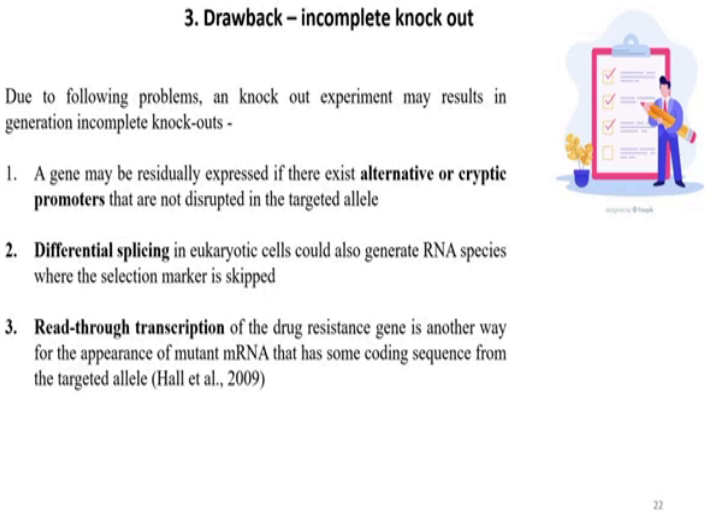

So, there are certain problems which occur in a knockout experiment and these are due to various regions. Number 1, a gene may residually expressed if there exist alternative or cryptic promoters that are not disrupted in the targeted allele. Then there may be different cell splicing in eukaryotic cells, which could also generate RNA species where the selection marker is skipped.

Another drawback is that the read through- transcription of the drug resistance gene is another way for the appearance of mutant mRNA that has some coding sequences from the targeted allele. The neomycin-r marker gene contains a strong promoter; therefore there is a chance of neo-r gene interfering with downstream genes after gene targeting via a replacement vector.

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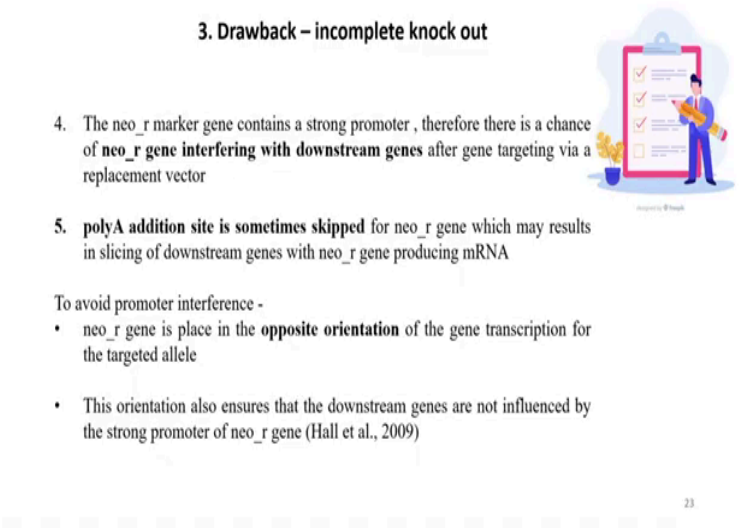

PolyA addition in site is sometimes skipped for neo-r gene, which may result in slicing of downstream genes with neo-r gene producing mRNA.

To avoid promoter interference neo-r gene is placed in the opposite orientation of the gene transcription for the targeted allele. This orientation also ensures that the downstream genes are not influenced by the strong promoter of neo-r gene.

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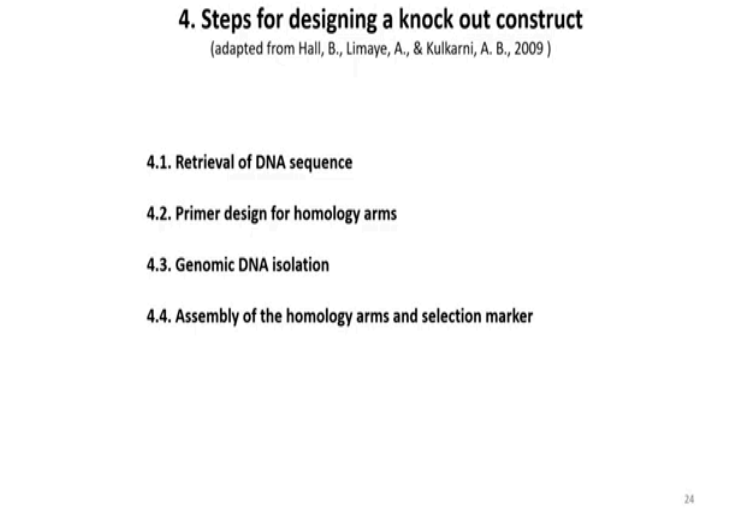

What are the various steps one needs to follow for designing a knockout construct in a laboratory setting? We have to start with the retrieval of DNA sequence, which contains the target or the gene of interest or the sequence of interest. Then we have to design primers for homology arms and then go for genomic DNA isolation and then assembly of the homology arms and selection markers.

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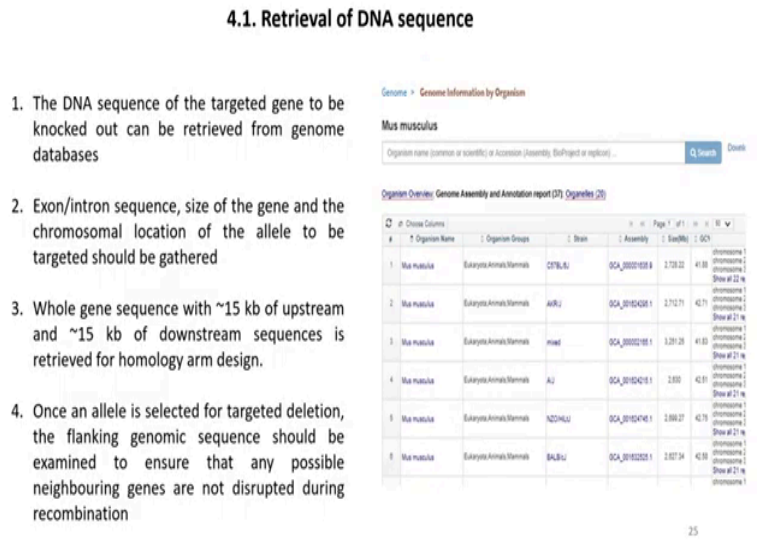

So, for retrieval of the DNA sequence of the targeted gene, we have to visit genome databases. Exon/intron sequence, the size of the gene and the chromosomal location of the allele to be targeted should be gathered, I mean the information need to be known. The whole genome or the whole gene sequence with a 15 kb of upstream and 15 kb of downstream sequences is retrieved for homology arm design.

Once an allele is selected for targeted deletion, the flanking genomic sequence should be examined to ensure that any possible neighbouring genes are not disrupted during recombination. So, for example, here we have Mus musculus database in which we may try to find out a target gene and while doing so we need to focus that we get the complete information about the location of the gene in the particular chromosome. And the generic sequence of the particular gene and 15 kb of upstream and 15 kb of downstream sequence, which will help us in designing the 2 flanking homology arms.

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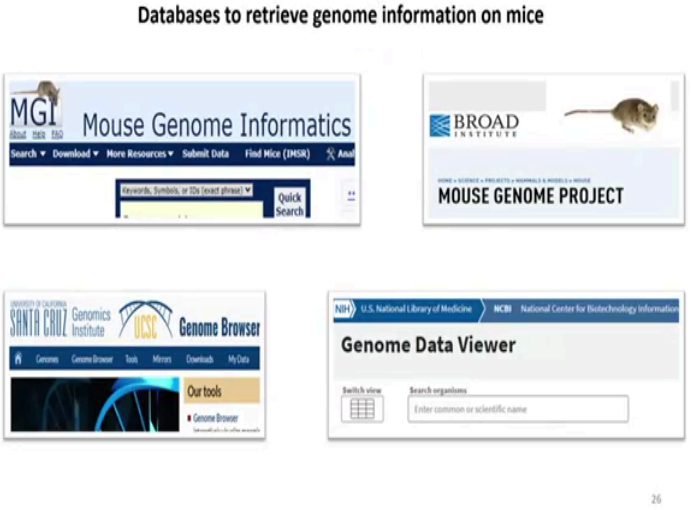

For this you may use different databases depending on the organism and these are some of the databases from which you can get lot of genomic information on mice. The Broad Institute Mouse Genome Project, then you have MGI the Mouse Genome Informatics and you have Genomics Institute Santa Cruz Genome Browser or the NCB Genome Data Viewer.

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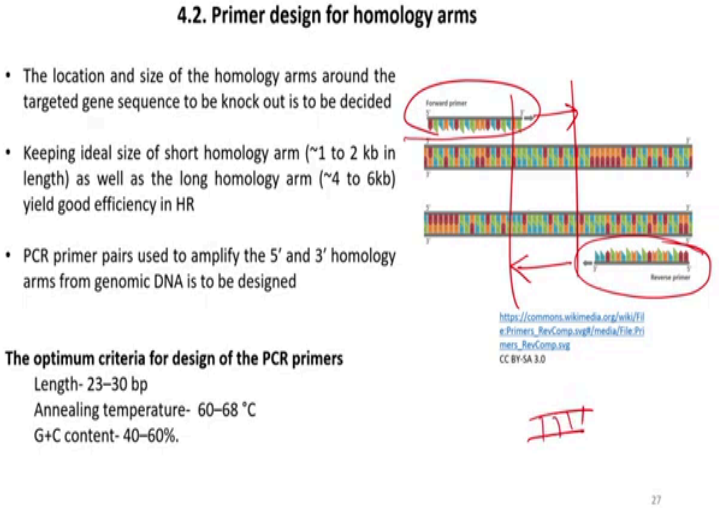

Let us start with the primer design for homology arms. So, you are all well acquainted I suppose with the PCR polymerase reaction which has certain requirements like the use of a forward primer and a reverse primer and they will amplify a genomic sequence in between

them. So, for designing this forward primer and the reverse primer we need the genetic information.

So, the location and size of the homology arm around the targeted genetic sequence to be knocked out is to be decided. Keeping an ideal size of sort homology arm 1 to 2 kb of the sequence is targeted and for the long homology arm 4 to 6 kb of sequence will yield good efficiency in the homologous recombination. And you have to remember the discussion on the short arm and the long arm we had in one of the previous slides and the requirement of the concept, which is used for primer design for homology arms.

The PCR primer pairs used to amplify the 5 prime and the 3 prime homology arms from the genomic DNA is to be designed and there are various online softwares to which you can go for the primer design optimization. In general; however, the optimum criteria for PCR primer design are as below- we select a length of around 23 to 30 base pairs and we target a annealing temperature of around 60 to 68 degree centigrade and the GC content should be ideally around 40 to 60 percent maximum.

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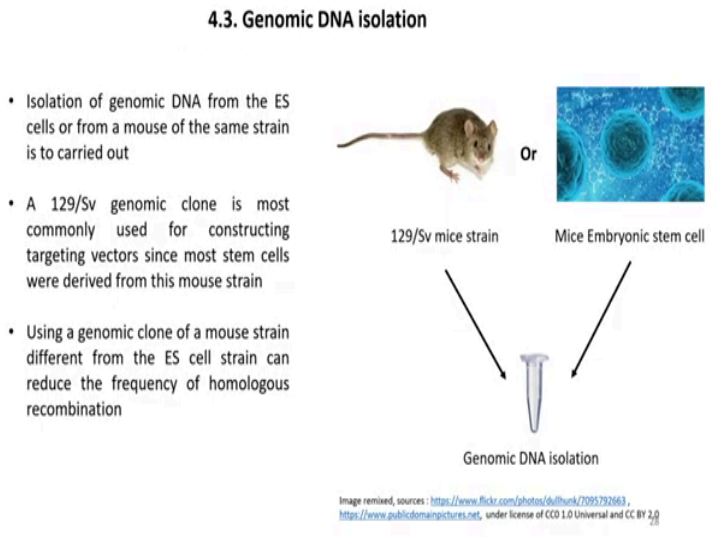

And the next step in this procedure is the genomic DNA isolation. So, there are various standard protocols available for genomic DNA isolation which you can find out from a laboratory manuals and some standard protocols. Here, we are focusing on the isolation of genomic DNA from embryonic stem cells or from a mouse of the same strain, which will be used for the knockout reaction.

A 129/Sv genomic clone is most commonly used for constructing targeting vectors since most stem cells are derived from these particular mouse strain.

Using a genomic clone of a mouse strain different from the embryonic stem cell strain will reduce the frequency of a homologous recombination. So, you need a model strain/ model organism, not only the model organism you also need the standard strain to have higher frequency of homologous recombination for successful gene knockout reaction.

So, either you use this strain or you use the embryonic stem cell for obtaining the genomic DNA by standard protocol. So, once the PCR primer is designed based on the retrieved genomic DNA and for the homology arms designing and then the genomic DNA is isolated, we go on to the next step of assembly of the homology arms and the selection marker.

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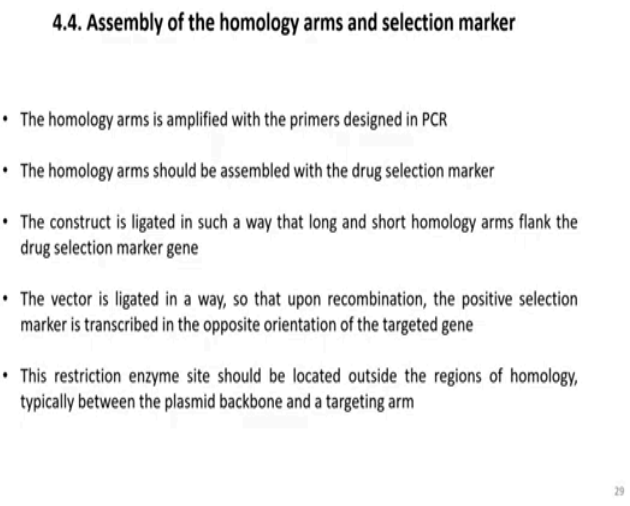

The homology arm is amplified with the primers designed for the PCR reaction and they should be assembled with the drug selection marker. The construct is ligated in such a way that long and short homology arms flank the drug selection marker gene. The vector is ligated in a way, so that upon recombination the positive selection marker is transcribed in the opposite orientation of the targeted gene.

The restriction enzyme site should be located outside the regions of homology, typically between the plasmid backbone and a targeting arm.

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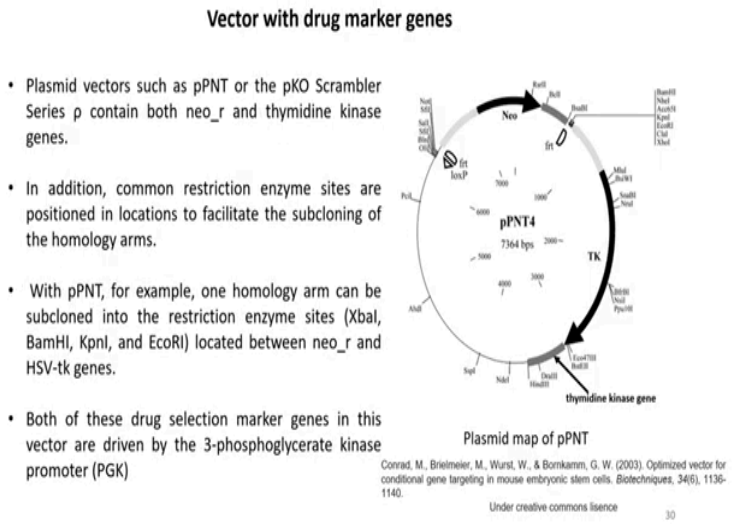

So, let us study these vector with drug marker genes over here and you can see here a site with many restriction enzyme, I mean multiple cloning site over here. And then you have another site of similar site over here and within a very narrow stretch ok.

So, now, you have this Neo located over here and TK located over here and then this is the pPNT4 plasmid. So, such plasmids like pPNT or pKO scrambler series contains both these neomycin and thymidine kinase genes. And then they have common restriction enzyme sites, which are positioned in locations to facilitate subcloning of the homology arms as shown over here.

With pPNT for example, one homology arm can be subcloned into the restriction enzyme site Xbal, BamH1 and then Kpn1 and EcoR1 this particular site, which is located between neomycin-r and HSV-tk genes. Both of these drug selection marker genes in this vector are driven by the PGK or 3- phosphoglycerate kinase promoter.

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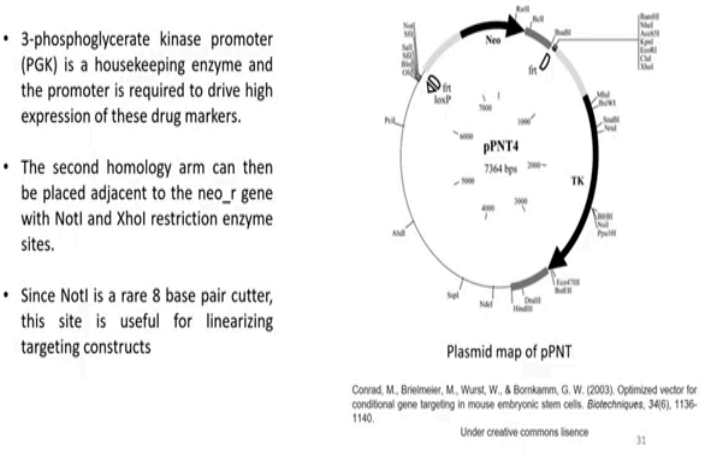

The 3- phosphoglycerate kinase promoter is a housekeeping enzyme and the promoter is required to drive high expression of these drug markers. The second homology arm can be placed adjacent to the neo-r gene with Not1 and Xho1 restriction enzyme sites here. Since Not1 is a rear 8 base cutter, this side is useful for linearizing targeting constructs.

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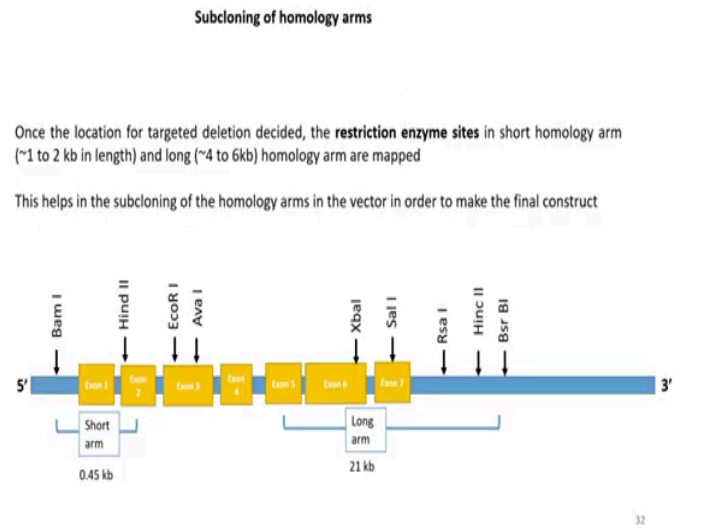

Let us now discuss about sub cloning of the homology arms. Once the location for targeted deletion is decided, the restriction enzyme sites in short homology arm around 1 to 2 kb in

length and the long homology arm 4 to 6 kb long are mapped. This helps in the sub cloning of the homology arms in the vector in order to make the final construct.

So, here this is the short homology arm around half kb, here in length and then this is the long arm around 21kb over here and you can see the various exons 1 2 3 4 5 6 7 in a contiguous way and then you have the map of the various restriction sites in these genomic layout.

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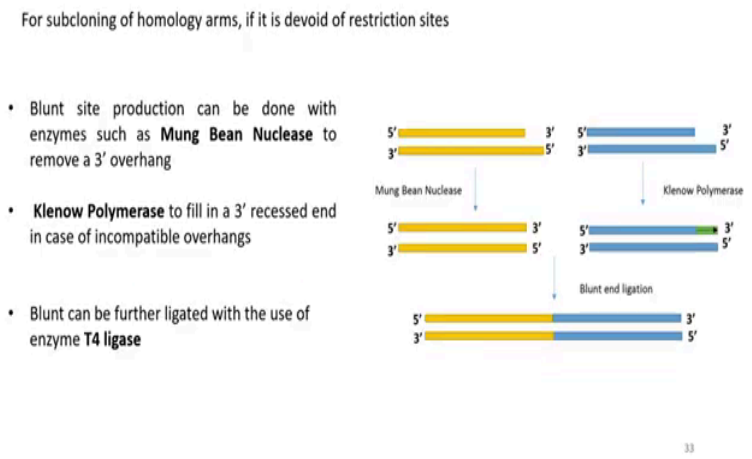

For sub cloning of the homology arms, if it is devoid of restriction sites, we will go for certain techniques. Blunt site production can be done with enzymes such as Mung Bean Nuclease to remove a 3 prime overhang. And Klenow Polymerase is used to fill in a 3 prime recessed end in case of incompatible overhangs.

Blunt ends can be further ligated with the use of enzyme T 4 ligase, as shown here in this particular figure.

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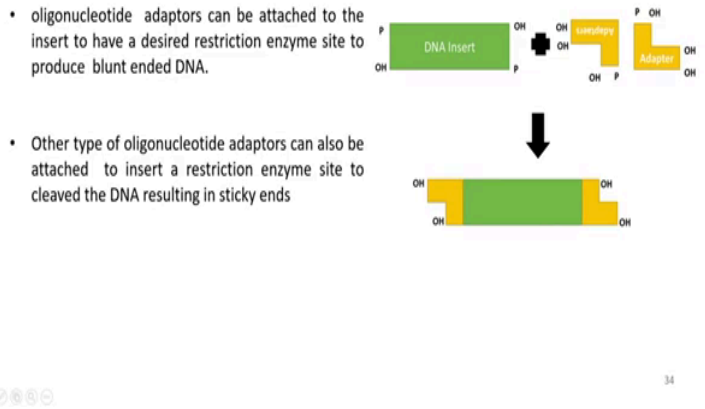

Besides these, oligonucleotide adapters can be attached to the insert to have a desired restriction enzyme site to produce the blunt ended DNA. So, this is a DNA insert and then we have the adaptors over here and we added the adaptors to the 5 prime and the 3 prime end of this particular DNA insert.

And these particular adaptors has certain restriction sites, which will be you know compatible with in the cloning reaction. Other types of oligonucleotide adaptors can also be attached to insert a restriction enzyme site to cleave the DNA resulting in the sticky ends.

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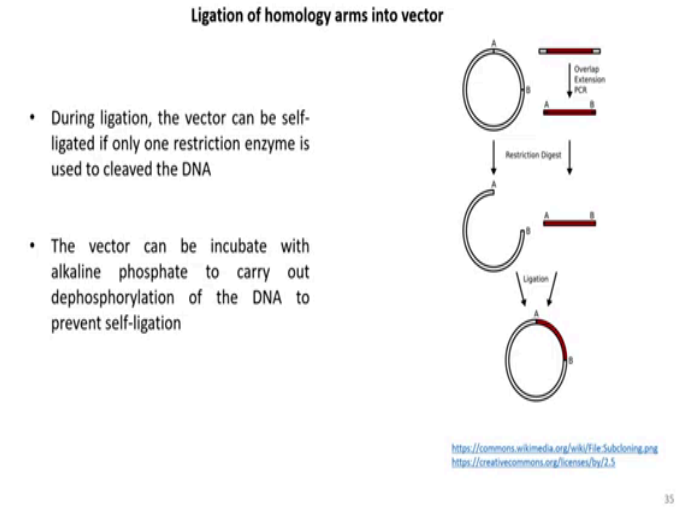

Now, let us focus on the ligation of homology arms into the vector as shown in this particular figure. So, here you have an overlap extension PCR and then this vector is opened up and then by restriction digestion. And these particular fragment is ligated to the open vector and then this gives the hybrid molecule over here.

So, during ligation the vector can be self-ligated if only one restriction enzyme is used to cleave the DNA. The vector can be incubated with alkaline phosphates to carry out dephosphorylation of the DNA to prevent this self-ligation and while discussing about the role of alkaline phosphates in our introductory classes we have discussed this point thoroughly.

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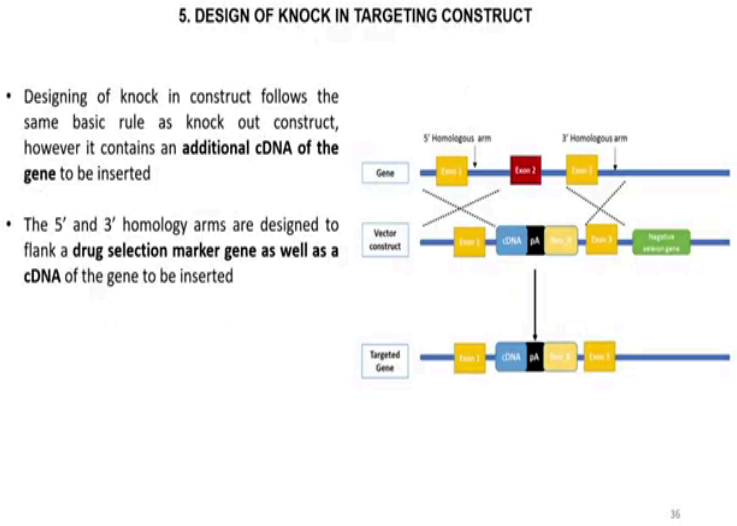

Next we go to the design of knock in targeting constructs. Let us now discuss about the design of knock in targeting constructs. So, designing of knock in construct follows the same basic rule as in the case of knock out construct, however here we have an additional DNA insert or cDNA of the gene to be inserted. So, we do not use a full gene with exons and introns, we only have a complementary DNA copy of the gene to keep the construct smaller.

The 5 prime and 3 prime homology arms are designed to flank a drug selection marker gene as well as a cDNA of the gene to be inserted. So, this is the homology arm on these DNA targeted DNA and then we have these vector construct over here and it has homology in these two regions exon 1 and exon 3. And in the center you can see the selection marker as well as the cDNA gene and there is another negative selection marker over there.

And as a result of these homologous recombination over here, this portion is replaced with these particular construct. So, this is the final product resulting out of this reaction.

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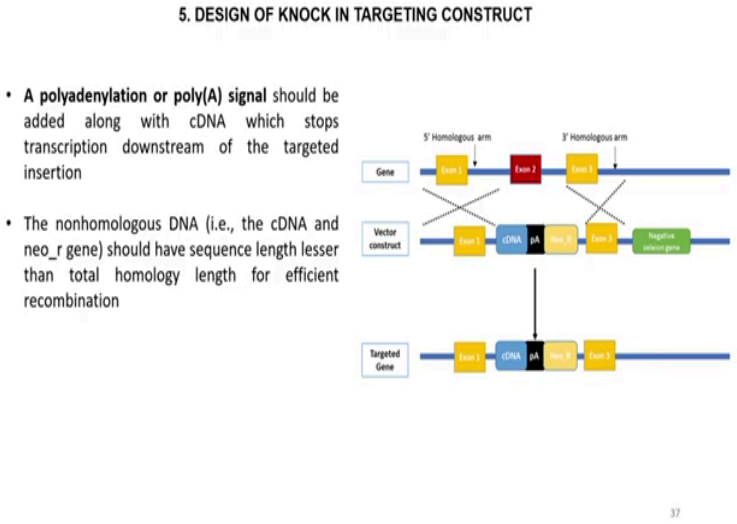

A polyadenylation or polyA signal should be added along with cDNA, which stops transcription downstream of the targeted insertion, this is the polyadenylation sequence. The nonhomologous DNA that is the cDNA and neo-r gene should have sequence length lesser than total homology length for efficient recombination.

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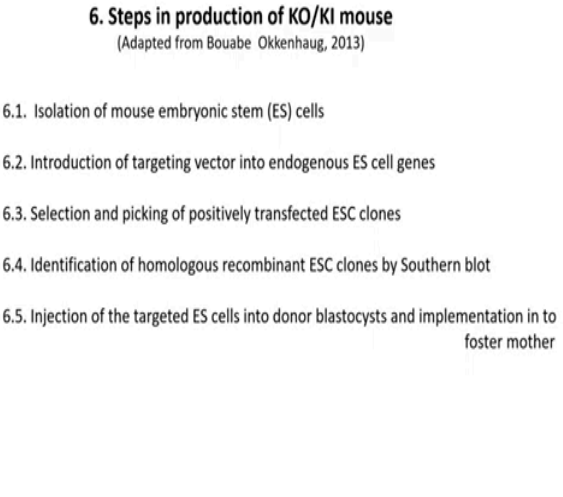

What are the steps in production of knockout and knock-in mouse? We have to start with isolation of mouse embryonic stem cells, then introduction of targeting vector into endogenous embryonic stem cell genes, then selection and picking of positively transfected ESC clones, identification of homologous recombination ESC clones by southern blot. Then injection of the targeted embryonic stem cells into donor blastocysts and implementation in to foster mothers.

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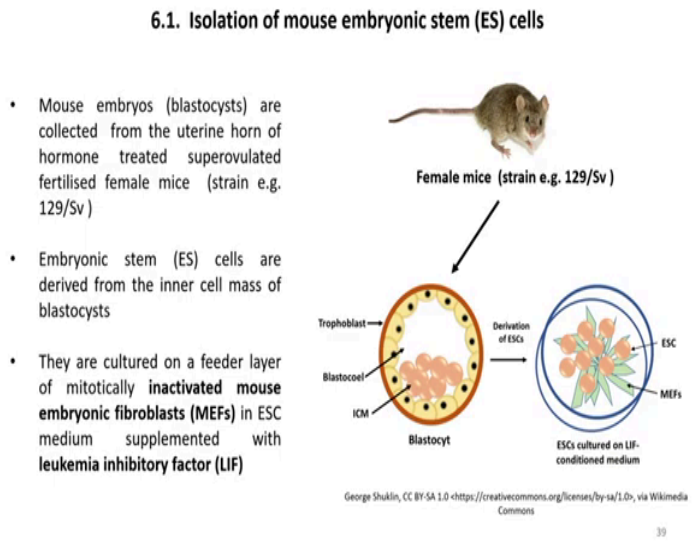

So, here you can see a female mice strain we are taking the standard strain of 129/Sv. So, the mouse embryos blastocysts are collected from the uterine horn of hormone treated super ovulated fertilized female mice. The embryonic stem cells are derived from the inner cell mass of the blastocyst. They are cultured on a feeder layer of mitotically inactivated mouse embryonic fibroblast MEFs in ESC medium supplemented with leukemia inhibitory factor LIF.

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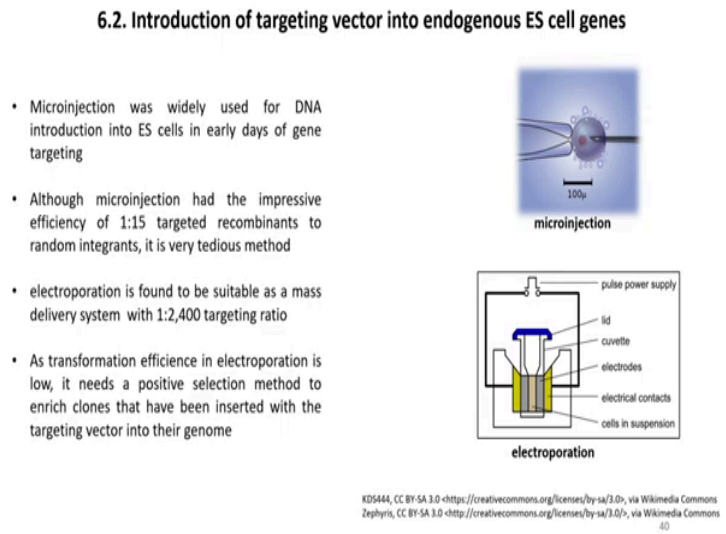

So, we use mostly microinjection for the introduction of targeting vector into the endogenous embryonic stem cells, which we have isolated and cultured in the first step. So, this is a process of microinjection and you can see here one glass pipette pointed glass pipette is being used to deliver the targeting vectors.

Although microinjection had the impressive efficiency of around 1:15 targeted recombinants to random integrants, it is a very tedious method. Now electroporation is found to be suitable as a mass delivery system with 1:2400 targeting ratio. As transformation efficiency of in electroporation is low, as you can see from these figure 1: 24 verses, 1:15 verses, 1:2400.

It needs a positive selection method to enrich clones that have been inserted with the targeting vector into their genomes. So, for your own understanding you may study about the electroporation method a little bit.

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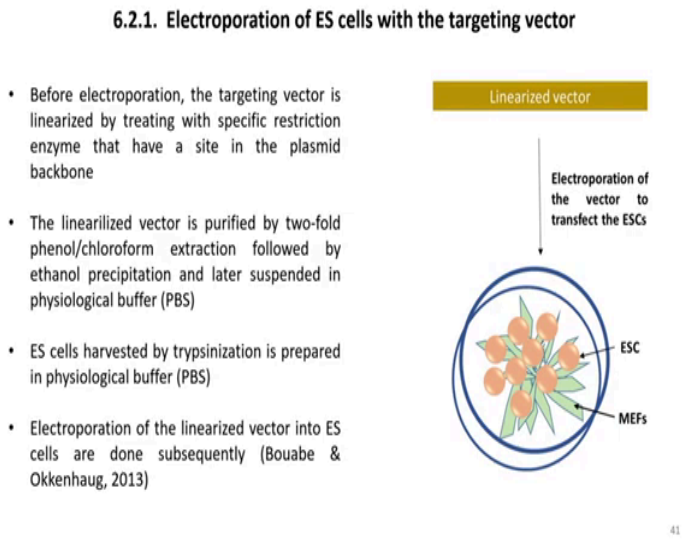

So, how we do electroporation of the embryonic stem cells with the targeting vector. So, before electroporation, the targeting vector is linearized with specific restriction enzymes that have a site in the plasmid backbone.

The linearized vector is purified by two-fold phenol chloroform extraction followed by ethanol precipitation and later suspended in physiological buffer. Embryonic stem cells harvested by trypsinization is prepared in physiological buffer as well. Then electroporation of these linearized vector into the embryonic stem cells are done subsequently.

So, here these embryonic stem cells as a result of this electroporation of the linearized vector will be having the transformation happening.

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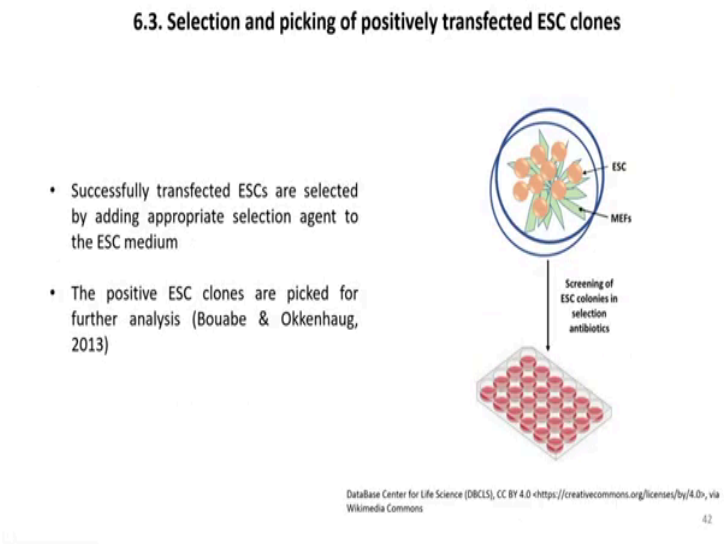

And after that transformation happens or transfection happens, we have to select the successfully transfected embryonic stem cells by adding appropriate selection agents to the embryonic stem cell culture medium.

The positive embryonic stem cell clones are picked for further analysis.

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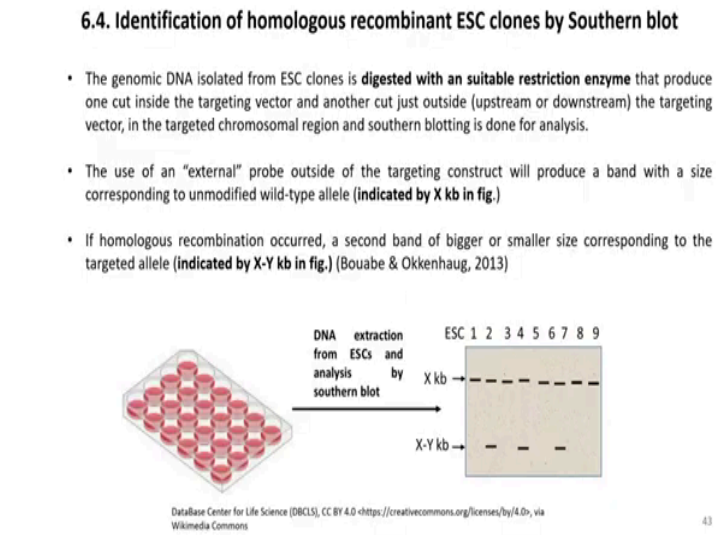

Now, we need to go for the identification of homologous recombinant embryonic stem cell clones by Southern blotting. The genomic DNA is isolated from the ESC clones and digested

with an suitable restriction enzyme that produce one cut inside the targeting vector and another cut just outside upstream or downstream the targeting vector, in the targeted chromosomal region and Southern blotting is done for analysis.

The use of an “external” probe outside of the targeting construct will produce a band with a size corresponding to unmodified wild-type allele, which is indicated by X kb in this figure. If homologous recombination occurs, a second band of bigger or smaller size corresponding to the targeted allele indicated by X-Y kb in this figure will occur.

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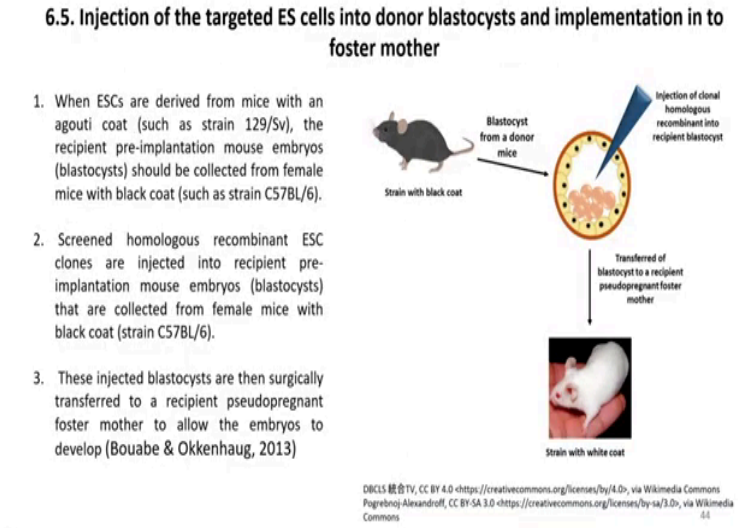

Now, we go for the injection of the targeted embryonic stem cells into donor blastocysts and implementation into foster mother once the successful transfection is confirmed. So, when the ESCs are derived from mice with an agouti coats, such as, the strain 129/Sv, the recipient pre-implantation mouse embryos should be collected from female mice with black coats such as strain C57BL/6.

Screened homologous recombinant ESC clones are injected into recipient pre-implantation mouse embryos or blastocysts that are collected from female mice with this black coat. These injected blastocysts are then surgically transferred to a recipient pseudopregnant foster mother to allow the embryos to develop.

So, you have these strain with black coat a blastocyst from the donor mice is taken and here we inject the clone of homologous recombinants into this recipient blastocyst and this is transferred to a recipient pseudo pregnant foster mother.

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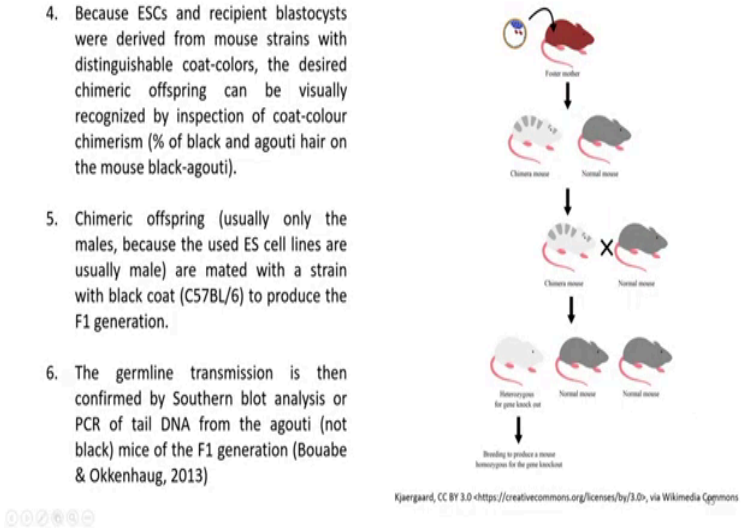

Because the embryonic stem cells and recipient blastocysts were derived from mouse strains with distinguishable coat-colors black and white, the desired chimeric offspring can be visually recognized by inspection of coat-color chimerism certain percentage of black and agouti hair on the mouse black-agouti.

The chimeric offsprings usually only the males because the used ES cell lines are usually male are mated with a strain with black coat to produce the F1 generation. So, this is the foster mother and this gives rise to a chimera mouse and you can see here the normal mouse and crossing between the chimera and the normal mouse will result in a normal mouse and heterozygous for gene knockouts.

And we carry out breeding of these heterogeneous gene knockout population to produce mouse, which is homozygous for that particular gene knockout. So, the germline transmission is then confirmed by Southern blot analysis or PCR of tail DNA from the agouti mice of the F1 generation.

So, by this process starting from isolation of DNA, then using vectors for carrying out the knockout or knock-in and then finally, implementing them into the mice blastocysts and then

or transferring them to a foster mother and then creating chimeric mouse and then crossing them with normal mice and obtaining a heterogeneous gene knockout population and by selfing or breeding within this population a homogeneous gene knockout or gene knock-in mice can be generated.

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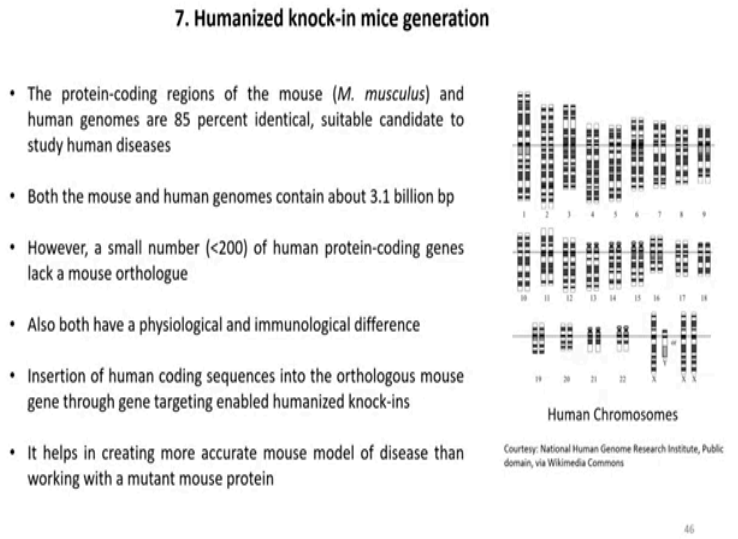

Now, let us discuss about one method, which is known as humanization of experimental animal models. So, in the beginning we discussed that we may have double knockouts, triple knockouts and so on and similarly we may have double knock-ins, triple knock-ins and so on.

Now, in certain cases mouse and humans have lot of homology, but some of the genes are not similar. So, we may knockout some of the genes which are not similar to humans in mice and then we may replace certain genes in the mice with human copies. So, for such a humanization program, we may require both kind of approaches a knockout as well as the knock-in approaches.

So, let us briefly find out some of the facts. So, the protein - coding regions of the mouse and the human genome are 85 percent identical and therefore, with this high identity or similarity a mouse is a suitable candidate to study human diseases. So, we may be able to draw lot of inferences with this 85 percent similar genes, but now they are 50 percent genes, which we need to take care of because they are different.

Briefly the mouse and human genome both contain around 3.1 billion base pairs. However, a small number of human protein-coding genes lack a the mouse orthologue. And both these organisms have different physiological and immunological features or properties or characteristics. Therefore, insertion of human coding sequences into the orthologous mouse gene through gene targeting would make us capable in obtaining humanized knock-ins. And this would help in creating more accurate mouse models for disease than working with a mutant mouse protein.

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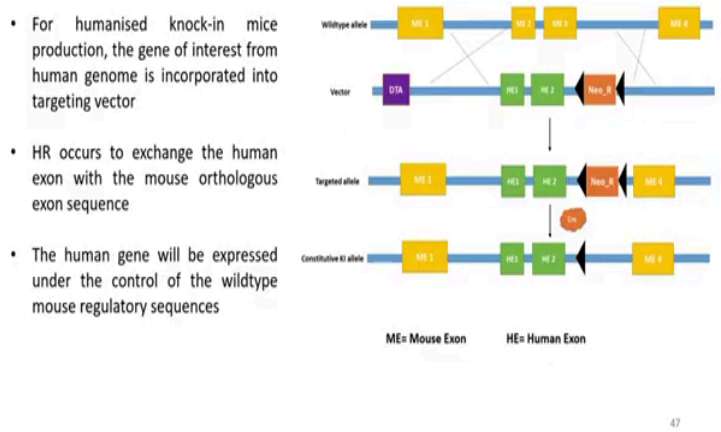

So, for humanized knock-in in mice production, the gene of interest from human genome is incorporated into the targeting vector. Homologous recombination occurs to exchange the human exon with the mouse orthologous exon sequence. And the human gene will be expressed under control of the wildtype mouse regulatory sequences.

So, here in this you can see ME stands for the Mouse Exons and HE stands for the Human Exons and this is the wildtype mouse. So, these are mouse exons 1 2 3 4 and this is a vector which is vector construct with all the elements required for knock-in. You have these human exon 1, human exon 2, the marker genes, the LoxP sites; ok, as we have already discussed.

And these are the stretches with homologous sequences and as a result of these wildtype mouse is replaced with human genes as well as a neomycin-r marker and we can select this and then at a later step using the Cre-LoxP recombinase system these antibiotic gene is got

rid of. And this is the constitutive knock-in allele which is the outcome of these entire exercise.

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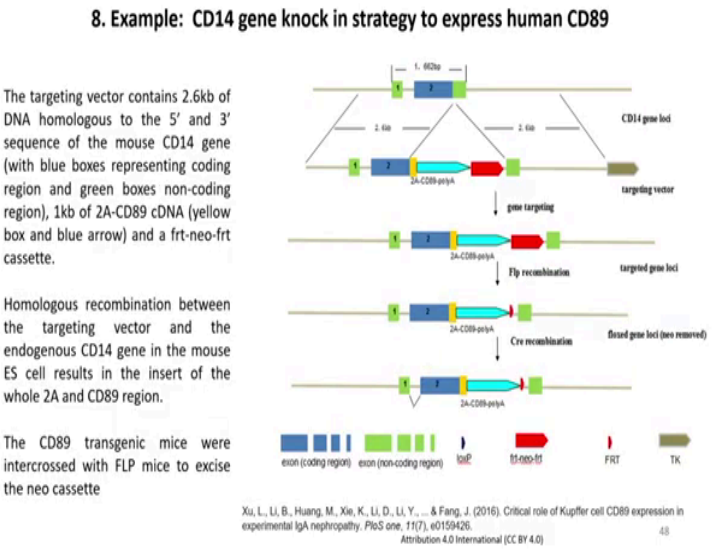

Let us have some example of some CD14 gene knock-in strategy to express the human CD89 in mice. So, the targeting vector contains around 2.6 kilo base of DNA homologous to the 5 prime and 3 prime sequence of the mouse CD14 gene, here with blue boxes you can see over here representing coding region and the green boxes the non-coding region. Then you have 1 kb of 2A-CD89 this is a yellow box here and a frt–neo-frt cassette.

Homologous recombination between the targeting vector and the endogenous CD14 gene in the mouse embryonic stem cells results in the insert of the whole 2A and CD89 region. And then you have these various recombination steps due to FLP then the Cre recombination taking place.

The CD89 transgenic mice were intercrossed with FLP mice to excise the neo cassette.

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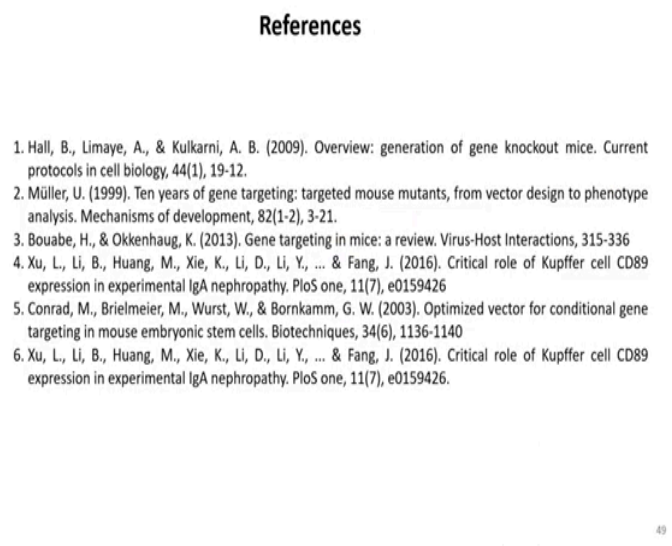

So, with this we come to end of this lecture.

Thank you for your kind attention.