**Host-Pathogen Interaction (Immunology)**
**Prof. Himanshu Kumar**
**Laboratory of Immunology and Infectious Disease Biology**
**Department of Biological Sciences**
**Indian Institute of Science Education and Research (IISER) - Bhopal**

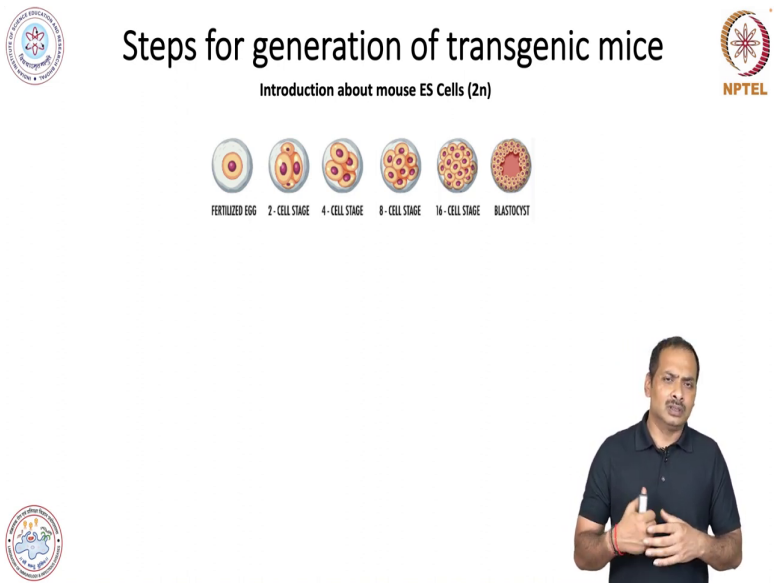
**Lecture: 13**
**Cells of Immune System-Transgenesis - 1**

Hi, so in previous session we have learned about the hematopoietic stem cell you have learned that how this hematopoietic stem cell is used for addressing various and biology or Immunology questions and how this hematopoetic stem cell is used for creation or generation of the transgenic mice. And you have learned what are the different kinds of transgenic mice you have you know this the role or the usage of knockout mice, conditional knockout mice.

I have explained you with some example experiments and today we will or in this session we will discuss how we will create these transgenic mice. So, this is a really very interesting as well as it is a very challenging or I can say it is a mammoth task to create a transgenic mice. So, I will discuss the technology which was used quite exhaustively or quite it was used by most of researcher.

And however recently there is a some group of or some group are using another technology which we call it as a Crisper Cast 9 method. So, today I am going to talk about the previous technology in which we exploit the concept or the phenomena of homologous recombination. So, let us begin.

**(Refer Slide Time: 02:17)**




So, there are some very important steps for generation of transgenic mice. So, in previous session you have learned that this embryonic stem cell which is pluripotent cells is used for the generation of transgenic mice but the question is from where you get the this embryonic stem cells. So, embryonic stem cell basically we get from the blastocyst. So, in this scenario what we are doing we keep the male and female mice for mating.

And after some duration we sacrifice that female mice and we take out the blastocyst as you can see in this image that there is a different developmental stage of embryo that is you can see there is a two-cell stage after fertilization then four-cell stage, eight-cell stage 16 cell stage and then there is a blastocyst and that has some cavity which we call it as a Blastoisescoeal. So, what we are doing we take out this blastocyst and then we take out the inner mass of the cell and this inner mass of the cell we use as a embryonic stem cell.

So, embryonic stem cells are very kind, of fragile. Fragile means its handling is very very important. If you are very poor handler of the cells then it is very difficult to create the knockout or transgenic mice out of this embryonic stem cell. So, for the prerequisite for doing this kind of work you should be excellent in handling the mammalian cells. And in addition, there is a several challenges and several things or several special things are needed for the maintenance of embryonic stem cell.

And the first and foremost important thing is that these embryonic stem cells they can grow in dishes or glass plates however if you use those embryonic stem cell which is growing on the dishes they will they cannot you cannot use it for making transgenic mice because they

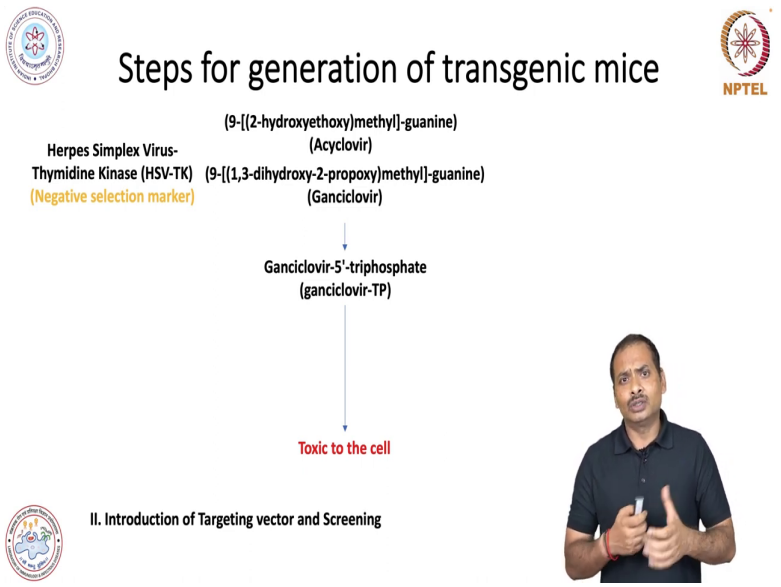
will differentiate. So, their culture work is quite different. So, I am going to explain few of those very common and essential steps although I am not going to explain you are deep experiments but I will give you the concept.

So, for growing embryonic stem cell you need a bed of mouse embryo embryonic fibroblast we in short we call it as a ~~meths~~MEFs Mouse embryonic fibroblast. So, this mouse embryonic fibroblast we basically prepare from the from the mice pups~~ffs~~ which is which is under the development which is still in utro it is present inside the uterus we basically sacrifice those pregnant female mice and then we take out the embryo and then from that we make the mouse embryonic fibroblast cell.

After that once you make this mouse embryonic fibroblast cell we treat with some agent for there are some agent which will arrest their cell division but the cells will be alive. So, after treatment we put this cell in the dishes and then over there we put the this embryonic stem cells. And these embryonic stem cells they grow in a in a colony form and in medium we add some special factor or you can call it as a cytokine or factor or hormone which is we call it as a LIF L I F. it is a ~~Leukemia~~Leukaemia Inhibitory Factor.

So, this ~~Leukemia~~Leukaemia Inhibitory Factor keep this embryonic stem cells in ~~D~~ dedifferentiation state. Please remember this is most important to keep these embryonic stem cells in ~~D~~ dedifferentiation step and then we grow it in more number and then we perform all all molecular experiments. Before that so, once you have embryonic stem cell then you need to plan how to target your Gene.

**(Refer Slide Time: 07:31)**




So, I will show you how to prepare the targeting vector. For example you have some Gene and how you will go for making this target making for this targeting vector. So, here I am just showing a gene you it is not important what is this Gene and you can see in this in this Slide the upper panel there is a wild type ~~A~~allel~~n~~l. So, this this is a this is a genomic arrangement of the gene.

Gene means that you know that in gene is basically over the DNA it is it is present in the form of exons and introns. And there are some another element such as 5 prime ~~utr~~UTR and 3 prime ~~utr~~UTR. So, these are the component of the Gene and it is present on the DNA and you can see that there is a the blue colour box which is just upstream to the ATG and there is a blue colour box and this blue colour is for three five Prime ~~utr~~UTR.

And there are the pink colour boxes they represent the exons and you can see there is a blue colour box towards and this is a three prime ~~utr~~UTR. So, this is a genomic arrangement of some Gene. So, what we do we basically try to disrupt this Gene and how you can do the disruption it is a very simple either you disrupt the UTR~~utr~~ or you can disrupt ~~utr~~UTR as well as a first Exon then you can make this Gene inactivated.

So, this is below just below that there is a targeting vector and here I am showing this targeting Vector has a two major component HSV TK and NEO. So, this is the kind of plasmid and below this NEO there is a quite big piece of DNA which is derived from the genomic DNA. So, in simple word you need to clone such a big piece of DNA in the targeting Vector.

And you will all you also need to clone a small portion of a stream to the NEO Gene and that will basically help in recombination I am going to explain what how this NEO work and how HSA TK work. So, once you will do successfully all once you make this targeting vector after finishing your all this thing you will get this kind of mutated allele. So, you can see in in bottom panel that the two exons and first Exon along with three prime 5 Prime ~~utr~~UTR is d~~estruc~~isrupted.

So, if you are successful then you will get it like that and please remember one more thing. So, this is a diploid~~deployed~~ system it is a not haploid system. So, story will not end over here. So, there is another level of a challenge how to make it diploid~~deployed~~ that is reasonably simple but making a hetero kind of thing means one allele is mutant and another allele is a wild type that itself is a big challenge.

So, now I will tell you about the various component of this targeting vector. First, I will tell you about the NEO. So, NEO is a basically neomycin resistance Gene and it is a positive selection marker. I will tell you what is the positive and negative selection marker after a while and this neomycin is a needs a drug which we call it as a G418. It is a nothing ~~et I think~~ it is a simple amino glycoside this drug is aminoglycoside and this is an antibiotic similar to the Gentamicin.

Probably you have you might have used if you are a researcher if maybe you have used the Gentamicin ~~P~~PB. It basically blocked the polypeptide synthesis by inhibiting elongation a step in both the prokaryote as well as in eukaryote. So, the concept of positive selection is that if your Gene is inserted into the genome then that that cell will grow over the G418 this will provide a resistance.

So, this is a concept of a positive selection marker and that needs in this system we need that positive selection marker. And there is a negative selection marker negative selection marker the concept is just other way around if the gene is inserted into the genome then if you put some drug which is which is needed for negative selection marker then those cells will die. So, this is a very simple concept.

So, here the negative selection marker is HSV-TK. HSV-TK is nothing it is a herpes simplex virus thymidine kinase we do have a thymidine kinase but this thymoidine kinase is needed in this experiment and they have a there is a drug known as a acyclovir which is a quite complex it is a derivative of guaoanine and there is a Glanciclovir this is also a derivative of this guanine and this drug when you have this Gene then this Gene will incorporate these modified derivative of guanine in the genome.

And that will result the incorporation of this derivative of guaninegoing in instead of guanine and then that once it will be incorporated it is toxic to the cell and cell will die. So, so, this is the way by which the negative selection marker works. If I will revert the slides you can see again this is the arrangement of these selection markers HSV-TK and NEO. And now you can understand if there is a perfect homologous recombination taking place in the cell, means, this targeting Vector will go to the to the same gene or same genomic Locus.

Then there will be the removal of HSV-TK. So, once it will be removed then this will the these cells if the homologous recombination is taking place in perfect way then HSV-TK will be removed and when HSV-TK will be removed then if you put the ganciclovir drug the cell will grow because that enzyme is not there and then the derivative of guaninegoing in will not incorporate it in the DNA while DNA synthesis.

So, in that way we can select the correct clone. So, once you make this kind of targeting vector and after that you need to incorporate in the cell, embryonic stem cell there are several ways by which you can do this incorporation one is the chemical way, and another is the electrochemical way. So, there are some agent which we call it as a for example lipofectamine and there are some molecules which makes a vesicle and in that vesicle this DNA molecule is incorporated.

And these vesicles fuse with the cell and then this delivers the DNA molecule. So, we can use that method or we can use another method which we call it as electrochemical method. So, if we apply very high voltage to the cells and when you will apply for very short moment then that will cause formation of a small pores in the cells and when these small pores will be formed the DNA molecule will be transported inside the cell by simple diffusion method.

So, so this is a another way and please remember in at that high voltage for that short duration the cell will not undergo any severe damage the cell will survive. So, in that way the DNA will be transported and then this can move this can be for the move in the cell and then there will be the homologous recombination. And if homologous recombination is perfect then you will get the mutant cell which will have only neomycin but not HSV-TK Gene.

Then you can collect those cells and then you can move further. Just here I would like to give a note. So, there are several possibilities you should know about this thing. So, when you when you put this DNA molecule inside the cell then there will be a several possibilities one of that possibility is as I explained you there will be a perfect homologous recombination and that is a very good situation or that is a kind of win-win situation but in general it is not the case.

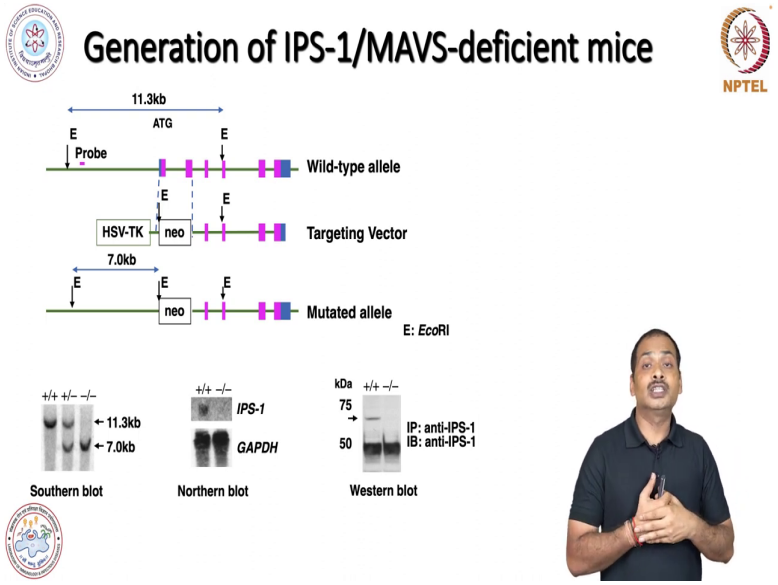
So, when you put the DNA molecule the DNA molecule has a property that it can integrate in whole genome anywhere. If it will integrate anywhere in the genome then those cells are useless it is not useful for making transgenic mice. So, that is why we have incorporated the HSV-TK the negative selection marker. So, if this DNA molecule will incorporate anywhere in the genome in that scenario HSV-TK will remain intact.

And when you put these cells over G418 that is positive selection marker drug that is neomycin resistance Gene drug that is G418 then they will grow very nicely but those cells are not true cells what you want. So, to select further we put ganciclovir in those cells. If those cells will grow in ganciclovir also then it is not correct cell why because the whole DNA fragment is incorporated into the genome.

And it means it is a not useful cell what you want is that your this targeting vector should present in correct Locus in the genome. So, if you see if there will be a perfect homologous recombination then this HSV-TK Gene will be kicked off and only neomycin Gene will be remained there. So, the cells which are growing on G418 but not growing on the cells which are growing on G418 as well as ganciclovier they are the correct cells. So, in that way we we can select.

It seems that it is a very simple but it takes some maybe six month or a year to conduct this kind of thing. So, I have already explained how you will introduce the targeting vector and you will screen. So, I gave the concept of a screening.

**(Refer Slide Time: 20:28)**

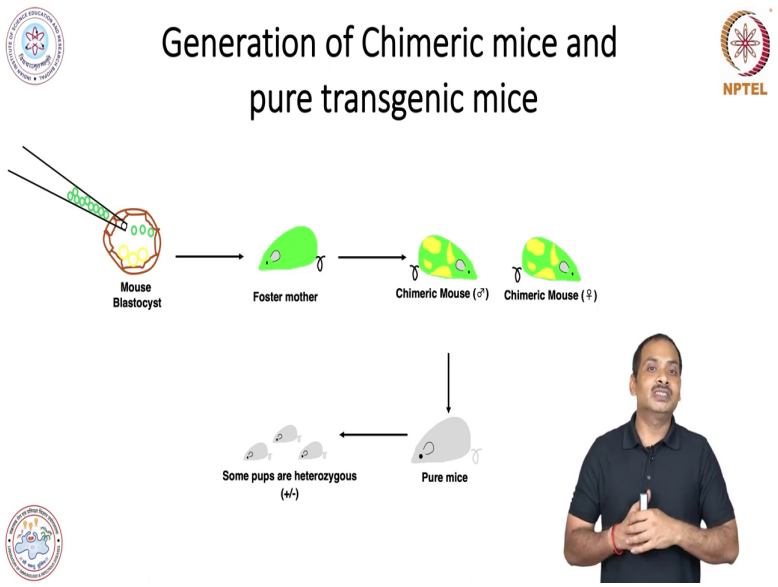



And now we will move that as a one example. So, this is a one gene which we call it as a IPS1 or also known as MAVS. So, this mouse I have created. So, here you can see that this is a genomic location of this Gene. So, we have created this kind of targeting vector and after that we have after doing all selection and all those things we got this kind of mutant allele. And once you get all this thing in perfect way then you have to prove it by Southern blot.

Southern blot is basically you; so, here you can see how we can screen there is a several method by which one can screen the correct embryonic stem cell one is that using PCR method you will design some primer very smartly and by that you can you can find out your clone is correct or not this is one way which is commonly used by many researchers another way is that you can do the southern blot.

So, in southern plot you here you can see there is a probe and if your allele is mutant then you will get this 7 kilo base a pair fragment and that you can visualize over the southern blot.

**(Refer Slide Time: 21:56)**




So, after doing all this thing then we move to the basically a cellular work. So, far you have done molecular you have done the cell screening and found out the correct cell and please remember during all this process you need to maintain the cells very very delicately and very nicely. Otherwise if this sells even if you made a mutant cell and if this cells will get differentiated then you can never get the transgenic mice out of this cells.

So, you have to maintain these cells you have to handle these cells very delicately. So, after that we basically introduced these mutant cells in the blastocyst which is again derived from another mice and then this is a blastocyst as you have seen in previously slide. And then this you will make you will perform the surgery in Foster mother which is a hormonally prepared Foster mother is just hormonally prepared to take this blastocyst and take it further for further development up to the Puffps.

So, you will incorporate into the foster mother and then this foster mother will give the birth of a chimeric mouse which may be male and female and here you just note that so the body of chimeric mice is consists of two kinds of cell one is the mutant cell and another is the wild kind of cells. Wild kind of cells means the cells which is normal. So, in blastocyst you can see there is a yellow cell this yellow cell is from the blastocyst which is a normal.

And the green cell which you can see here these green cells are only mutant cell. So, the body of the mouse is made up of two kinds of cell one is derived from this blastocyst cell and another is from embryonic stem cell. So, that is why we call it as a chimera. So, you take this

chimeric mice and then you mate it with some pure line generally we use some strain of mice for example C57 black six or BALB/cbulsi mice.

And if the reproductive organ of that chimeric mice is derived from the embryonic stem cell the mutant embryonic stem cell then you will see the germ line pass means so, for example you are taking the male chimeric mice which can produce a sperm of two kinds one is mutant derived from mutant cell and another is normal cell. So, this mouse will transfer this mutant sperm to the to the another female mice that is pure mice then you can see there will be a heterozygous germ line transmission.

This is heterozygous germ line transmission and once you get this heterozygous mice after that everything is very easy-peasy peasy what you can do you just you know very well the mendelian law. So, you can just make this male heterozygous with female heterozygous and then you will get the knockout mice you know the very well the you will get in one is to 2 is to one ratio. So, one is to 2 is to one ratio one is the wild type another is heterozygous wild type and one will be the knockout mice.

So, you can use this mice and perform your all those interesting experiments. So, in this session I will stop here and in next session I will discuss about the conditional knockout mice how you create the conditional knockout noise and I will also tell very briefly how you can make a double knockout and all those things, thank you.