Eukaryotic Gene Expression
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Lecture No. # 37
Knockout mice

Welcome to this Lecture series on Eukaryotic Gene Expression: Basics and Benefits. This is our Lecture number 37. Today, we are going to talk about a very interesting and very important topic entitled Knockout mice. So, basically you are going to try to understand how we can knock out the Expression of a specific Gene in a mammal whether it is a mouse or a Rat or embryonic stem cells.

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Lecture 29 Cloning and Expression vectors
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The reason, why this topic is very important is that if we look at the Lectures that we have covered in the last 7 or 8 Lectures, we started this part of Expression systems among series of Lectures by looking at how we can express genes in equally and then, we discussed about how we can express genes in leaf cells and insect cells then we discussed about various eukaryotic protein Expression system, how we can express genes in mammalian cells.

Then we talked about Human Gene therapy, where we have talked about expressing genes in humans with the aim of curing certain genetic disorders. Then, we discussed

about a very interesting and exciting area of research called DNA vaccines, where a failed Gene therapy technique about what is called as a naked DNA injection, which actually failed as a Gene therapy technique, found a new application in the form of development of DNA vaccines.

Then in the last two classes we discussed about generation of transgenic animals and transgenic plants how we can express genes in mice and other farm animals to make useful recombinant proteins as well as for understanding certain basic aspects of biology and we also discussed about transgenic plants how people are trying to use plants as factorize for producing a variety of proteins recombinant proteins including what is called as a oral or edible vaccines and so on.

So, in all these lectures our emphasis has been primarily on expressing genes that is how to express an exogenous Gene, how to express as transgene in a variety of Expression systems. From today's lecture, we are going to cover about how to inhibit or how to repress the expression of a gene in an animal model that is what we call as a knockout or knocking out the Expression of a gene or knockout mice.

So, this lecture is different from what we have been discussed in the last 8 lectures the last 8 lectures focus has been primarily on expressing a gene in various Expression systems, but today we are going to talk about inhibiting the Expression of a specific gene. This is not an easy task, because as we know if we take either mouse or a Human genome, we have more than about 30 to 50,000 protein coding genes. And, if you want to specifically inhibit the expression of one gene, that to in animal model it is a very challenging and daunting task. And in fact, the people who develop this technology of knocking out gene in a mouse actually got a noble prize.

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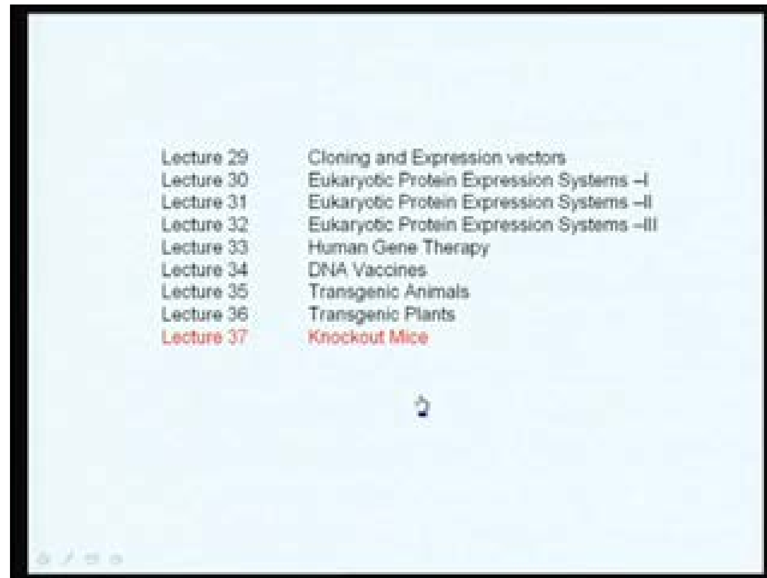

So, let us now try to discuss and see what exactly are these knockout mice. So, how knockout mice are generated and how you can specifically knockout the expression of a particular gene in a mouse.

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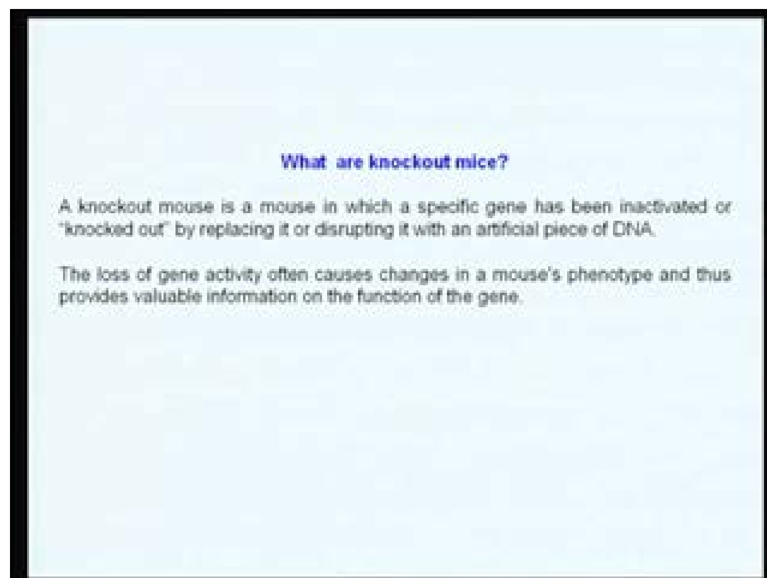

So, what are Knockout Mice?

A knockout mouse is a mouse in which a specific gene has been inactivated or knocked out by replacing it or disrupting with an artificial piece of DNA. So, the loss of gene

activity often causes changes in the mouse phonotype and thus provides valuable information on the function of the gene.

So, the difference between a transgenic mouse model and a knockout mouse model is that in a transgenic mouse, we are actually expressing a transgene over the normal genes which are actually present in the animal, we are trying to over express a particular gene and see what happens to the phonotype, where as in the case of knockout technology we are actually inhibiting the or repressing the Expression of a particular gene so that the gene product is not made. And ask the question if the gene is not expressed, what happens?

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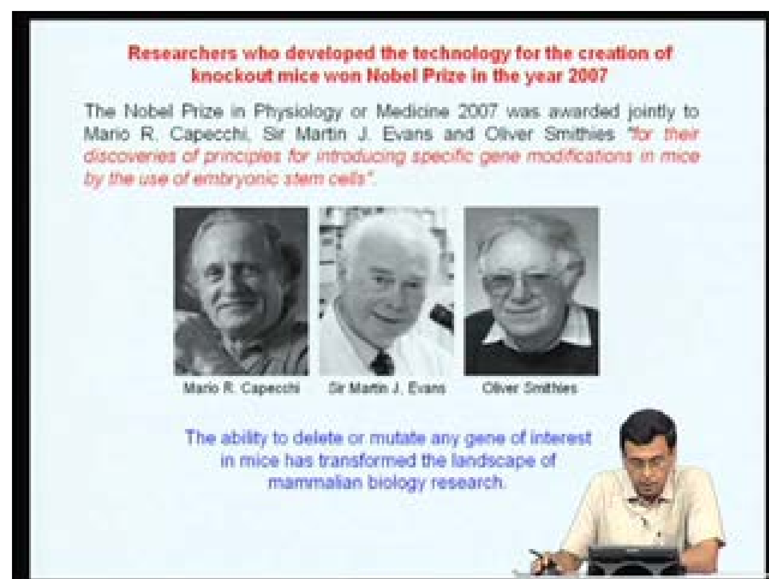

So, we will discuss as we go about, what are the exact differences between the transgenic mouse and a knockout mouse. Now, the topic we are discussing today is very very important because the researchers, who developed this technology to create knockout mice, actually won the noble prize in the year 2007.

So, the noble prize for physiology and medicine in 2007 was actually awarded jointly to Mario Capuche, Martin J Evans and Oliver Smithies for their discoveries of principles for introducing specific gene modifications in mice by the use of embryonic stem cells. Why were they given this noble prize for this discovery?

Because the ability to delete or mutate any gene of interesting in mice has transformed the landscape of mammalian biology research. So, what I am going to discuss in the next few minutes is to give you evidence that how this technology developed by these three people about the ability to knockout genes in embryonic stem cells and then take this embryonic stem cells in which the genes have been knocked out and then put them in a mouse embryo and create a mouse what is called as a knockout mouse in with that particular gene is not expressed.

This creation of this knockout mouse technology has changed the way the biological systems functions, the way gene functions can be studied and so on. And that is why these knockout mice considered as a very important discovery in biology and these people got noble prize.

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So, what exactly this people did?

Martin Evens actually spent a lot of time and published the number of papers and standardized the technology for the Cultivation of embryonic stem cells. And Oliver Smithies was the first who used this embryonic stem cells and developed techniques for targeting a transgene to a specific region in the ES cells. He could demonstrate that it is possible to target a transgene to a specific locus.

As we have discussed in the earlier lectures, especially in Human Gene therapy the problem with things like hetero viral vectors is that the hetero viral goes and randomly integrates anywhere in the genome and causes lot of problems and in fact, it even caused cancer in couple of instances.

So, it is very very difficult to specifically target a gene to a specific region on the locus in mammalian genome and because whenever you put a transgene, the chances of random integration are much higher than the targeting to a specific region by homologous accommodation. So, Oliver Smithies and Mario Capecchi actually developed technologies to selectively identify those cells in which gene targeting has taken place compare to those in which the Gene has gone and randomly integrate elsewhere in the genome.

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So, some of the key papers published by Oliver smithies are just mentioned here where for example, in 1987, he actually published a paper on targeted correction of a mutant HPRT Gene in mouse ES cells and he stated that this modification of a chosen Gene in pluripotent embryonic stem cells demonstrates the feasibility of this route to manipulate mammalian genomes in a predetermined way.

And, in 1985, he published another paper where he actually showed insertion of DNA sequence into the Human chromosomal betaglobin locus by homologous recombination and then germ line transmission of a planned alteration made in a HPRT Gene by

homologous recombination in embryonic stem cells. We will discuss some of these papers in detail as we go long.

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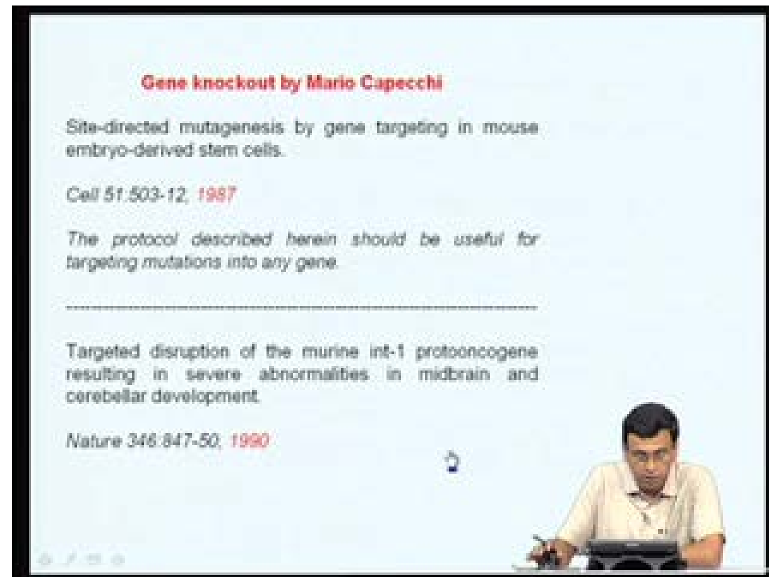

Similarly, Capuche published many land mark papers where in 1987, around the same time as Oliver Smithies published, he showed actually side directed mutagenesis can be made by gene targeting in mouse embryonic derived stem cells and three years later he actually demonstrated that using this technology, it is possible to disrupt the function of a protooncogene called Int-1 leading to severe abnormalities in midbrain and cerebellar development. And Int Gene is actually a protoonco gene, which plays a very very important role in development and, if you knockout the expression of this Int-1 gene it results in several development abnormalities. So, these are some of the key papers published by these noble laureates in the area of Knockout Mice.

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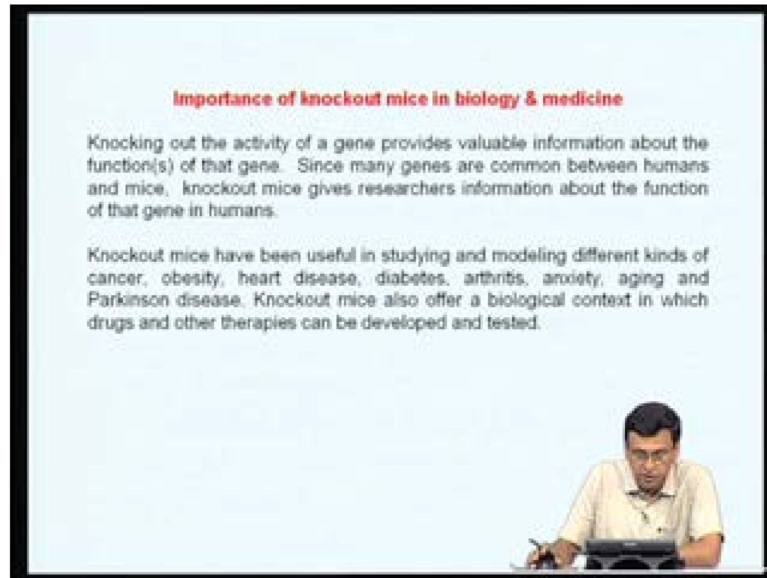

Now, what is the importance of Knockout Mice in biology and medicine and why this technology became so popular and why these individuals got noble prize? Now, knocking out the activity of a gene provides valuable information about the functions of that gene. Since, many genes are common between humans and mice, Knockout Mice give the researchers information about the function of the Gene in humans.

So far, the difference between the transgenic mouse and a knockout mouse is that in the case of a transgenic mouse, we are over expressing a gene over and above that of a normal or a mutant crop which is present, where as in the case of knockout mice, we are blocking the expression of their particular gene and asking the question what happens if the gene is not expressed. Does it have any important role, what happens so and so forth.

So, Knockout Mice has been very useful in studying and modeling different kinds of cancer, obesity, heart disease, diabetes, arthritis, and number of diseases. And Knockout Mice also made offer a biological context in which drugs and other therapies can be developed and tested. So, I am going to give a few examples as we go along to see how the development of this technology for knocking out genes in mice revolutionizes research in biology and medicine.

So, let us spend some time to understand what is the main difference between a transgenic mouse and a knockout mouse? Now, a transgenic mouse typically expresses one or more copies of a gene that is integrated into genome in a random fashion. This is

what we are shown because many injected transgenes into the fertilized egg of a mouse, it goes and randomly integrates in a head to tail fashion in anywhere in the mouse genomes. So, we have no control over it, whereas in case of a knockout mouse, both alleles of a gene are deleted in a targeted fashion by homologous recombination. So, in the knockout mouse since you have to specifically knockout a specific gene, you have to target your transgene construct in such a way that it goes and recombines with the gene present in the chromosome of the cell. So, that genetic recombination takes place and it goes on specifically knocks down the expression of that particular transgene.

So, while traditional transgenic mice are generated to express a protein, much information can be learned from the elimination of a gene or deletion of a functional domain of the protein. So, this is what the main distinction between a transgenic mouse and knockout mouse. In a transgenic mouse model we are trying to understand the function of a gene by actually over expressing the gene, by over expressing the protein coded by the gene and ask the question what happens when you over express the particular Gene or protein.

For example we had learnt in the last class that if we over express the human growth hormone the rat growth hormone gene, in the mouse it grows much bigger because growth hormone promotes growth using metallothionein promoter.

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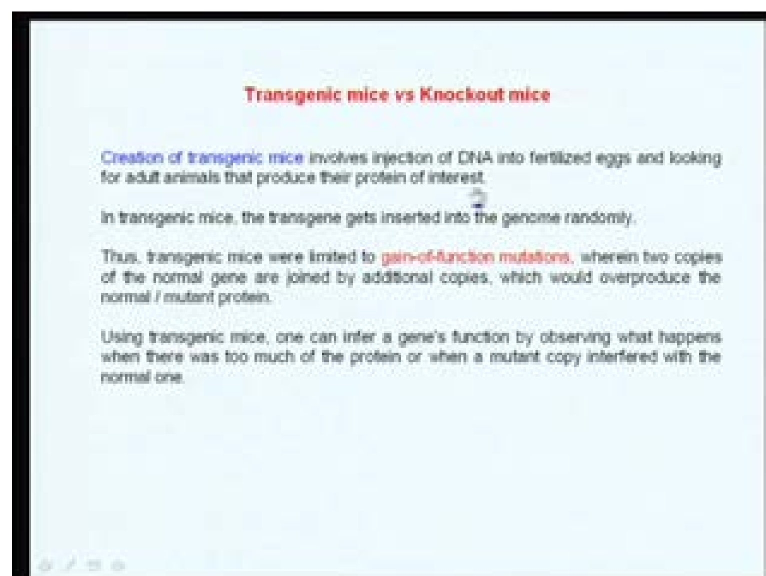

So, the creation of a transgenic mice involves injection of DNA in to fertilized eggs and looking for adult animals that produce their protein of interest that is what we discussed in the previous class, whereas in the case of transgenic mice, the transgene gets inserted into genome randomly, these are the two important features of a transgenic mouse. So, transgenic mice are actually used primarily to gain-of-function mutations,where in two copies of the normal gene are joined by additional copies which would over produce the normal or mutant protein.

So, this is what we understood by the transgenic mice. So, the normal copies or mutated copies are still there, but over and above this normal copies are present in their normal location in this genome, we are adding extra copies by over expressing the Gene and generating transgenic mice and ask the question what happens when you over express the gene what is the phenotype you generate.

So, it is a gain of function mutation that we are studying. So, using transgenic mice one can infer a genes function by observing what happens when there was too much of a protein or when a mutant copy interfered with the normal one.

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So, by this basically what knowledge you gain by making transgenic mice where as in the case of Knockout Mice the knockout technology allowed research to delete specific genes. So, in this case the transgene which is usually a mutant or deleted version of the

normal gene is introduced in the embryonic mouse cells and culture and then allowed to recombine with this sister DNA in the genome

So, you make a what is called as a knockout construct in which the Gene is said disrupted or mutated and when you introduce this mutated version or a deleted version of a Gene inside DNA cells by using specific techniques you make it you select for those cells in which this transgene has recombined with the endogenous chromosomal copy and therefore, as deleted both the normal alleles in the chromosomes only your transgene or the mutant version of the gene is expressed.

So, you do not have the normal copies of the gene anymore. It is a primary distinction between a transgenic mouse and the knockout mouse. So, the recombination cuts out the resident DNA and insert the mutant DNA which can then form the damage protein or does not produce any protein at all, depending upon how you have designed your transgene construct.

The embryonic stem cells then grow into a whole mouse with a specific mutation exactly where it supposed to be in the genome. So, this is the major difference between transgenic mouse and knockout mouse that in the case of a knockout mouse your construct has gone exactly the same place where the normal counterpart is present, whereas in the case of a transgenic mouse your gene may go and randomly integrate anywhere and the normal copy may be still there in the elsewhere in the normal place of the genome but your transgene may be getting expressed somewhere else in the genome. So, the gene knockout technology allowed researchers to study loss-of-function mutations wherein one can infer gene’s function by observing what happens when the gene is absent or when mutant copy of the gene expressed instead of the normal one.

So, by generating transgenic mice you understand the Gene function by gain-of-function mutations, whereas, by generating a knockout mice for the same gene you are asking the question what happens when the gene is not expressed when the function of the gene is lost. So, it is a loss of function mutation. So, transgenic mice are a gain-of-function mutation, knockout mice is a loss-of-function mutation. And the first human disease models created this technology were cystic fibrosis process from the labs of Smithies and Evans. So, we will discuss some of these things.

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So, I am going to give a series of slides just to emphasize the point that what kind of experiments were actually done, what kinds of genes have been knocked out. There are number of genes that have been knocked out. I am going to give you a selected list of examples and I am going to show some very nice pictures of how knocking out specific genes, what kind of phenotype people got, and how you can demonstrate that this gene has a particular function by using the knockout technology.

Here is for example, one picture where it shows a laboratory mouse in which a Gene affecting hair growth has been knocked out, the one on the left. So, it has much less hair compare to the wild type mouse. So, clearly saying that this gene is very very important for the hair growth. If you knockout the gene, you get a mouse which does not have as much hair as the normal one.

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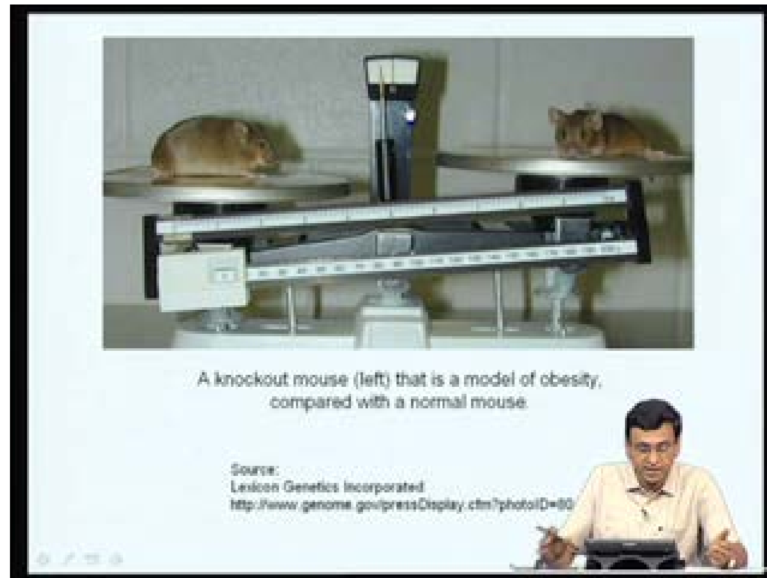

It is another example, it is another Gene, if you knockout this gene in this mouse, the mouse becomes obese. You can see this is the normal mouse and you can see this mouse is much bigger. So, this is a knockout mouse model for obesity and you knockout that particular gene, the mouse becomes obese or fat compared to normal, clearly telling that this gene has a very important role in obesity.

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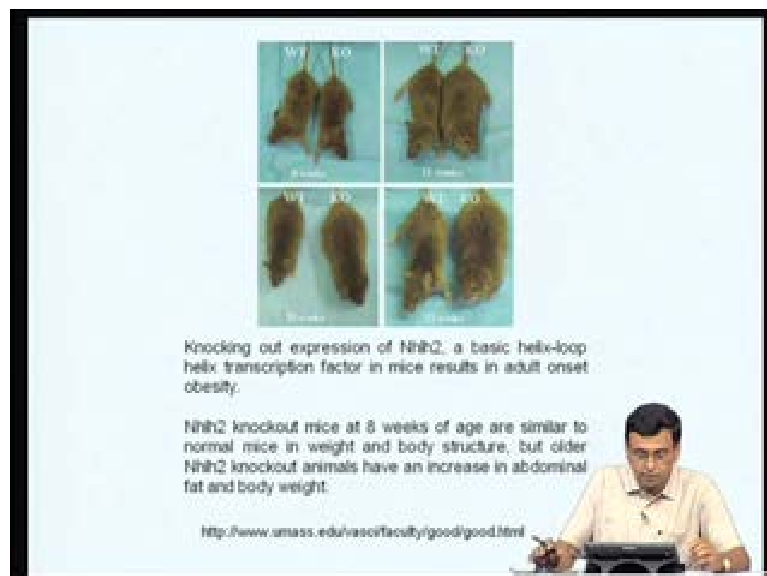

It is another very interesting gene, a gene called Nhlh2, which is actually a transcription factor and if you knockout this expression, it results in what is called say adult onset obesity. Now, what is this?

You can see when the mice this wild type mouse and a knockout mouse, when they are 8 week old, they were both look normal, but as the age 12 weeks, 28 weeks, 52 weeks, as you can see the mouse in which this particular gene has been deleted is becoming fatter and fatter, it is becoming more obese compare to the normal mouse. So, as the mouse age, they become more and more obese. So, this is called as an adult onset obesity indicating that this transcription factor somehow plays in the very very important role in this particular disease.

So, it gives an idea understand what kind of genes are getting affected by knocking out the expression of this particular gene. So, Nhlh2 knockout mice at 8 weeks of age are similar to the normal mouse in weight and body structure, but older knockout animals have an increase in abdominal fat and body weight.

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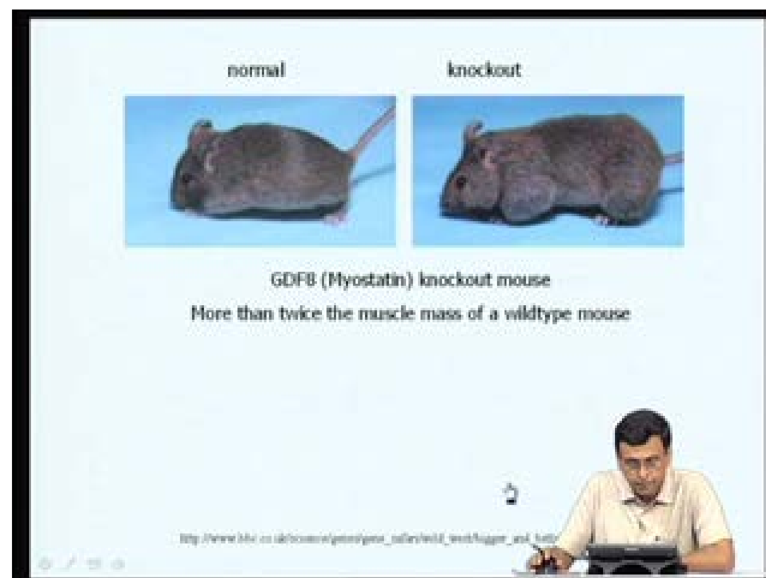

So, very very interesting phenotypes we can observe by knocking out this very very important genes. It is another example if you knock out a gene coding for a protein called GDF8 known as Myostatin and this mouse has twice the muscle mass compared to the wild type. So, the muscle mass increases, very very interesting, clearly saying that this protein plays a very important role in myogenesis or muscle formation.

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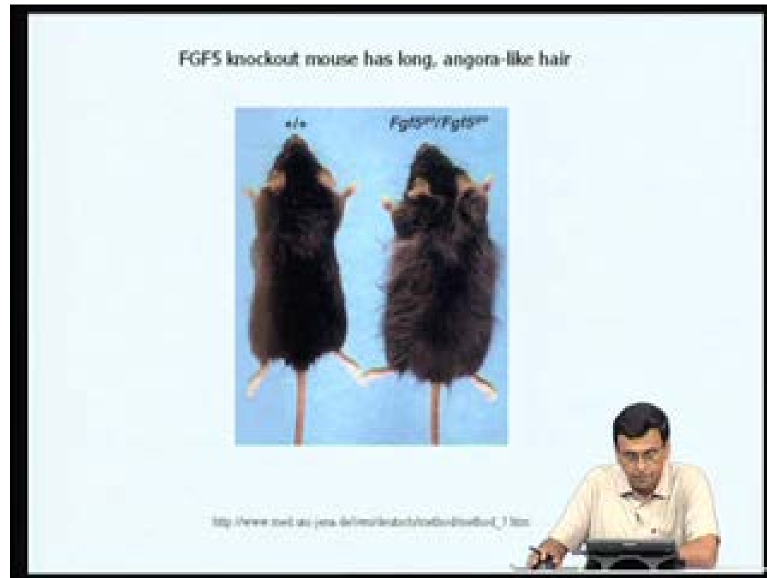

Another important mouse FGF5 if you knock out this particular protein the mouse has long angora like hair. So, very interesting phenotypes.

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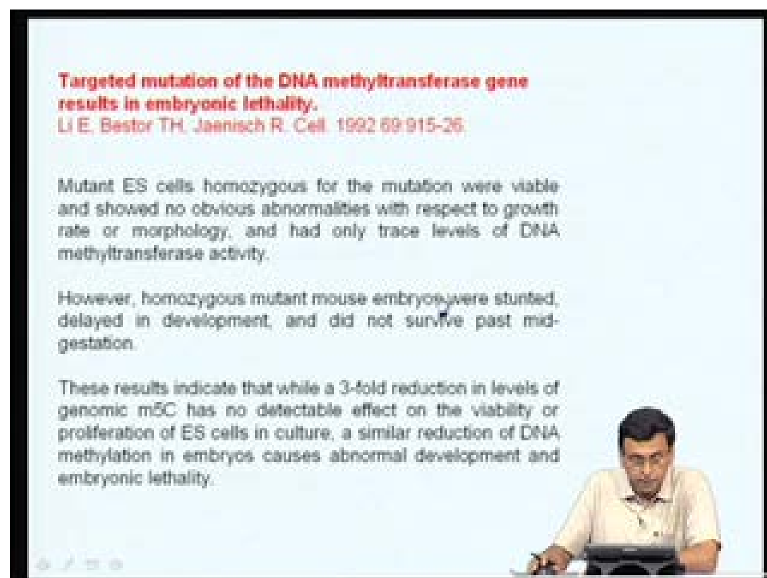

Again, if you knockout a gene that codes for a DNA methyltransferase gene, it results in embryonic lethality, again published by rule of Rudolf Jaenisch groups in sale in 1992. As, we have studied how epigenetic plays a very very important role in embryonic development, we have discussed essentially how DNA methylation plays very very important role especially denovo methylation takes place in the fertilized egg and then a

new methylation pattern is established as the fertilized eggs starts dividing during early embryonical development stage.

And there are what are called as DNA methyltransferase gene which actually plays very very important role in methylation of the promoters and turning on or turning off what specific genes during embryonic development and to actually demonstrate this DNA methyl transferase or DNA methylation plays a very important role in embryonic development, they actually knocked out the DNA methyl transferase gene. And although the embryonic cell did not show any phenotype, they were normal, that is if we knockout the DNA methyl transfers Gene embryonic stem cells they still divided and behave normal, but when they will take this ES cells into the DNA methyl transferase gene is knocked out and you know develop a mouse model the mouse did not develop beyond a certain stage of development clearly indicating that if you knockout DNA methylation is not proper, embryonic development is totally abolished, you cannot get an adult mouse out of it indicating DNA methylation plays a very very important role during embryonic development.

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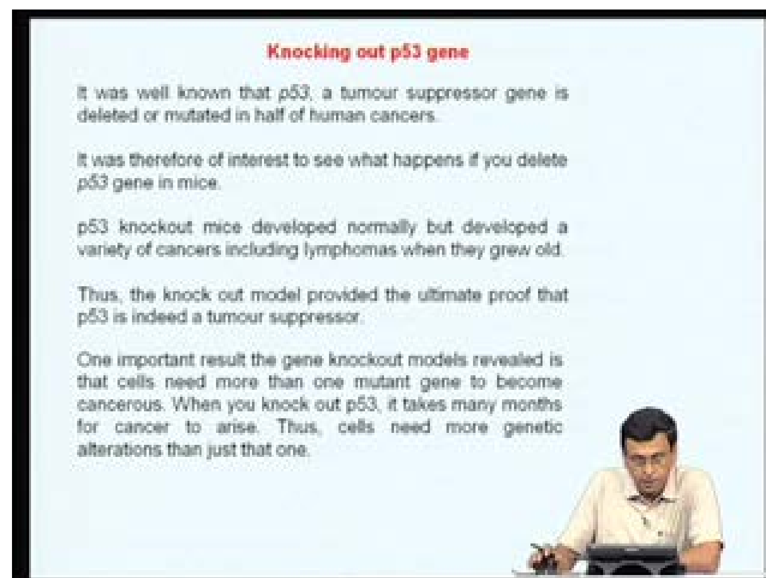

Again p53 gene. So, p53 gene is a tumor suppressor gene, and we all know people have done number of experiments in number of cell lines and then shows if you knock out p53 gene, the cell line becomes cancerous, it becomes tumorogenic, but when they actually

knocked out the p53 gene in mouse, the p53 knockout mouse developed normally, but developed a variety of cancers including lymphomas as they grew old.

So, by actually demonstrating in an animal model that if you knock out p53, it ultimately develops cancer, it provides ultimate proof that p53 is actually tumor suppressor gene and is an essential for the normal phenotype of an individual, if you mutate or if you delete the p53, it leads to cancer.

One important result of the gene knockout models revealed is that cells need more than one mutant gene to become cancerous. When you knockout p53, it actually took many months for cancer to arise. Thus, cells need more genetic alterations than just one.

So, although ultimately the mouse developed cancer, but in the initial stages of development the mice were pretty normal, all the p53 was knocked out, but as they age as they grew older, more and more mutations accumulated and therefore, along with mutant p53 or deletion of p53, other mutations are also required for getting a cancer phenotype.

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Similarly, another very important tumor suppressor gene called the retinoblastoma gene, when they actually knocked it out, people actually expected that these mice should form tumors in the eye because that is what retinoblastoma is, but instead of eye tumors these

animals actually got pituitary and thyroid gland cancers, which was totally unexpected result.

So, this knockout technology actually gave phenotypes, which were not expected, many of these knockout mice, there are phenotypes which were very very interesting and which were totally contradictory to what was actually expected of these knocking out of these genes and it was later found that a second gene actually protect the eye cells from cancer and both mutations were required to form tumors.

So, the knockout models, animal models gave tremendous knowledge on the function of these genes and in many cases, either substantiated or provided the ultimate evidence of all the work, that was done in cell lines and, many times it actually gave up some new very interesting and surprising results making you to develop new lines of research.

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Similarly, in another study when the gene encoding telomerase, telomerase is a protein that is responsible for maintaining the tips of the chromosomes. And as long as telomerase is there, the chromosomes replicate normally and the tips replicate, but if you knockout this telomerase, then the ends of the chromosomes cannot replicate anymore. And in such mice, cancers arose when the chromosomes tips shorten over the course of lifetime of cell division and the failing tips cause chromosomes to break and recombine incorrectly, until finally the cells which are large number of mutations needed for transition to malignancy.

But, what all these knockouts in this genes involved in cancer actually told is that the most important factor that direct cancer is age. So, whenever they mutated many of these oncogenes or tumor suppressor genes, it is not that the animals developed cancer immediately, this is only a period of time as the mouse age, they started developing cancer indicating that accumulation of mutations require more than one mutation for cancer to arise.

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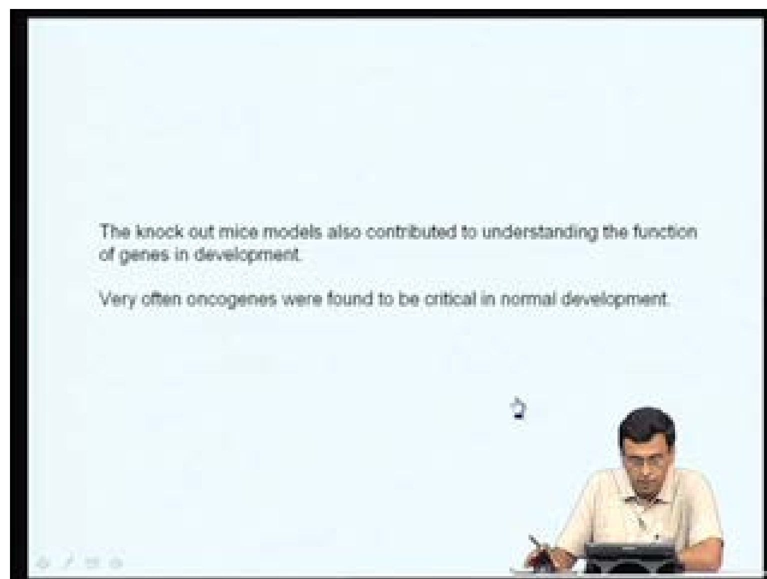

So, the knockout mice models also contributed to the understanding the function of genes in development. In fact, very often oncogenes were found to be critical for normal development. As, I told you in the one of the first slides, Mario Capuche actually knocked out int-2 gene which is actually a proto oncogene, it led to development of lot of abnormalities.

So, it became very clear that many of these proto oncogenes, which actually are involved in cancer development, they play very very important role during embryonic development. So, these kinds of a very interesting results came out of knockout mice research.

I have so for discussed with you, what is the importance of knockout technology and how the knockout technology is different from that of it transgenic technology because of an transgenic animals, we are actually over expressing the gene over the normal gene which are present at the chromosomal copy and gene that we are introducing, it can

actually randomly integrate anywhere in the genome and express it, whereas in the case of a knockout technology the transgene that you are introducing, goes specific to a particular locus in the genome, recombines with the normal copy present in the chromosome, and replaces the normal gene present in the chromosome. So, your transgene is present exactly in the same place as a normal gene is present. So, it is very very specific. So, side specific recombination, the transgene goes and replaces the endogenous gene by side specific recombination and so, it is very specific.

In the transgenic mice, we are looking at the function of a gene by gain-of-function mutations whereas, in the case of knockout mice, we are trying to understand the function of a gene by loss-of-function mutations. These are the two major distinctions between transgenic mice and knockout mice and I have given you several examples as how to generate knockout mice or by knocking out specific genes, how very important, very interesting phenotypes could be developed, whether you knockout a transcription factor or a knockout a proto oncogene, you can see that mice developed very very interesting phenotypes and by knocking out these kinds of a genes, one can also understand the biology of the disease much better and it can also develop animal models for a number of diseases.

So, now let us spend some time to understand what the technology is, how can you knockout the specific gene, it is almost like needle in a hairstyle. There are so many genes in the genome, but you want to specifically knockout a particular gene.

How do you do that?

Because genetic recombination is a very rare event and the transgene that we have introduced has to go and specifically recombine with the endogenous gene which is in the chromosomal copy, it happens only one in thousand or one in ten thousand. It usually goes and randomly integrates anywhere in the genome. So, the challenge in developing a knockout mouse is to identify those rare recombination events over the background of random integration events. So, you need to kill those cells in which your transgene or your knockout construct has got randomly integrated all over the genome, and select only those cells in which your knockout construct or transgene has specifically gone to replace the endogenous gene by genetic recombination.

So, we have to score for this rare recombination event over the large background of random integration events. That is the key feature of this knockout technology. So, if we want to generate knockout mouse, the gene, that you have introduced has to undergo what is called as a homologous recombination.

So, what are the various steps in generating a knockout mouse, the first is you need to design what is called is a knockout construct just as you if you want to express a gene and generate a transgenic construct, there you need a promoter and you then you put your gene of interest downstream of the promoter and put the proper polysequences and then you inject them into the fertilized eggs, it goes and randomly integrates and the promoter will now drive the expression of a transgene and your proteins are produced.

Whereas in the case of the knockout construct, the gene that you have introduced actually should be a mutated version, or certain regions of the gene should be deleted so that it is a nonfunctional gene. So, the knockout construct is different from a transgenic construct. In the transgenic construct, actually the normal version of the gene is usually expressed to make the wild type protein, whereas in the case of a knockout construct, usually the gene that you are introducing will either carry a mutation or it will actually carry a deletion of certain bridge.

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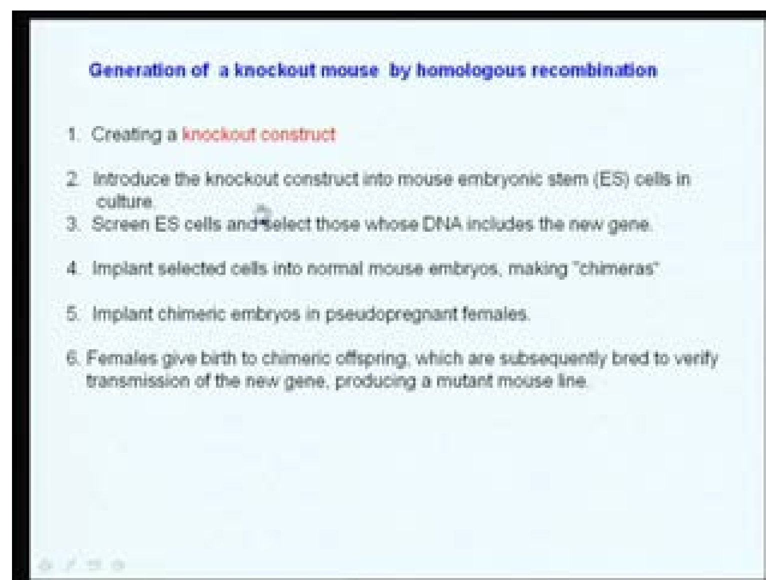

And once you make a knockout construct, you introduce the knockout construct into a mouse embryonic stem cells in culture and then screen the embryonic stem cells and

select only those whose DNA includes the new gene and then implant the selected cells in to normal mouse embryos making a chimeric mouse and then you breed this chimeric mouse to generate homozygous, implant the chimeric embryos into pseudo pregnant females.

And then once is females give rise birth to generate chimeric mice, you then breeding them to generate a pure line or a homozygous knockout mouse. These are the various steps in generating the knockout mouse, but now let us go step by step and try to understand how exactly go about developing a knockout mouse.

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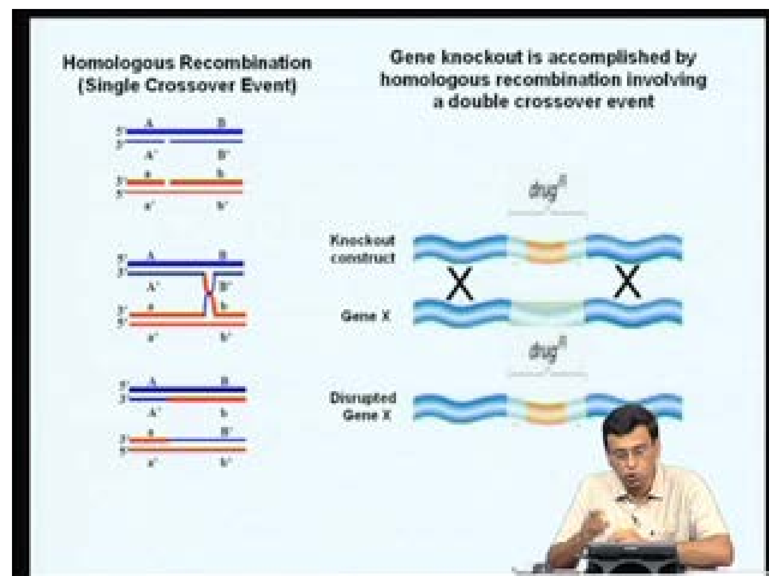

So, the key mechanism involved in generating a knockout mouse is homologous recombination. I am sure; all of you had studied what is homologous recombination. Homologous recombination normally takes place when the maternal paternal chromosome come together especially during meiosis and exchange parts of their chromosome.

This is what is the molecule basis for the differentiation, which usually happens during the meiosis in the germ cells. So, you can see that the paternal maternal chromosome come together what is called as genetic recombination takes place that the very famous holiday junction is formed and segments of DNA gets exchanged between the two chromosomes and you get a recombined chromosome.

But this homologous recombination is a very very rare event. So, what happens during the development of a knockout? The gene knockout is actually accomplished by homologous recombination involving a double cross over event, that means, you need to have cross overs at two different regions of the genes. Now, I am going to explain in a schematic manner, in a very simple manner how exactly the gene knockout takes place.

Now, let us see this is the gene of your interest, let us say, you want to actually introduce a mutation in this particular region of the gene. Now you have to design a knockout construct where you take either pcr amplifier or isolate the gene of your interest and then in the very crucial region of this gene which is very very important for the function of gene, you replace that portion of a gene with an expression cassette that codes for a resistance for a particular drug.

It could be neomycin or usually an antibiotic resistance gene. So, you replace the essential region of your gene of interest with an expression cassette that codes for a antibiotic resistance mark, this is what is called as a knockout construct. Now, once you have a knockout construct, you then introduce this knockout construct into embryonic stem cells.

So, in the embryonic stem cells inside the nucleus, you have the normal copy, let us say which is homologous to this gene, now homologous recombination takes place now between your transgene, the gene you have introduced knockout construct and the endogenous gene. So, this crucial region of this gene will now get replaced by the drug marker and therefore, you now get a disrupted gene.

So, this normal gene now got disrupted, because the region which is very crucial for a gene function has now been replaced by an expression cassette coding for a drug resistance marker, and then you can now select those cells which have undergone recombination by screening for drug resistance.

These are very very general strategy involved in generating a knockout embryonic stem cells. Now, you take this embryonic stem cells, screen for those in which the recombination takes place and then put them in pseudo pregnant females, and you get a knockout mouse,

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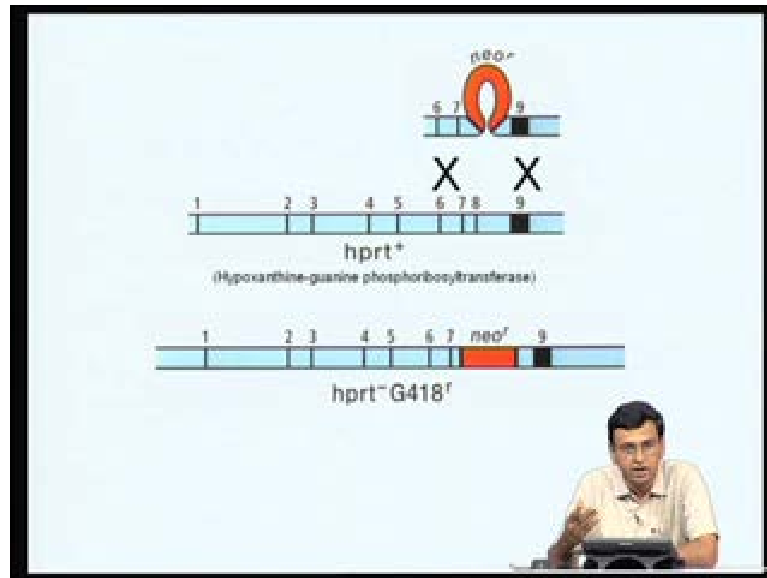

I will give a specific example, if you want to now knockout the hypoxanthine-guanine phosphoribosyltransferase (hprt) which is very important in the salvage pathway of purine metabolism.

What you design is that you design a knockout construct, wherein you introduce a neomycin resistance expression cassette between seventh axon and ninth axon so that the eighth axon is missing. So, this is your transgene construct. Now, what happens when you take this transgene construct and introduce into embryonic stem cells? Recombination takes place between your transgene and the endogenous chromosomal copy of the gene. Due to the double cross over, you now get a knocked out endogenous chromosomal copy.

So, in the place of the eighth axon, now you got a neomycin resistance cassette, you can now this becomes hprt minus and G418 resistance; that means, these cells now are expressing neomycin resistance gene marker therefore, they become resistant to G418, but since the eighth axon is missing, they cannot make the function hprt protein. Therefore, they become hprt minus.

So, this is the basic strategy for knocking out a particular gene. So, certain crucial regions of the axons are replaced by a selection marker and then you screen for particular cells in which the selection marker is expressed and those cells are the ones, in which the gene has been knocked out.

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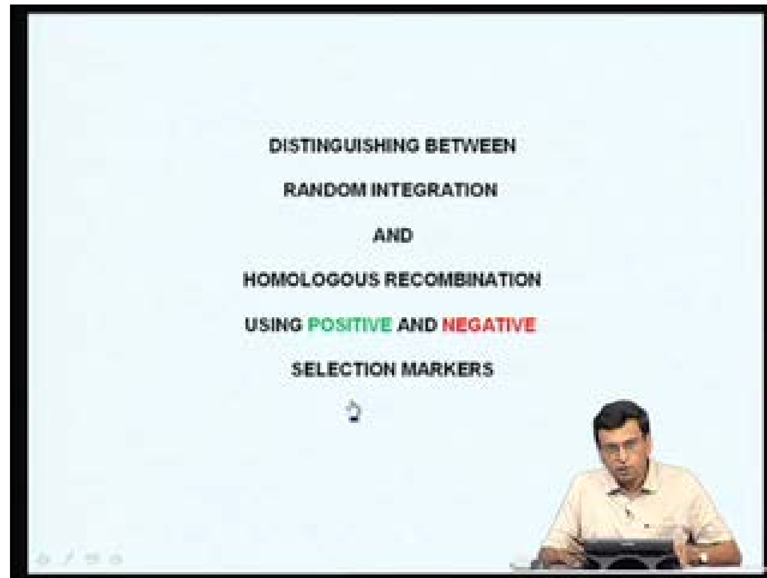

As I said the crucial step in generating a knockout mouse is to distinguish a random integration event from cells, those have undergone homologous recombination. As I said homologous recombination is a very very rare event. Usually whenever you introduce a transgene into these cells, it goes and randomly integrates all over the genome.

So, the key to developing a knockout cell, ES cells or knockout mouse is to score for those cells which have undergone homologous recombination and kill those cells in which your transgene has got randomly integrated elsewhere in the genome. This is carried out by using positive and negative selection markers, very very important features.

So, I am going to spend some time to explain to you how do you do this positive and negative selection and how you can selectively select those cells which have undergone genetic recombination, and kill those cells in which the gene has got integrated randomly. So, how do you do distinguishes between random integration and homologous recombination, this is carried out by doing what is called as positive and negative selection markers

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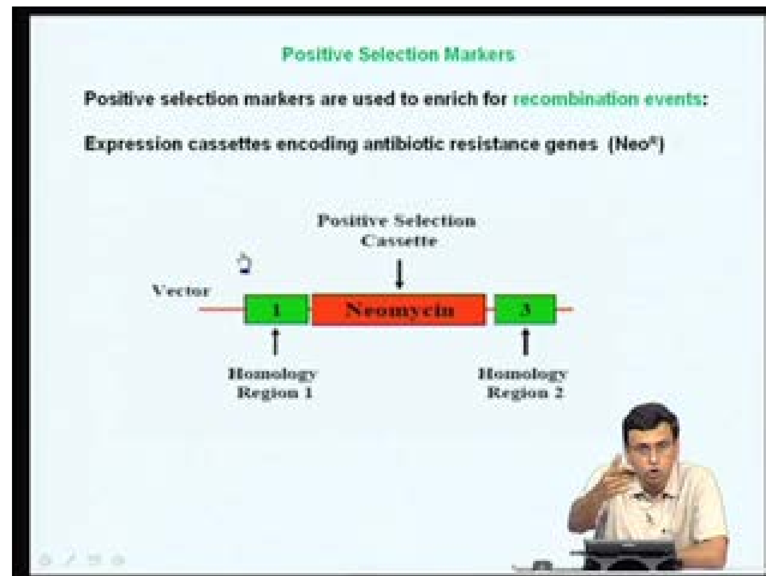

Now, what is this?

Positive selection markers are those which are used to enrich recombination events.

The Expression cassettes encoding antibiotic resistance are usually employed as positive selection markers. So, suppose we have a gene which has number of axons and suppose say axon two is crucial for the function of the particular gene, what you do is that you design a knockout construct in such a way that the axon two is now is replaced by a positive selection cassettes and in this case, it is a neomycin expression cassette.

So, the axon two of this gene has been replaced with a neomycin resistance cassette. So, you have on either side of a neomycin resistance cassette you have axon 1, axon 3 . So, these are the regions of homology and these are the regions which are crucial for recombination, the double cross over that has to take place between the endogenous chromosomal copy when you introduce this construct into the cell.

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Now, what are Negative selection markers?

Negative selection markers are used to enrich for homologous recombination events over random insertions. Usually, the Herpes Simplex Virus Thymidine Kinase coupled to gancyclovir treatment is actually used as a negative selection marker. Now, what is this? Gancyclovir is actually a synthetic nucleoside analog of deoxyguanosine, which is phosphorylated by this viral thymidine kinase, and once it is phosphorylated and when this phosphorylated form of guonosine nucleoside analog gets incorporated into the elongating DNA chain during a DNA replication, it inhibits the DNA by chain termination and therefore, DNA replication stops.

So, gancyclovir is a nucleated analog which when incorporate into elongated DNA, DNA synthesis cannot proceed further and therefore, the cells die, cells cannot divide anymore. So, HSVTK gene preferentially incorporates gancyclovir into the DNA and the host thymidine kinase or the mammalian thymidine kinase has no affinity for gancyclovir.

So, only those cells which express the viral thymidine kinase, gancyclovir will get incorporated into DNA and those cells die because they cannot divide. And, these kind of negative selection markers are usually incorporated into a knockout construct outside the region of homology, very important. The positive selection marker is incorporated into the trans knockout construct within the regions of homology, whereas a negative

selection cassette is incorporated into the transgene in the knockout construct outside the region of the homology. So, this is basically the knockout construct that we have to make if you want to knockout a particular gene.

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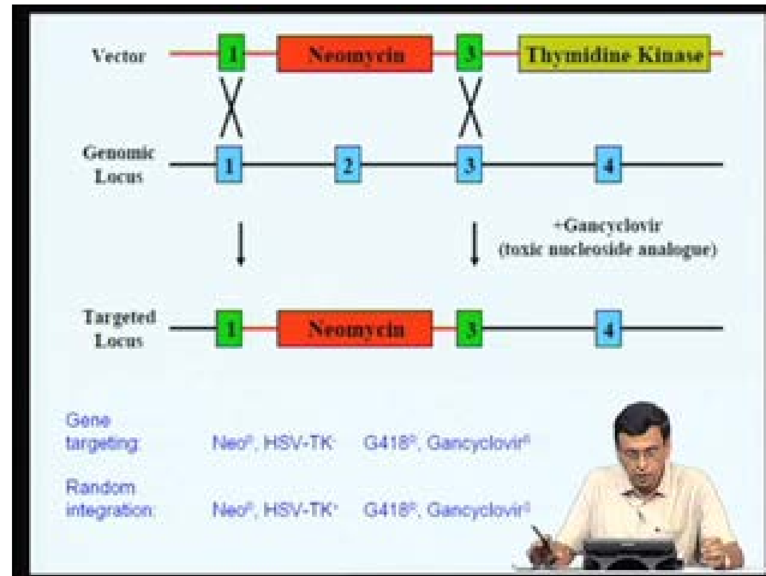

So, what happens, once you make this knockout construct, you inject this knockout construct embryonic stem cells, recombination now takes place between these two regions of homology and as a result only this region now gets replaced.

So, you will now the thymidine kinase gene will not get incorporated into the chromosomal locus only the neomycin resistant cassette will get incorporated if the cells have undergone homologous recombination. So, such cells will be neomycin resistant, but since they do not carry the HSV thymidine kinase gene they are resistant for G418 or gancyclovir resistant.

So, if we have gancyclovir, these cells will not get cleared. On the other hand, if random integration has taken place, if there is no homologous recombination here, if the entire transgene has got inside, got integrated by randomly, then both neomycin gene as well as the thymidine kinase gene will get incorporated and as a result these cells in which this entire cassette has got integrated because of random integration, will be both neomycin resistant as well as HSTVK positive, they will also be expressed in thymidine kinase.

Therefore, they will be resistant to G418 or gancyclovir G418, but they are sensitive to gancyclovir. So, if we now take those cells which have undergone random integration; that means, in these cells both neomycin resistant cassette as the thymidine kinase have will express both the resistant marker as well as the thymidine kinase and therefore, such cells if you now treat with G418 and gancyclovir, gancyclovir will now get incorporated into the DNA and these cells will get killed. This is what is called as a negative selection whereas those cells which have undergone homologous recombination, thymidine kinase gene will not get incorporated, only the neomycin cassette will get inside. Therefore, there will be G418 resistant, but also resistant to gancyclovir because they do not express HSVTK and therefore, gancyclovir will not get incorporated into DNA. Therefore, the cells cannot be killed with gancyclovir

So, you can see by making this kind of a knockout construct, you can now select only those cells which have undergone homologous recombination, because these cells will be resistant to both G418 as well as gancyclovir whereas in those cells where the transgene has got randomly integrated everywhere, they will be expressing both neomycin resistant marker as well as HSVTK and therefore, there will be G418 resistant because of the neomycin resistant they will also be sensitive to gancyclovir because they are expressing thymidine kinase.

And when you treat them with these two compounds, all those cells in which the random integration taking place, they all die. So, you can see by using this very clever strategy, you can select only those embryonic stem cells which have undergone targeted disruption of your gene of interest whereas all those cells, in which the genome as got randomly integrated they get killed. So, this is called as a positive negative selection marker and using this kind of a technology, you can identify only those ES cells which have undergone genetic recombination or which have undergone targeted disruption. And once you have those ES cells, you can actually confirm that target disruption actually taken place by actually designing primers and doing a pcr by usually you design one primer outside the region of homology, one primer within your selection marker and by doing the pcr and if you generate the expressed size of fragment, you know that your transgene has really gone to the right place.

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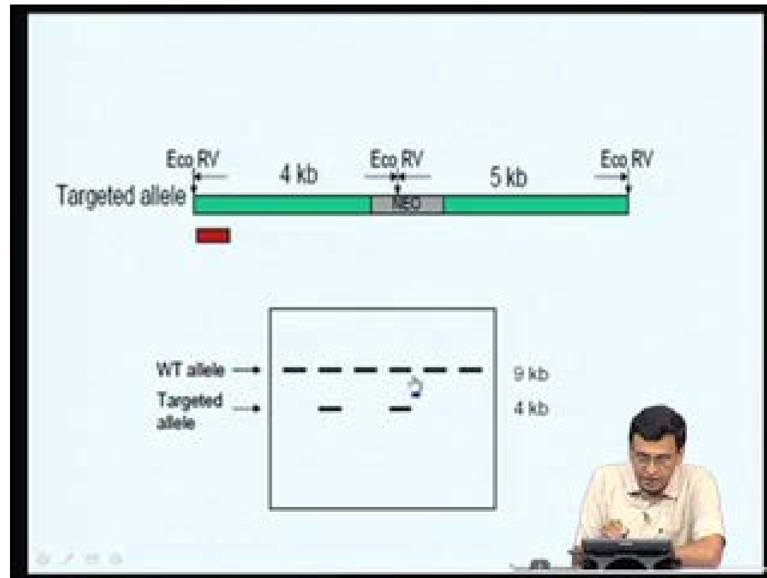

You can also confirm by doing what is called as a southern blot analysis. For example, here let us say there is an Eco R1 side and Eco R5 side flanking the positive selection marker then suppose the antibiotic resistant gene has an Eco R5 side then, if you now isolate the genomic DNA from this ES cells and then do a southern blot analysis using the probe somewhere in this region this probe will now hybridized to a 4 kb region as shown here, whereas if the disruption has not taken place, it will generate the entire length because this Eco RV site is not there. Therefore, 4 plus 5, there are only two Eco RV sites, this probe will now hybridized to a 9 kb region; that means, these are not knocked out, the recombination has not taken place, whereas the recombination has taken place, because we are introducing an extra Eco RV, this probe will hybridise only to a 4 kb band therefore, all those which shows a 4 kb band in addition to a 10 kb band, remember you have knocked out only one allele. So, these are heterozygous.

So, in addition to the normal copy of 10 kb, you will also generate an additional 4 kb indicating that one of the alleles has undergone homologous recombination and therefore, you can confirm target disruption. So, then you take these ES cells, now introduce into the mice and you get generate a chimeric mice and then if you breed chimeric mice then you get a homozygous knockout and in such knockouts now do a southern blot analysis you will not get the 9 kb, you will see only the 4 kb indicating that both alleles has now been knocked out.

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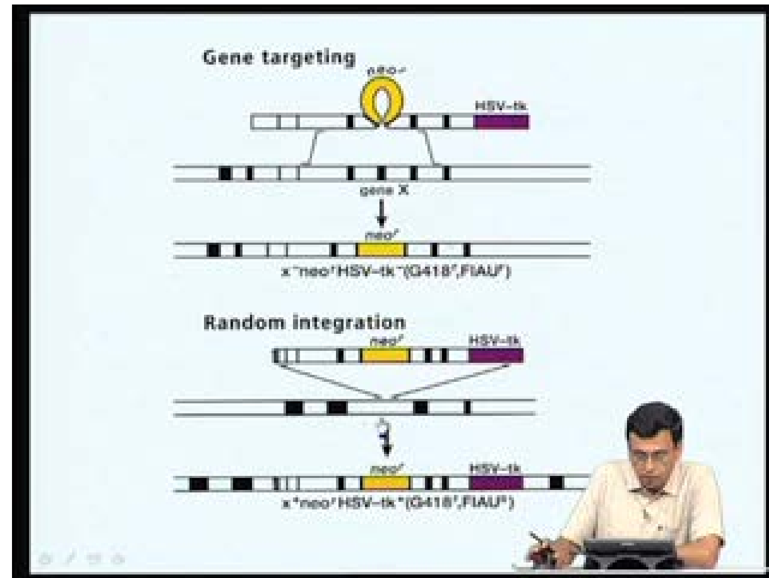

So, this is the summary of the gene targeting strategy, which was developed by Mario Capuche, the positive negative selection protocol. So, the targeting construct usually contains a neomycin resistant cassette in a crucial axon that is required for the particular gene and if the cells are neomycin resistant and HSVTK minus; that means, those cells have undergone gene targeting.

The gene has been disrupted and such cells will be resistant to G418 because they are expressing neomycin, but they will also be resistant for Ganciclovir or FIAU, an analog of same or both are more over same, because they are not expressing the HSV thymidine kinase gene, whereas in those cells where the transgene has got randomly integrated, both neomycin resistant marker and HSVTK marker will get integrated in the chromosomal copy, and therefore, they will express both neomycin resistance as well as HSVTK and such cells will be G418 resistant, but sensitive to Ganciclovir or FIAU. So, they get killed. So, by using this positive and negative selection, you can score for only those ES cells which have undergone targeted disruption of your gene whereas, in all those cells in which they have undergone random integration, they are eliminated. So, this is basically the technology.

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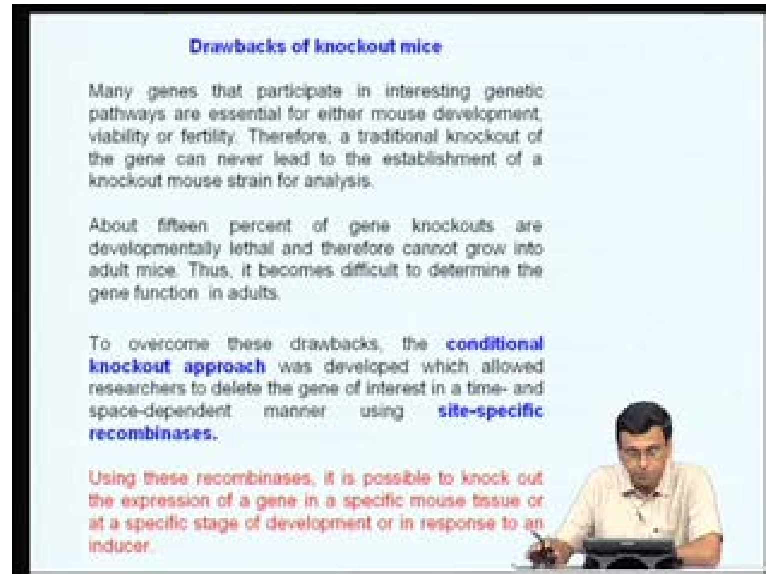

Now, what are the major drawbacks of this knockout mouse?

One of the major drawbacks of generating knockout mouse is that many genes that participate in interesting genetic pathways are essential for either mouse development, viability or fertility. They are all very very crucial. Therefore, a traditional knockout of a gene can never lead to the establishment of a knockout mouse strain for analysis. For example, I want to understand how important is the homeotic gene, as you know homoerotic genes are very very important for normal development, you have studied it in detail in some of the previous classes. But, if you now knockout a homeotic gene, since without that homoerotic gene, the embryonic development will not proceed, I will never get a normal embryo. So, it is very very difficult format to establish a mouse line, a knockout mouse line because, those mice will never develop beyond a certain embryonic stage, because the homoerotic gene is crucial for it. So, one of the major drawbacks of this knockout mice is that genes, which are crucial for embryonic development, in such cases you cannot develop this knockout mouse alleles, because they are all embryonic lethal and such embryos die very early during their development. So, about 15 percent of the gene knockouts are developmentally lethal and therefore, cannot go into adult mice therefore, it becomes difficult to determine the function of the genes in adults. Since, many of these genes are not only important in development stage; they may have a different function during the adult life.

So, you can never understand the complete function of these genes, because they are all embryonic lethal. So, to overcome this major drawback of this knockout mouse, you develop what is called as a conditional knockout approach, which allowed researchers to delete the gene of interest in a time-and space-dependent manner using what are called as side specific recombinases.

So, instead of knocking out the gene in all the cells of your body, you use what are called as side specific recombinases so that your gene gets knocked out only in select tissues or your gene gets knocked out only when you want them to be knocked out. For example, I want to knockout the function of a gene at specific stage during development, it is possible. This is what is called as a conditional knockout.

Let us now see how is this conditional knockout is done. So, using these side specific recombinases, it is possible to knockout the expression of a gene in a specific mouse tissue or at a specific stage of development or in response to a specific inducer. So, I am going to spend some time to explain to you how this conditional knockout is done, because of very very important technology.

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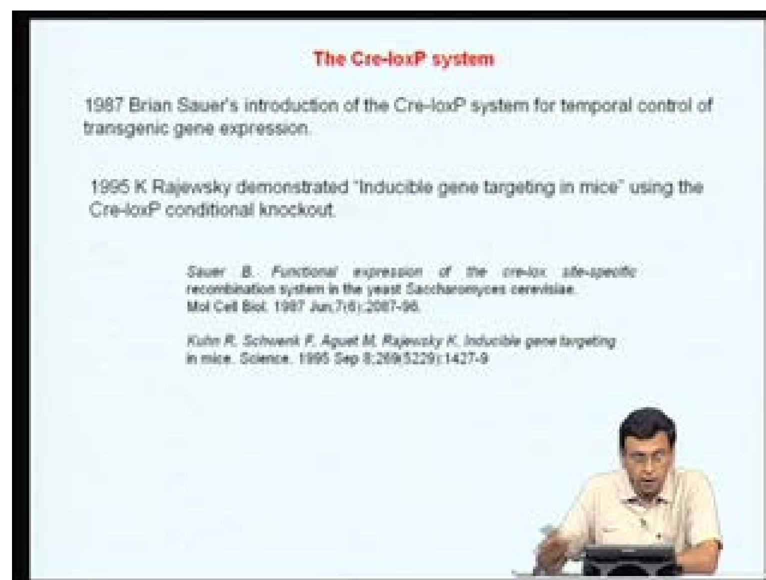

Now the key to this development of this conditional knockout mouse technology is they develop identification of side specific recombinases. One such side specific recombinases were identified very early during the 1980s by Brain Sauer, who identified

what is called as a Cre-loxP system. It is actually a recombinases which is present in a bacteria hpv.

What actually showed is that, by using this Cre-loxP system, you can actually knockout genes conditionally in transgenic mice. This was shown by Rajewsky lab in 1995. The original papers are actually shown here. So, let us now see what is the Cre-loxP system, how exactly you can knock down genes specifically in select issues or at specific period of development using this side specific recombinases.

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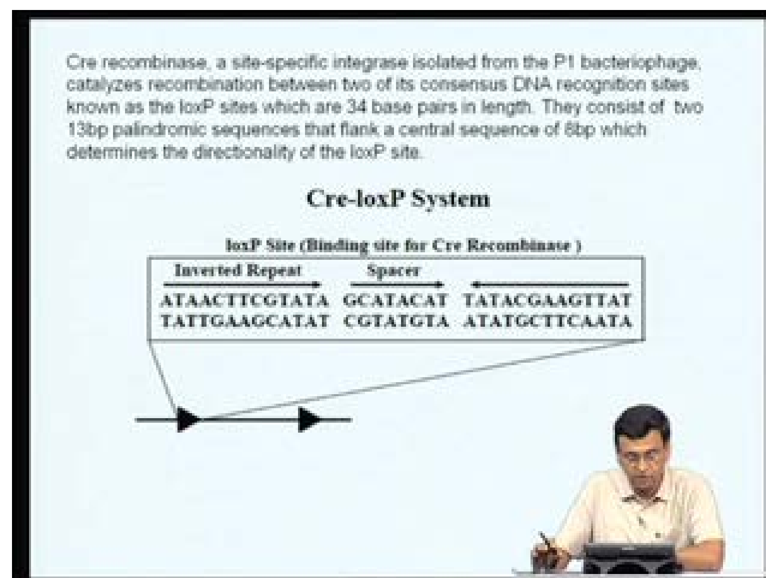

Now, Cre recombinase is a side specific integrase isolated from the P 1 bacteriophage. It catalyzes recombination between two of its consensus DNA recognition sites known as the loxPsites, which are 34 base pairs in length. Here is the loxP site. So, the loxP site, that is the site that is recognized by the Cre recombinase, consists of two 13 base pair palindromic sequences. I've shown here, these are palindromes you can see ATAACT T, ATAACTT. So, they are in an inverted fashion just like the steroid hormone response elements, which you have studied in earlier classes. So, the recognition sequence for this Cre recombinase known as the loxP site consists of an inverted repeat or a palindromic sequence separated by an 8 base pair sequence. Remember, the steroid hormone response elements. For example, if you take the glucocorticoid response element, which you have studied, also consists of an inverted repeat. AGAACDGDCC separated by a 3 base pair spacer so that two monomers of can bind to this sites and then activate transcription. In

the same way the recognition sequence for the Cre recombinases also consists of two inverted repeats separated by an 8 base pair spacer.

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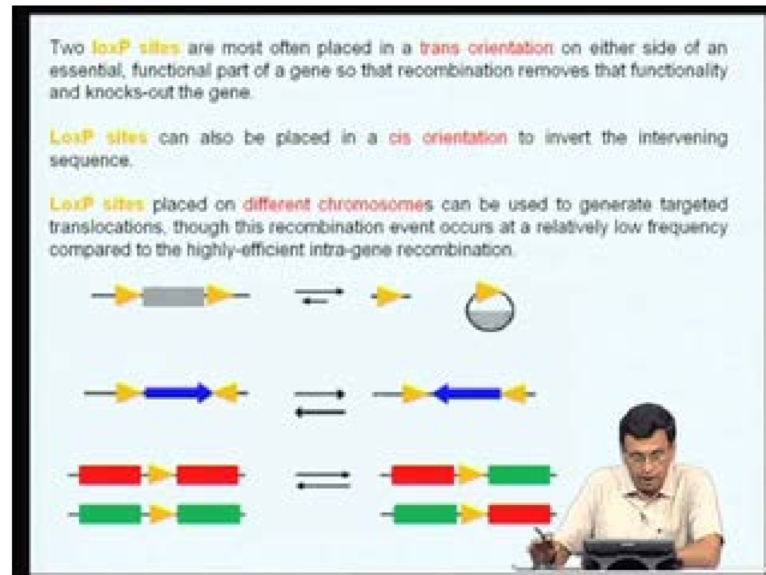

So, what happens when your Cre recombinases by into the sequence. Now, when this 2 loxP sites are placed in a trans orientation; that is when they are present as direct repeats, the functional recombination removes the functionality and knocks out whatever is there in between. So, anyhow 2 loxP sites in the same orientation, when Cre recombinases is expressed in such cells, now the Cre recombinases promotes homologous recombination and whatever region that is present between the two sites is removed and therefore, that region is lost.

So, if you introduce 2 loxP sites in this orientation in a gene, this region of that particular gene will be deleted and therefore, you get a nonfunctional gene. On the contrary, if the loxP sites are present a cis orientation; that is the opposite orientation, now when the Cre recombinase expression in such cells, it now inverts the gene sequence, the gene which is present direction now gets inverted on the opposite direction.

On the other hand, if you place the 2 loxP sites in two different chromosomes, then it can bring about chromosomal translocation. Thus, you can see. So, depending upon how the loxP sites are arranged whether they are arranged in a cis orientation, trans orientation or on two different chromosomes, you can get wide different types of genotypes. In one case the region between the loxP sites can be deleted or the region between the 2 loxP

can be inverted or chromosomal translocation can be brought about the by the Cre recombinases depending upon how this loxP sites are arranged in a particular genome.

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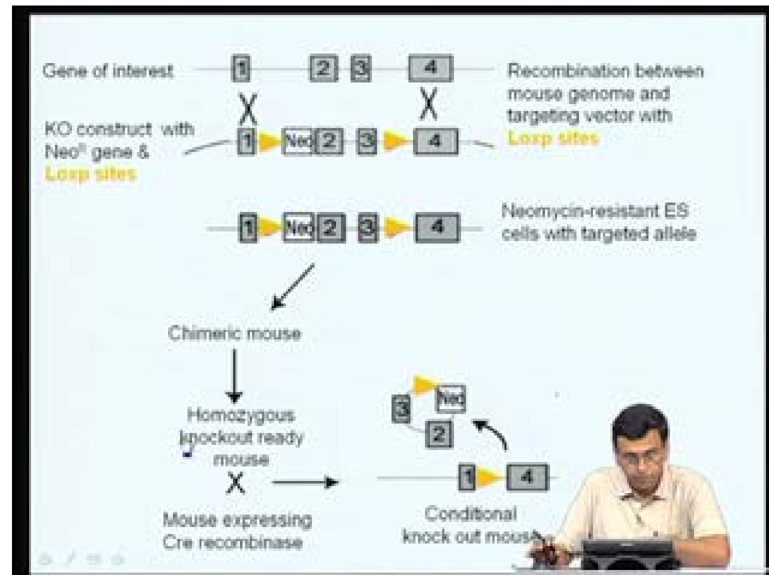

So, this is a cartoon, where I have actually cued what exactly happens. So, if you want to now knockout using a Cre recombinases, what you do: you introduce the 2 loxp sites along with a neomycin resistance marker usually in the intronic region of gene of your interest. Now, recombination now takes place between the mouse genome and this is the mouse genome containing the normal gene, this is your knockout construct where you have the 2 loxP sites and a neomycin resistance marker and now you get embryonic stem cells in which the neomycin expression cassette is incorporated and you have the loxP sites. Remember the gene is still normal, because all the axons are still there, but since they are expressing neomycin resistance, you can actually select for these cells. Now, take this ES cells, now introduce into the pseudo pregnant females and you generate a chimeric mouse then you breed this chimeric mouse and you get what is called as a homozygous mouse in which both the alleles now contains the loxP sites. These are called as knockout ready mice.

Remember the gene of your interest is normal because it still as all the axons. In addition, it is also expressing neomycin because the neomycin resistance cassette is in the intronic region. Now, if you now cross this homozygous knockout ready mouse with a mouse that is actually expressing Cre recombinases then Cre recombinases now will

remove the loxP sites between the two and now the axonic region is now removed, the two and three are removed now. So, now you have a nonfunctional gene.

So, now you can disrupt a gene. So, the mice are normal as long as Cre recombinase is not expressed, but once you breed this mouse with another mouse which is expressed in Cre recombinases, now Cre recombinases now promotes recombination and removes the region in between the 2 loxp sites and you now get a conditional knockout mouse. This is conditional because the key here is that you can now express the Cre recombinases in a specific manner. You can put that Cre recombinases under a tissue specific promoter or a promoter under a inducible promoter or in a developmental space specific promoter.

So, depending upon how you express Cre recombinases, target target disruption can be brought about only those situation. For example, you express the Cre recombinases only in select liver tissue, then only in liver tissue your gene will be disrupted or if you express the Cre recombinase in a inducer specific promoter, in your inducible promoter only when you add the inducer your gene will be disrupted. So, this is what is called as a conditional knockout.

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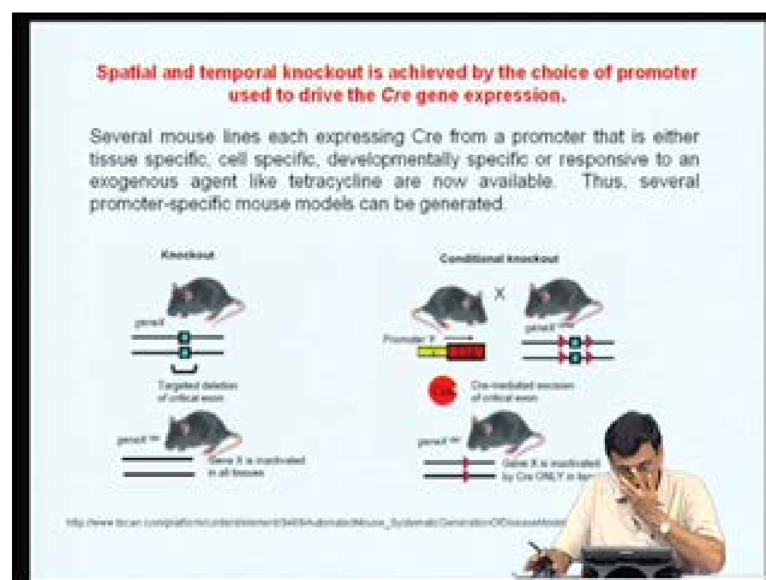

So, spatial and temporal knockout is achieved by the choice of the promoter used to drive the Cre gene expression. Several mouse lines expressing Cre from a promoter that is either tissue specific, cell specific, developmentally specific or responsive to an

exogenous agent like tetracycline are now available and thus, several promoters specific mouse models can be generated.

So, these are all available. For example, there is a consortium which actually generated all these. There are now mice conditional knockout mice models where the Cre recombinases is expressed under a tissue specific promoter and therefore, only in those genes when you now cross these mice with the mice containing the loxP sites, only in those tissues in this Cre recombinases is expressed, the knockout takes place. So, this is a difference between the conditional knockout and the regular knockout. In the regular knockout mice in all the cells of the body whereas in the conditional knockout only those tissues in which Cre recombinase is expressed, the gene will be knocked out. So, depending upon whether using cells plus tissue specific promoter or a development specific promoter or inducible promoter, you can you can bring about knock out only in a conditional manner

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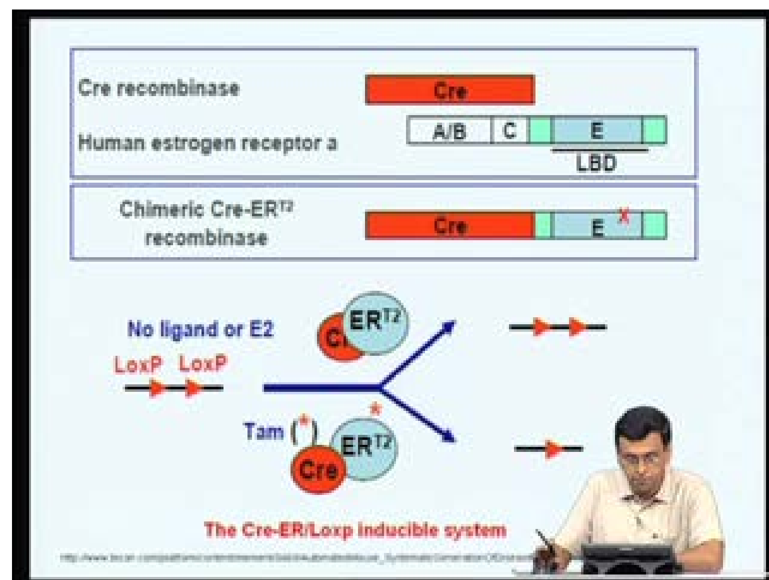

For example, instead of using Cre recombinase in tissue specific manner, we can also take the Cre gene, the recombinase gene and fuse it to the ligand binding domain of estrogen receptor.

So, when you now express, now develop those mice in which the Cre recombinase is bound to the estrogen receptor, now this Cre recombinase will be present in the cytoplasm when there is no hormone. The moment you inject hormone to such mice,

now the Cre recombinase goes inside the nucleus and brings about recombinase with the loxP sites and you get targeted disruption.

So, estrogen inducible knockout can be brought about. So, in these mice your target gene of interest will be knocked out in an estrogen or a tamoxifen dependent manner. So, either by using tissue specific or development specific or using this kind of inducible system, you can conditionally knockout your gene of interest in select tissues or whenever you want.

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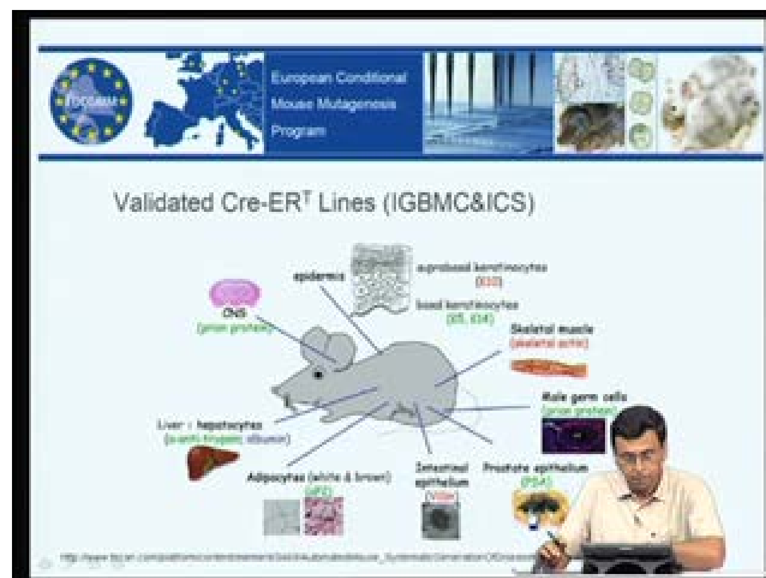

So, this is what called as a conditionable knockout. There are actually consortia now. For example, here is a European conditional mouse mutagenesis program, where they developed several such mouse lines which are expressing Cre under in a wide variety of tissue specific promoters. For example there are mice which express Cre only in the central nervous system, there are mice, which express only in the liver using the alpha antonio trips or albumin promoter, there are mice in which the Cre recombinases expressed only in adipocytes promoter or only in intestine epithelium or only prostate or male germ cells, skeletal muscle, keratinocytes. So, using this kind of tissue specific promoters, you can regulate the expression of the Cre recombinase and when you take such mice and cross with another mice in which you have your gene of interest in the 2 loxP sites, your gene will be disrupted only in those tissues in which Cre recombinase is expressed.

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So, this is how conditional knockout can be brought about. In addition to Cre recombinases, there are also several other recombinases. Here, I am not going to the details, rather we will come up with Flip recombinases what are called FrtDNA sites, there are also many other recombinase like Dre, phlc31 and so on and so forth. The mechanism is more or less similar to what I have expressed for Cre recombinases.

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So, you can see this development of this conditional knockout as revolutionized the way, we can study genes and understand their functions in animal models. I just want to

introduce this one more technology, the terminology. So far, I have been talking about knockout that is also what is called as a knocking.

Now, in contrast to knockout in which gene or part of a gene is deleted, in the case of knocking, the gene is replaced by mutant version of the same gene using homologous recombination. So, far we discussed about deleting, but in the case of knocking what you do, you actually instead of deleting that gene, you introduce a mutant version of gene.

This is very very useful, because if you want to actually study a disease model, for example, if you want to identify how a particular aminoacid mutation of a particular protein is affecting the function of the protein. So, instead of deleting they gene, you can actually introduce between the two loxP sites, a mutant form of the protein so that on recombination, wild type will be replaced by the mutant copy, now you can ask the question, what happens in the mutant protein is expressed, will it produced the same kind of a disease as appears in the disease model. So, knocking is also very very useful in understanding the function of certain proteins that lead to certain diseases.

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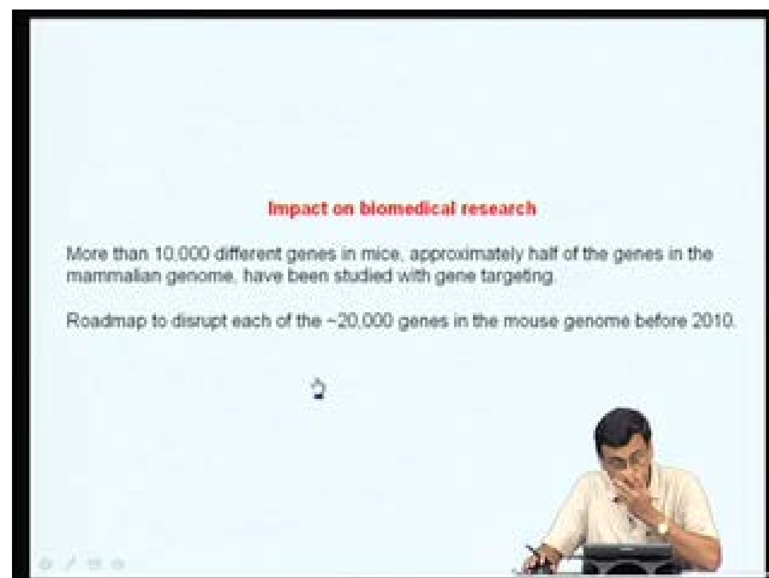

So, more than 10,000 different genes in mice and approximately half of the genes in the mammalian genome have been studied by using this kind of a knockout technology and there is a actually a roadmap to disrupt almost 20,000 genes in the mouse genome before the end of this year.

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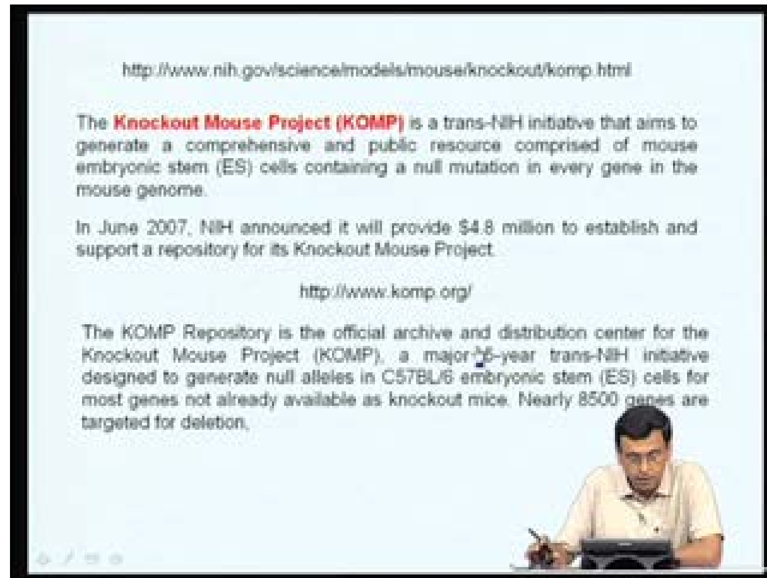

This is what is called as knockout mouse project(KOMP), which is actually funded by the National institute of Health in United States, almost 4.8 million dollars has been provided in June 2007 and if you can go to the website www.komp.org, you can get what all the progress that is being made in this knockout mouse project.

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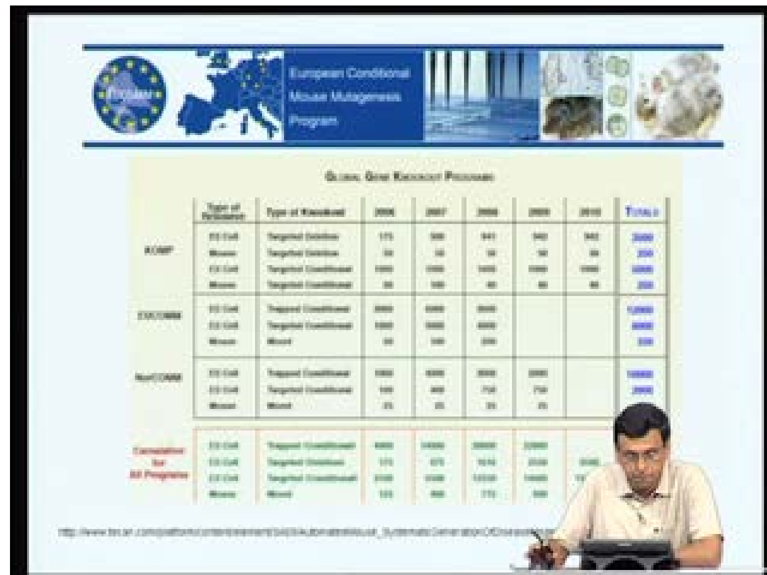

There is another example, the European conditional mutagenesis program. They have year wise target of how many genes has to be deleted in the mouse embryonic cells in a

conditional manner or in mixed manner and year wise they have given statistics on how they are going to disrupt genes one by one in the entire mouse genome.

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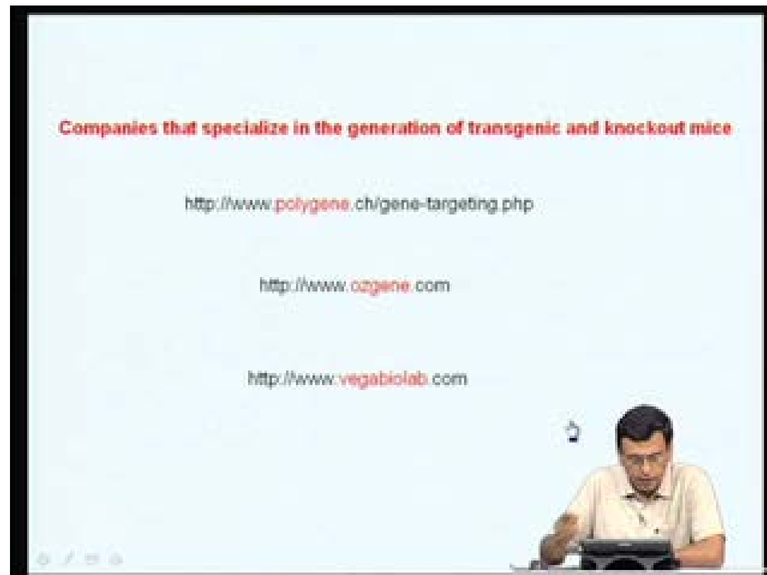

There are now a number of companies, which can generate transgenic or knockout mouse. You just pay them; they develop the transgenic mouse or knockout mouse for you. Polygene, Ozgene, Vegabiolab and so on and so forth.

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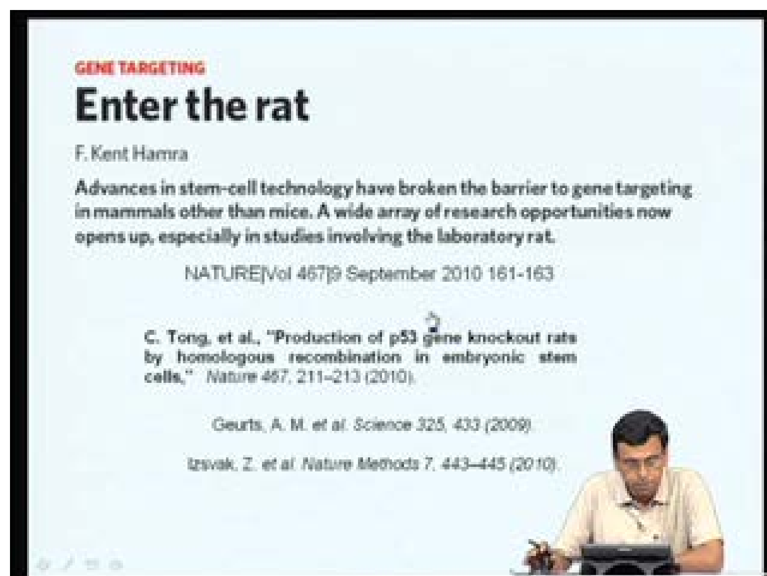

And more recently just this year, September 2010, the rat knockout. So, far knockout has been only for mice, now a knockout rats also been developed using homologous

recombination technology. For example, first paper appeared in the this year, production of p53 knockout rats by homologous recombination in embryonic stem cells, there are many other papers of similar nature

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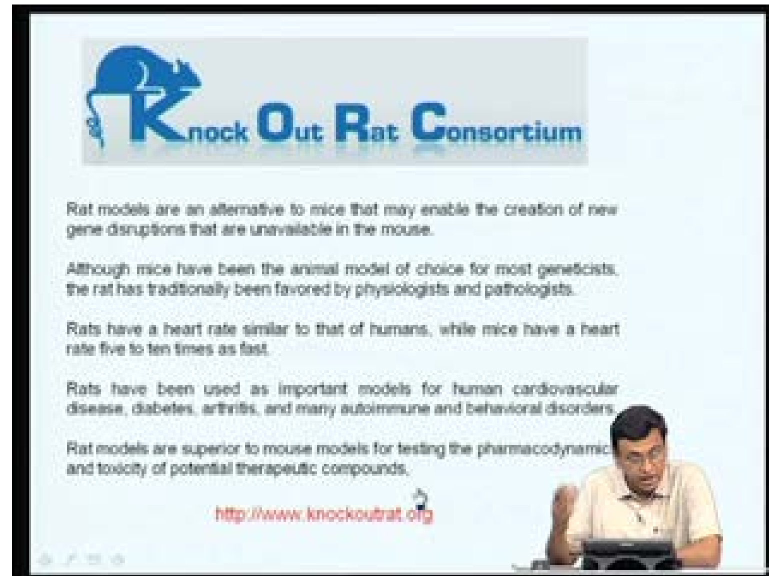

Why Rat?

Just like we have a Knock Out Mouse Consortium, now you have a Knock Out Rat Consortium, because many people think that rat is a better model especially for certain physiologists and pathologists, for certain diseases rat is a better model than a mouse. For example, rats have a heart rate similar to that of humans whereas, mice have a heart rate 5 to ten times faster. Similarly, rats have been used as important model for human cardio vascular diseases, diabetes arthritis. So, if you want to study some of the genes and diseases, you would prefer knockout those genes in rat rather than in mouse.

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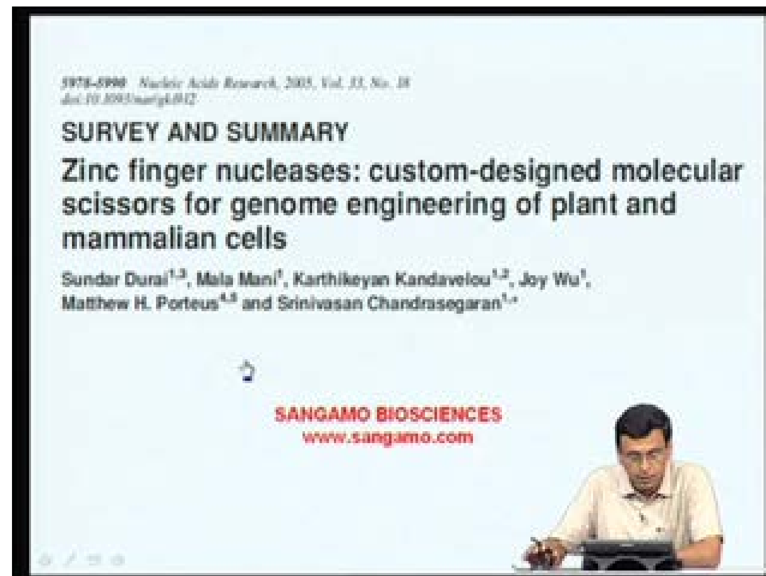

So, new technologies have been developed to knockout genes in rats. There is another exciting development that has taken place what are called as Zinc finger nucleuses that have been developed. As we studied in our earlier Lectures, Zinc finger transcription factors or sequence specific transcription factors they have zinc fingers which go and specifically bind to target sequences and activate transcription, example: hormone receptors. So, what they have done very cleverly, they take the DNA binding domain which consists of two zinc fingers and fuse it to the catalytic domain of the restriction enzyme called a flock wound restriction nucleus.

So, this fusion between a sequence specific transcription factor, sequence specific DNA binding domain of a transcription factor and the catalytic domain of the restriction creates what is called as side specific nucleus is called as zinc finger nucleases. When you want to express these nucleases, this nuclease goes and specifically cuts near the target sequence.

So, you can specifically introduce double strand breaks depending upon the sequence which is zinc finger recognizes and once introduces the double standard rake by what is called non homologous end joining, these ends are repaired and as a result, you introduce extra bases and you lead to shift in the open raining frame and you disrupt the function of the gene.

This was again has revolutionized, there is actually a company called Sangamo biosciences, you can go to the company website and get more and how the Zinc finger nucleases are revolutionizing the knockout technology.

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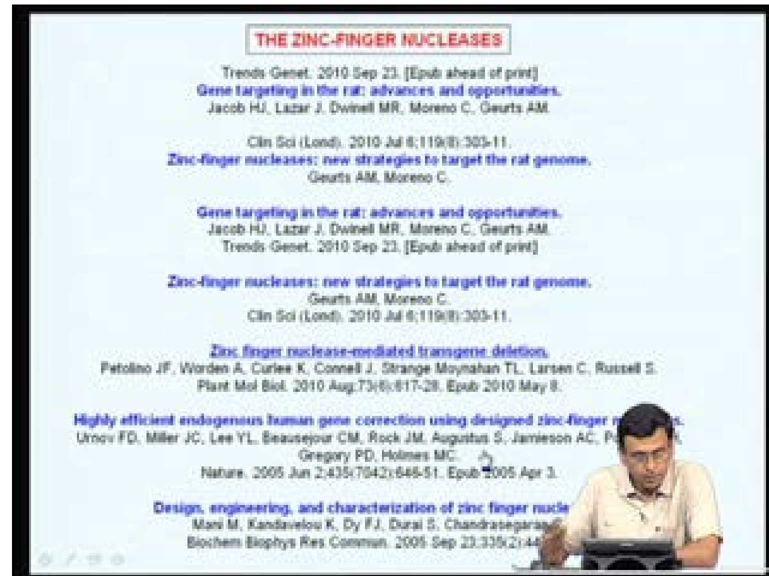

There are number of papers on Zinc finger nucleases. Due to positive of time, I am not going to discuss. One can go through a number of review articles and number of research articles that are developed how this Zinc finger nucleases have been used for knocking out genes across the animal and plant kingdom.

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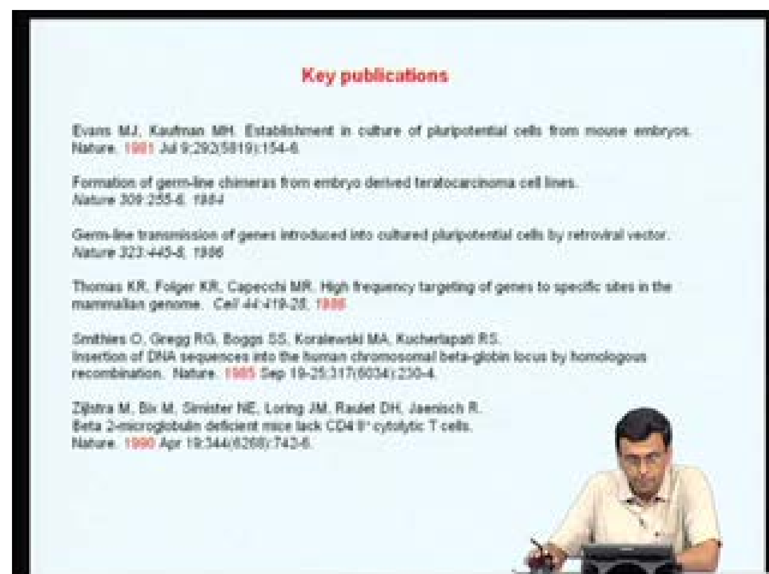

I am also listing a number of key publications especially from the Evans, Coffman and Capecchi's lab, very very important papers that actually revolutionized and actually lead to the award of the Nobel Prize for the researchers. Some of the review articles are listed here and I am also listing the Nobel Lectures of Mario capecchi, you can just cut and paste this website, the entire pdf articles gives you how he went on to develop this kind of knockout technology, the noble lecture of Martin Evans, you can just click on this website and you can get the article and again Nobel lecture of Olivier Smithies, very useful how they went about and developed this technology.

Also very very important websites, which gives you lot more information about gene targeting and gene knockouts.

I think I will stop here and hope I have convinced you that gene knockout has revolutionized the way research is done in biology medicine has given wonderful information about the function of various genes and has tremendous implications in the biomedical research. Thank you.