Human Molecular Genetics
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Module - 03
Lecture – 11
Animal Models for Human Diseases

Welcome to the third lecture of the third week of this course human molecular genetics. In the previous two lectures we have looked at two, we have looked at two distinct group of mutations. One you call as a gain of function mutation, other one is a loss of function mutation and in the gain of function mutation, we ended with a note that it is often, it is difficult to understand how a given mutation changes the function of a protein, in terms of the function of the protein in the cell, tissue or organism, right? It becomes very challenging, because you do not know the function of the protein. So, then if you know the function of the protein, then you can ask how a mutation altered the function of the protein. So, for such kind of approaches, it is going to be very extremely difficult to study humans.

So, we need models. We need models, meaning you have to use some other organism and create the condition, wherein the organism also has got a defect very similar to what you see in human and then ask, now the question, what goes wrong in this organism and study what are the events, chronological events, which happened first, which happened second and so on and then try to understand this is the physiological pathway and this is what it is abnormal and this is where the genes function and so on. So, that is where the model system for human disorders becomes very, very important. So, that is what put on the slide here.

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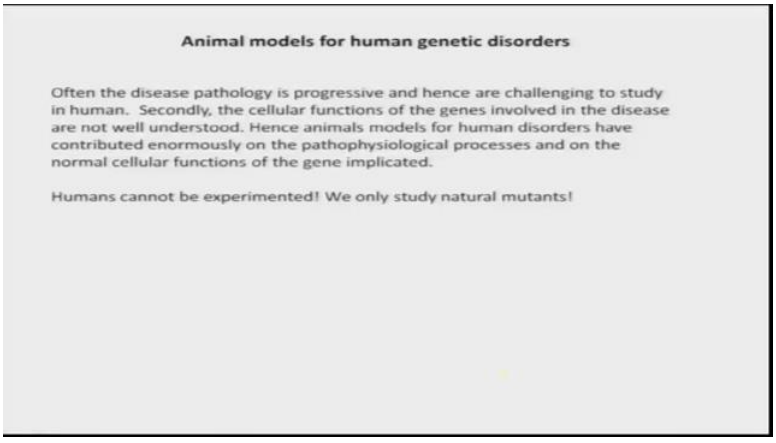

Often the disease pathology, pathology is progressive, meaning to begin with you are normal, like we have seen Huntington's disease. You know, you have different generation to begin, to begin with the individual is normal. At certain age you have symptoms. It becomes severe and severe and therefore it is very challenging to study it in human, because to begin with, we do not know that individual is going to have the disease or not.

Secondly, even if you know, how are you going to really look at what happens inside the human body. You cannot do any experiment with the humans, which is ethically not allowed and second for many of the genes that we know that they are defective and results in the disease, we do not know the function; that is something that we have already discussed. So we need to understand. So, here we use animal models for the human disorders and such kind of study using the animals models have contributed enormously to understand the pathophysiology; what goes wrong and what is the function of the protein and with that, we also try to go for some therapeutic attempts, can we, you know, reverse the phenotype, right and that can have a medical implications in human.

So, really, you know the animals really help us to understand the biological function of the genes and their protein and how defects results in the disease condition. So, simply because humans cannot be experimented, we only look at individuals that have had normal mutation, meaning natural mutation and we try to study them and try to understand as much as possible. So, what are model systems?

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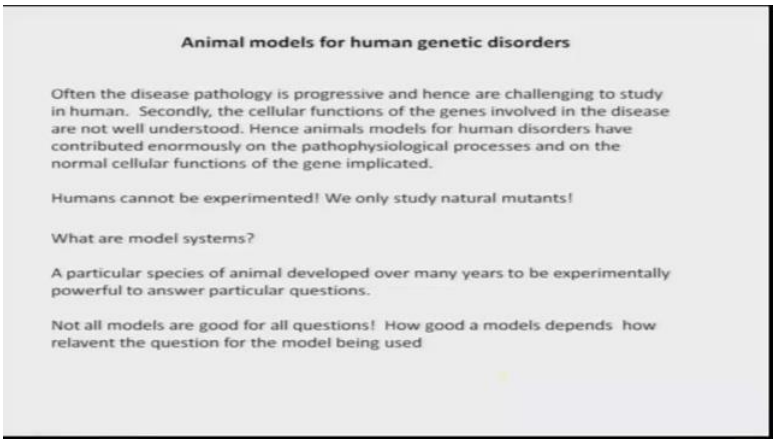

So, the model systems are, you know, when you talk about model animals, these are particular species of animal developed over many years, because you have to choose and you

have to characterise, understand how to you can, you know, maintaining in the lab. You cannot go and take a lab wild animal and then study on that, because they are like humans. There are lot of variations and whatever effect that you see at the end of any experiment, in a given set of individuals or organism, it is going to be very difficult to infer like humans, right. Therefore, you need to have lab build animals. So, that takes years to come up with certain system and these are experimentally powerful, very powerful to answer some of the very important questions with regard to the disease biology, disease pathology or even understanding how a gene functions.

Now, what is important is that not all models are good for all questions. The models are as good as the question. So, you need to know what question you have to ask with what model system. We are talking about cognitive ability, let us say. We were talking about how the brain functions. Now, these kind of questions you cannot go and ask using, you know, a cognitive function very, say unique to mammals or unique to primates, because the way the brain is formed and you cannot go and question it using some other model system, which is not even vertebrates. It becomes difficult, because the way the brain is organised is different and so on. So, only to know what question you are asking, accordingly you have to identify suitable model.

I am listing here some of the model system. People popularly use it for many biological questions.

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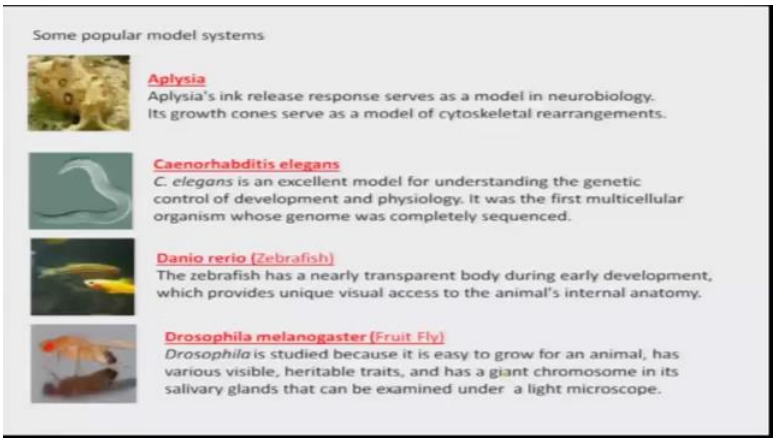

For example, *Aplysia*, this is a mollusc, a soft bodied animal on, which is a kind of a model which really helped us to understand the neurophysiology. So, all the information that we know about how the neurons functions, for example the *Aplysia's* ink release response, right, because there is a signal that releases ink; all these information have come from this models; very very powerful in neurophysiology. You have another model which is the *C elegans*, a nematode, a worm, which is, you know, very good model for understanding the development and physiology and as we know many of the model systems have led to discoveries that were awarded Nobel Prize. Every model we can count on that, they contributed to the Nobel Prize. That is the significance of their contribution.

You have Zebrafish again, which is a vertebrate. They have transparent body and now, you know, this is a very very powerful model system to understand the metabolic process. People are trying to model them for diabetics and obesity and many, many different conditions and you have the fly, fruit fly, *Drosophila melanogaster*. Again, it is a very very powerful species for genetic manipulation, for developmental biology, they are very very good and in all these model that are there in the, you know, except the *Aplysia*, you can engineer; we can change the genome. You can, you can make the change in the genome the way you like. So, you can model them beautifully.

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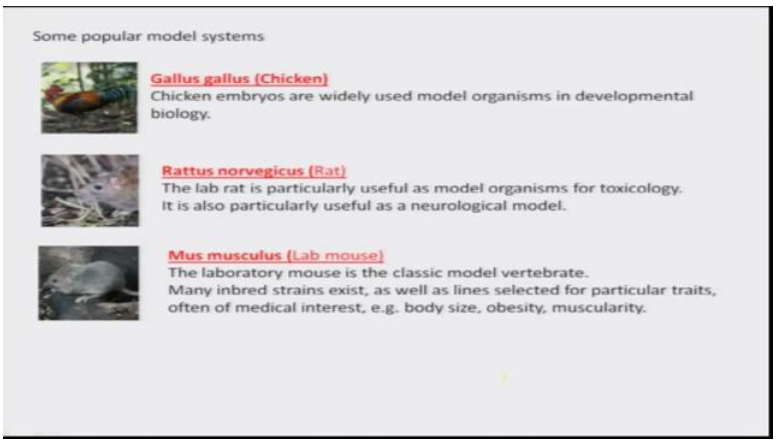

The model that belong to the vertebrates that are very close to the humans are the chicken, for example, *Gallus gallus*. These embryos are very very good for developmental biology. Majority of our understanding with regard to how the embryo grow, what are the signals that contribute to the embryonic growth, have come from studies from chicken and then you have rat, the lab rats and they are particularly useful as model system for many of the neurophysiology, toxicology, for example any of the drug safety or all these things, people use this model system to see that the compound is good for human applications. So, they are very, very good. Especially for neurological studies, rat has been the most popular model; because the brain size is bigger we can investigate different regions of the brain.

But, when it comes to human genetics, you know, modelling the human disorders, the most forward model is *Mus musculus* or the lab mouse. You must have seen the small white mouse that people use in the lab studies. These are cute animals, easy to handle, they do not really bite you and, and important thing is that you can change the genome. You can change the genome the way you want. Therefore, you can model it for a given human disease. That is very, very powerful and majority of the human, human genetic disorders are modelled using the mouse. So, we are going to look into some of the approaches people use to model mouse to understand human genetic disorders. So, we will take, you know, maybe two or three approaches; we will not get into all the approaches. We will show some examples as to how that has been used to, to understand the disease biology.

Let us look into two distinct approach people use when they use mouse as a model system. One is called as transgenic animals, meaning you are putting some extra copy of DNA and this DNA need not be from mouse, it could be from the human or it could be some engineered DNA sequence, you can put it inside the mouse genome and let that foreign DNA express and give a phenotype that you want the animal to develop.

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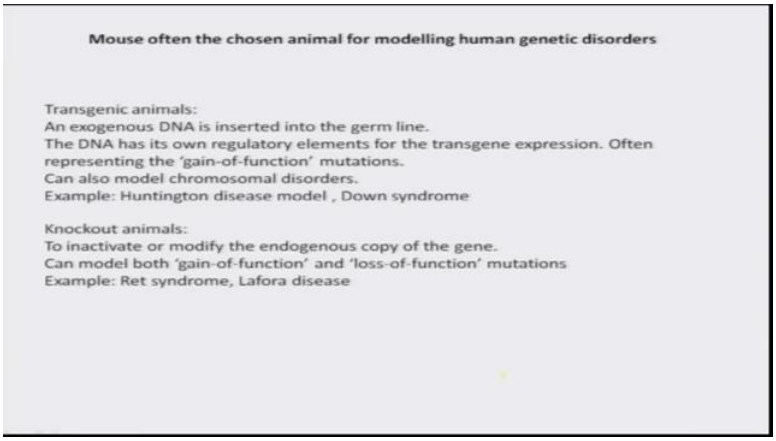

So, these are, you know, as I told you, exogenous DNA inserted into the germ line. What do you mean by germ line? These are transmitted via the germ cells. So, you can maintain a line, you can breed them, you can maintain the line and the DNA has its own regulatory elements. So, when you put a DNA, it has its own regulatory elements, meaning it has its own promoter. So, it would tell you where it should express, when it should express, what should be the quantity of expression and so on and that drives the expression. Often such models, that is transgenic, when you put an extra copy of the DNA inside the mouse genome, they represent what is called as gain of function mutation. Remember, we discussed a gain of function mutation, like for example Huntington's disease. You have the CAG repeat, when the repeat length goes beyond, for example 40, 50 60 and so on, you end up having disease. Now, you can insert a gene sequence having expanded polyglutamine and this is a gain of function. If that gene is expressed and if a protein is made, you are going to have the symptoms, at least you can expect that to the animal to have that.

You cannot model loss of function using transgenic. It is not impossible, but it is difficult. Reason is, for any gene that we talk about in the human, you have a homologous gene in the genome. So, even if you put an extra copy, which is representing a loss of function mutation, it is not abolishing or it is not competing with the, the normal gene that the mouse has. So, it is not going to really express that; so, that is difficult, right? So, normally we are going to have extra DNA, like we can model beautifully Huntington's disease. You can put a gene that has got expanded polyglutamine coding region or you can even model, for example conditions like, you know, Down syndrome, where you have, you know, three copies of certain genes. So, you can pick those genes that are three in copy in the human and we can make a transgenic animal in the mouse and that may develop a Down syndrome like

phenotype and you can study why, you know, your Down syndrome, when you have three copies of certain gene sequence, you have mental retardation. So, you can model the animal and see, is there anything can be done to rescue the phenotype. So, that is about transgenic.

So, you have the other group, which is called the knockout which is the most powerful approach you have. You have another approach that has come recently called,, cre-lox gene editing; we will not get into that, but this is something which is well established, you have it in many other textbooks. So, we will restrict our discussion to the knockout. So, what is that knockout? So, basically we try to inactivate or modify an endogenous copy of the gene. So, the mouse has got its own gene, so we are going to remove the gene or its function or alter the function of the gene to mimic what you see in the human. So, you do not put any extra copy of DNA here, copy of gene here, but you change, certain changes you bring about in the genome of the animal that results in the condition.

So, that you can model both gain of function and loss of function mutation here. For example, if you talk about a missense mutation that could result in a gain of function or a loss of function. You can by using this approach, you can even create such kind of a certain differences in the genome. So, now a missense mutation could be loss of function, you can model it or it could be a gain of function, again you can model it. So, that is the approach of knockout animal, a variant of which is called as the knock-in, which is used for it, this kind of application. But we will be talking about a knockout, which is generally for loss of function effect.

Let us see transgenics. How do you put an extra copy of DNA inside the mouse and make it express? So, what do you need?

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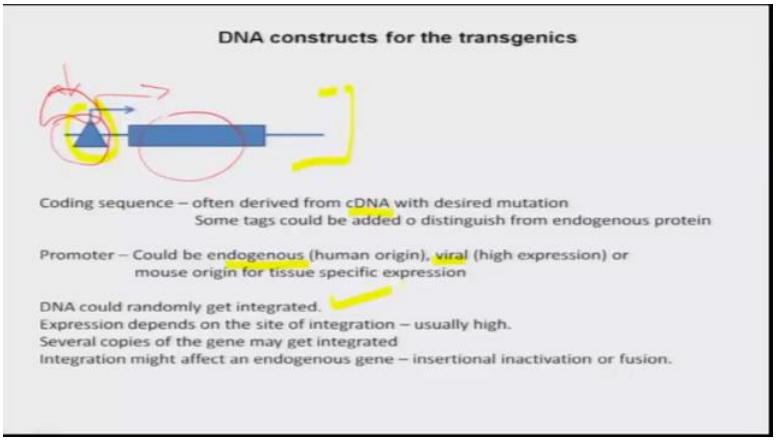

You start with what is called as construct. The construct is given here. So, what is this construct? This construct has got an element which you call as a promoter. So, what is promoter? This is an element which informs the DNA or the gene as to when it should express, where it should express. So, you may have a DNA binding protein, which is a transcription factor, comes and binds here. As long as the transcription factor is there, this gene will be turned on, so you will have the protein being made. So, what do you put here? So, this is the coding sequence. So, often the coding sequence is derived from cDNA, because your gene in humans could run into several kilo bases, hundreds of kilo bases. One of the genes can even go up to one mega base. So, you have multiple exons, ten of them, twenty of them, spreading large segment. So, that, you know, such big DNA to extract and put inside, is going to be extremely difficult.

So, what people do is, they take the cDNA, which is the processed DNA, meaning it has all the exons joined together, so it has the coding sequence, you fuse it with a desired promoter and make a construct. So, the cDNA would express, it will make the same protein, otherwise the endogenous gene would make, right? What are the promoters? The promoters could be the endogenous, meaning you use the human promoter. The gene that you got from human, obtained from the human DNA, you can use it with its own promoter, it may express, right, if it has got all the elements or you can use a viral promoter. For example, the virus has got its own promoter, which would function, because it uses very few transcription factors that we make. So, they are able to successfully make their own protein. So, we can use those promoter, fuse to whatever sequence that we want and then make a construct. So, now they will express very high or we can usea promoter of a gene of mouse origin to drive your transgenic expression, only in certain tissues.

For example, I want my, for example the Huntington protein which is having the expanded polyglutamine structures to express only in the neuron, so what I will do? So, I will go for any gene whose expression I know is restricted to the neuron, take that gene promoter, fuse it with for example my cDNA coding for the Huntington. So, what would happen? When you make a transgenic, although this particular gene copy would be present in all the cell, it would express only in the neuron, because it has got a promoter of a gene that express only in the promoter. So, this is a way I will be able to control the expression

So, DNA, what are the, how the DNA gets integrated? So, when you inject the DNA, we will come to that, how does it, how it is done, so it may get integrated anywhere. You do not really tell that this is a place in the genome you have to go. So, it is random. It goes and gets integrated anywhere. Now, the expression of the gene copy that you put in depends on the site of the integration. There could be regions, for example centromere region, where the spindle comes and binds and pull the chromosome apart during cell division and these regions are not gene rich. Normally they are very condensed. If your DNA gets in there, it may not express or it could be a region which has got many genes. So, if it gets integrated, it may express. While doing so, it may also disrupt the function of a gene that is located there.

For example it can go and land in a region where there is a gene, the transgene is expressed, but your normal gene that was present there could be affected. So, you know, you have to look at several lines there. So, that is what you inferred, the last point is integration might affect an endogenous gene. So, you know, it happens. So, you have to look at several animals and select one that is really good. So, that is a disadvantage and advantage of transgenics.

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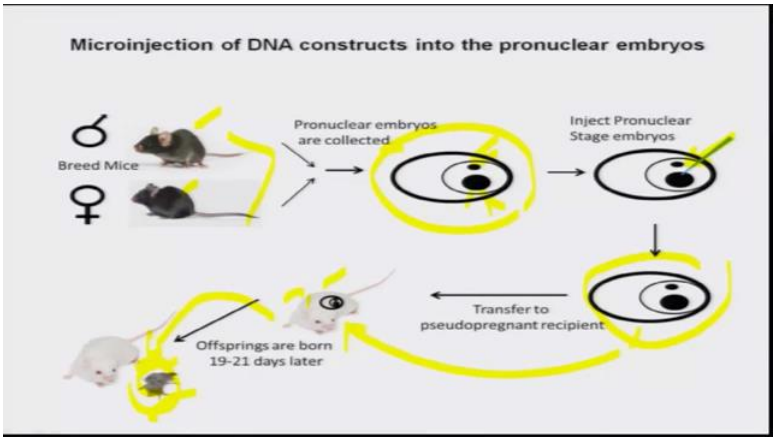

Let us see how you do it. So, what you do is that you, cross a male and female to get embryos. These are embryos in which you have, still they are single cell, you know, zygote. You have nucleus from the egg and the nucleus from the sperm yet to fuse. So, that is the stage at which you integrate the DNA that you want. So, you basically, you set the crosses and then you anesthetize the female and then take out all their embryo that are just fertilized, but yet the nucleus, the male and female nuclei yet to fuse and that is the stage in which we use a very thin capillary kind of needles to introduce your DNA. It pretty much inject the DNA inside the nuclei, right. So, when you inject, allow it to grow for sometime.

In this process, when the male and female nuclei fuse together there will be opening up of the genome and during this process, this DNA may get integrated somewhere and then, you take the embryo, put into what is called as the foster mother; the mother is not the biological mother for the embryo, but you condition her and put the embryo inside and allow her to deliver the pups and you have the animal that are born and some of them would carry their DNA in every part of the body including their germ cells. If it so happens that some of the germ cells of these animals have these transgene, if we cross them, then they are going to go to the next generation and you can characterise.

So, how do you know these are derived from this embryo, not from this mother? Because, you can use the fur colour; for example, white is the foster mother, her fur colour is white, but the embryos are derived from a brown or black mice and if the pups are black or brown in colour, that they are derived from this embryo, because you know this coat colour is dominant over white and, and they are no white here and that means that they are not from this foster mother. So, this is the way you characterise the transgenic animals. So, that is how

you use the foster mother to create animals that, that harbour the transgenic genes that might express some foreign protein.

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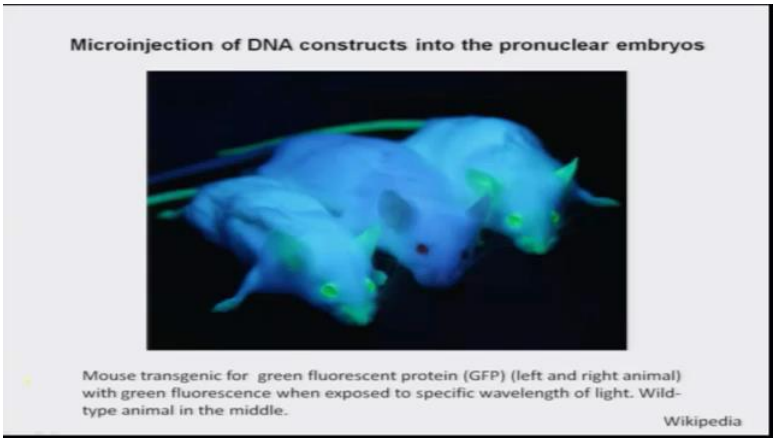

So, what you are going to see is an example of how a transgenic can be a powerful tool to express a protein that are not native to the animal. So, what is shown here, is three animals, born to a faster mother and the one on the extreme left and right are the, you know, so the one on the left and right are the animals that express a foreign protein. This protein is called as green fluorescent protein or GFP. So, what is the property of the protein? So, when you excite these proteins with certain wavelength of light, they fluoresce. They give you a fluorescent colour and that is what is shown here.

So, you can see that the, the animal on the left and right they have green colour in their ear, in their tail, in their nose and then eyes, you see that it is a glittering green colour, whereas animal that is in the middle is not showing such green fluorescence, because it does not have this particular gene. So, it clearly tells you the power by which you are able to express a protein,which is native protein of a jelly fish, a marine species present in sea water that gene you have obtained, you have used that, engineered it and you are expressing it in animal and it express the protein which is otherwise not present in your cell. So, that is the power of the transgenic animals.

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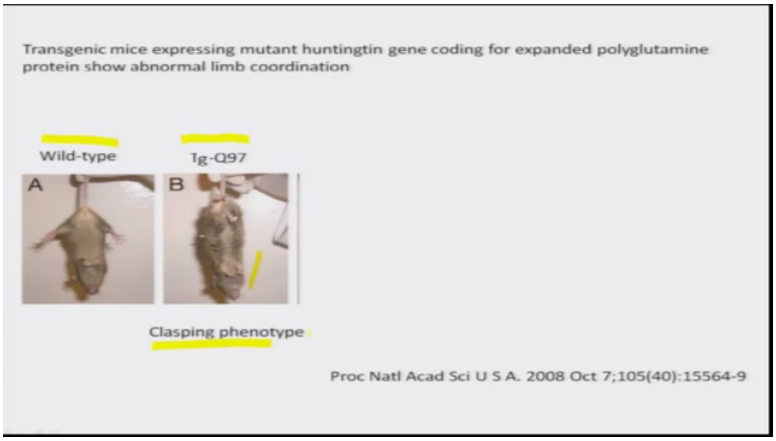

So, you can, for example express Huntington protein, you know, with expanded polyglutamine and see whether it develops disease like Huntington disease and that is what is shown here. So, here what we are seeing is a transgenic mice, wherein we have added extra copies of the gene having expanded polyglutamine repeat is now expressed and then, you are comparing it with a wild type which is a normal animal and this is the animal that expresses the mutant Huntington protein. So, what you can see here is that, when you lift the animal using a tail of the animal, then the animal normally try to explore what is around, try to escape. But, but when they have a very very poor coordination of their hands or limbs and you know, what you call hands and legs, then they try to clasp. This is called as a clasping phenotype. So, they, when they do not have the motor coordination ability, they clasp like this. This is one of the classic phenotype of motor deficit in motor coordination. So, this is an animal that develops Huntington phenotype.

Now, why, you know, you should develop animals? So, it really helps us to understand what happens at the level of cell or neuron and why you have the disease. So, what is known now is that, by studying such kind of animal models the polyglutamine protein form aggregates and these aggregates recruit a large number of proteins that are otherwise required for the normal function of, proteins, cell. As a result, the neurons do not function properly and they slowly die. If you are able to clear this aggregates, then the neurons function better.

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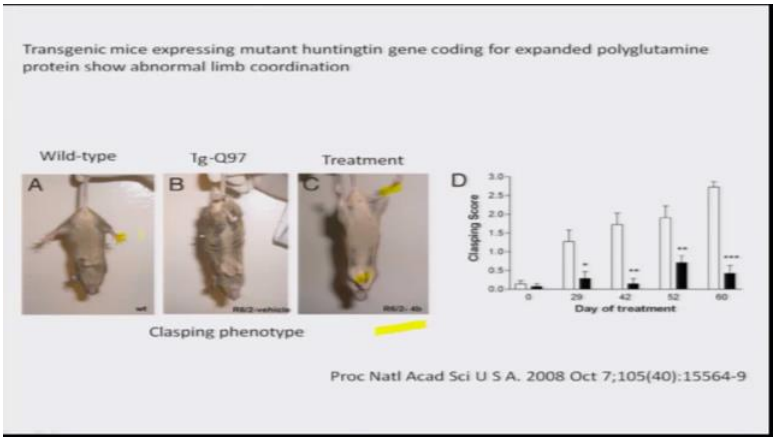

So, what is known is that, is there a way by which I can help the neuron to overcome such kind of toxicity, stress and people have used various ways and this is one such lead molecule a group has identified. When they have treated the animals that are expressing again the mutant Huntington and you can see that they are able to behave the way just like the wild type behaves. So, this animal model helps us to come up with some therapy, you know, at least in principle, a kind of a proof of concept, approach, using the animals we can screen and then you will be able to take it to the human application. So, that is where the model animals really help us. They understand, we understand the, how a defective protein results in the phenotype and we can also come up with certain ways to eliminate the abnormal function, a gain of function protein has offered to the cell, wherein it is expressed. So, that is the power of these animals.

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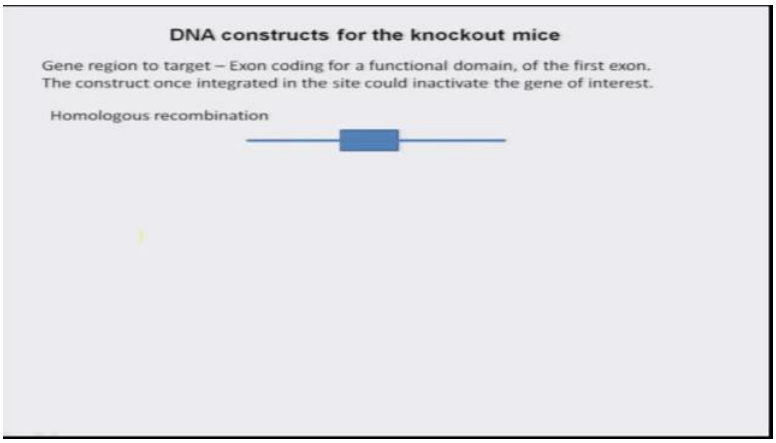

Now, you are going to go to the next approach what you call as knockout mice, wherein we generally remove the function of a gene that you are looking at, normally to generate a

condition which is a loss of function, right? So, let us look into how do you make the construct for the knockout, for generating knockout mice? So, the way you are able to destruct a gene function in the knockout gene approach is by homologous recombination. What do you mean by homologous recombination? So, you identify a region of a gene which you want to destruct or you know, delete or inactivate. So, which region you would identify for such kind of deletion? So, it depends on the gene that you are looking at, the information that you have about the gene.

One safe side would be to delete the first exon, because the first exon would have the promoter sequence. So, if you delete the promoter sequence along with first exon, the gene is less likely to be expressed. So, you pretty much brought in the loss of function mutation. If you are not sure about which is the first exon, you can go about deleting, you know, another exon. The reason being, at times certain genes could have more than one first exon, different promoters. Even if you delete one, the other promoter may still function. Therefore, you may not see the desired phenotype.

Therefore, you want to go for certain exons, which code for the functional domain of the protein, let us say a transcription factor. So, what is the functional domain for that particular protein? It could be the DNA binding domain, because a transcription factor needs to bind to the DNA for activating the gene for its expression. So, if you know the exon, which exon codes for the DNA binding domain, you can decide to delete that particular exon. So, let us have a look at here. How do you really do this?

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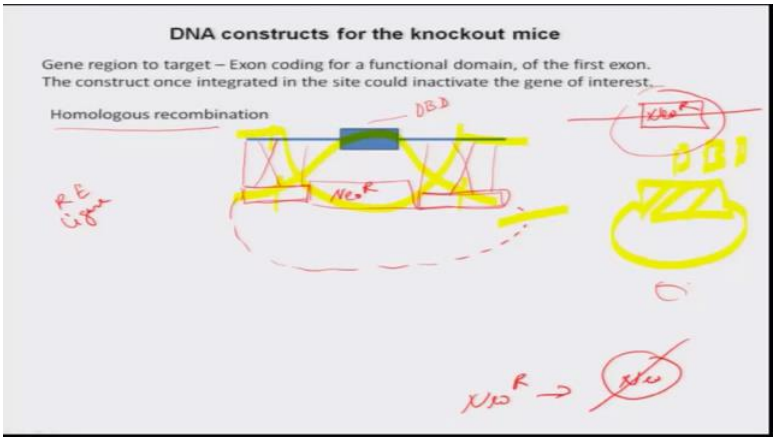

So, here in this particular cartoon that we are seeing is a exon, which codes for, let us say, DNA binding domain, so I have decided that I would knockout or I would delete this particular exon. So, what I would do? So, I will go for the so called genomic DNA library for this particular strain of mouse that where I am using and identify the fragment that represent this region of the gene. Likewise, identify the fragment that represents this region of the gene, right? How do I, you know, cut these fragments? So, I use those enzymes that we described, that is restriction enzymes, which would cut the DNA at desired region and you are going to use another protein which you call as ligase which joins the DNA fragments. So, we use this to identify and clone a small region that represents these two segments of the, the gene.

What I do? I fuse these two segments with another segment which is not from the mouse origin. It is a synthetic construct which codes for a protein called neomycin resistance protein. So, what is the function of this neomycin resistance protein? So, this protein codes for a protein which is able to degrade the antibiotic called neomycin. So, in the presence of neomycin resistance protein, the cell can grow, even when you have neomycin there. So, in the absence of this protein the cell cannot grow, it will die. So, why this is important, we will come back to this little later.

So, what you have done is that this whole thing is put in a plasmid. You remember, we discussed that these are circular DNA, which is present in E-coli and so on, which is used for the cloning purpose. So, you have used this and what you do is, you use a restriction enzyme, cut here, you make it linear DNA and use it for transcription. What is that? You introduce the DNA inside the mammalian cells derived from the mouse. What cell type, we will come back little later. So, you can introduce that. So, this DNA getting there and once it gets in, you

expect a process called as homologous recombination, something that is shown here. So, you may expect a homologous recombination happening here and here between the homologous sequence, just the way we discussed in meiosis recombination.

In somatic cells, although very very rarely such events happen. So, if that happens what you expect? You expect, you know, if there is a double strand break here, double strand break here, double strand break here, double strand break here; so, this DNA would get joined with this and this and you will have this DNA getting joined with this and this. As a result, you may have a plasmid having the exon of the gene that is coding for the DNA binding domain, whereas your gene now has got the neomycin. So, your gene would look like this. Now, in that exonic region, you have the coding region for neomycin resistance. So, once it gets in, you grow the cells in the presence of neomycin.

Now, all the cells that carries this DNA fragment would survive in the neomycin. They will multiply, grow. Those who do not have such neomycin resistance gene, even if they contain it in the plasmid like this, it will be degraded very fast, within couple of cycle and then the cells would die, because they cannot survive in the presence of neomycin. But what is, this is called as positive selection. You select for a particular phenotype that is resistance to neomycin. But, this kind of integration that is the integration in exactly the region that you want that to happen is a rare phenomenon, right? It can also happen by random integration.

For example, the DNA gets integrated somewhere in the genome and still the neomycin resistance would be there. The cells would code for that protein and therefore, they would grow. So, how do you know whether the cells that survive under neomycin resistance would have the same kind of integration that you desired? So, what people do is they go for what is called as a double selection.

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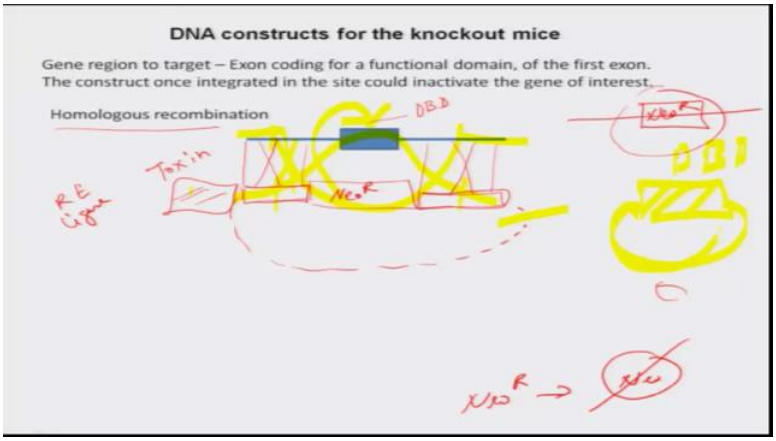

So, when you make a construct, for example this region you add another coding region. Now, this coding region codes for a protein which could be a toxin, meaning if this protein is expressed, the cell would die. So, if that DNA, like, though having the toxin and the neomycin resistance gets integrated anywhere in the genome, then that would result in the death of the cell, because the toxin is expressed and therefore, the cell would die. But, if the integration took place because of what is called as the homologous recombination, happens here, now the neomycin would replace this DNA binding domain coding exon, but in that process the toxin will not be getting integrated or the coding sequence for the toxin will not be getting integrated into this genome.

As a result, they would not die because of toxin, because that is not expressed, because that is excluded that remains in the plasmid and gets degraded, right. So, this is called as a double selection. You select for a phenotype that is survival and with neomycin and you also select for another phenotype that is toxic, you know, if it gets integrated with the genome, then, you know, which is not a homologous recombination process this cell would be eliminated. So, in this process you are able to get groups of cells, wherein such kind of exchange, the exchange of the DNA binding domain coding exon with neomycin resistance could happen and, and that would have an allele, in which the exon is deleted. So, that is how you generate cells in which you are able to create the mutation the way you want. So, that is the first step.

So, what do you do next? You have to create the animal. So, how do you create the animal having this mutation? So, it all depends on the cell in which you are making such kind of change in the genome.

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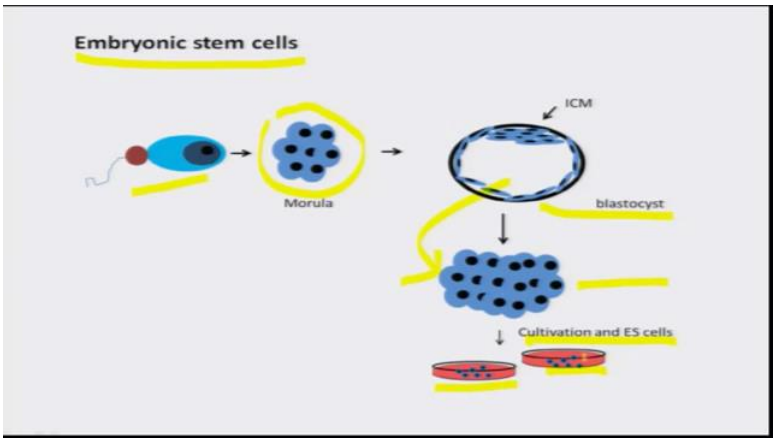

So, normally for generating knockout mice people use what is called as embryonic stem cells. What are they? Because these are called as stem cells, because they have the potential to develop and differentiate into any cell tissue type in your body. Because these are embryos, every cell has the equal potential to develop any part of your body. So, that is where you derive. So, in this case what happens is that the egg and the sperm fertilize and you have a stage of embryo called as morula and then you have a blastocyst stage called as blastocyst. The blastocyst stage you harvest the cells and cultivate them in the culture dish and which you call as embryonic stem cells, because they have all the potential. Now, you can culture them in this, in the lab and then, you transfect them with the construct that we discussed, allow such recombination to take place and then you identify cells that, wherein you have had such recombination took place. So, you have created the mutation, right?

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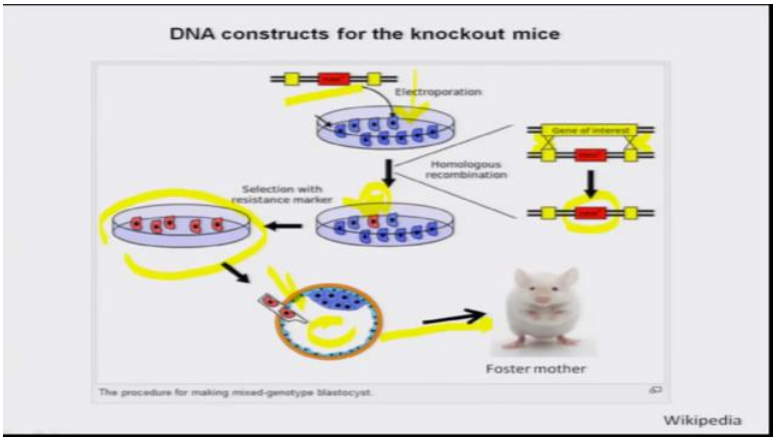

So, once you have that mutations created, you can generate animals. How do you do it? That is what shown here. So, as we have shown here, we have a construct in which you have a neomycin gene. You have transfected using a process; here it is shown as electroporation, there are more than one method, wherein you expect, you know, this kind of homologous recombination, which replaces the exon of a gene with neomycin resistance gene. That could happen in one of the cells that you have seen here, because of the selection, either because of or both because of the antibiotic resistance and because toxin not getting integrated into the genome, you would allow only those cells in which such integration took place and they are preferentially grown in a plate.

So, you would have now all the cells having the kind of change that you expected in the genome. Once you have grown enough, then you take few cells, these embryonic stem cells, wherein you have confirmed that you have altered the genome and inject inside the same embryonic stage, if you remember, we have just discussed about blastocyst. This is a stage from which you have taken the embryonic stem cells. Now, we can put their cells back into the same stage of embryo, using a fine needle. So, still you have some space here. Normally people add some 12, 13, 15 cells inside and once allow, then what happens, these cells get integrated inside the embryo, they grow and form different organs. Eventually that embryo is put into a foster mother, the mother who is not biological mother, but put inside a uterus, allow it to grow and then you have the embryos coming, right.

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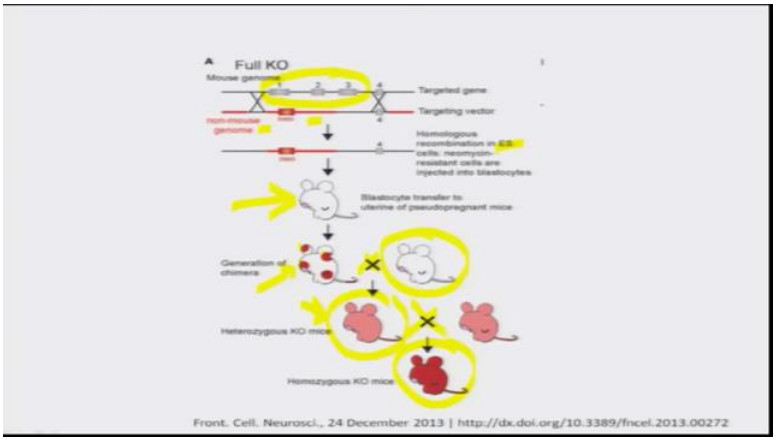

So, that is the embryo comes in. That is what is shown here. So, what you, just to summarize so far what we have discussed, so you have, you know, a neomycin cassette which you use to, for example remove certain exons. Here, what is shown here is more than one exon, exon 1, 2, 3 and then you have, you know, put that inside the blastocyst. You have derived the embryonic stem cells, put that blastocyst having these embryonic stem cells inside a foster mother and then these embryos grow. So, in these animals, you would have some tissues, some part of it would be derived from the embryonic stem cells in which you have changed the genome. So, that is what is shown here.

For example, if you have derived the embryonic stem cells from a mouse strain that has given you brown colour coat, coat colour and you have put that colour. cells into a blastocyst of animals that have the white fur colour, then you would have patches of, you know, your skin having brown patches that shows that it is a chimera, meaning it has the cells that are derived from the embryonic stem cells that you have engineered. Now, you cross it with, for example a white colour animal and you look at all those animals that are brownish in colour, because that is dominant over the white that would indicate that the germ cells that give this phenotype is derived from the embryonic stem cells that you have put into the blastocyst. So, that is the way you identify an animal which now carries 50% of the genome coming from the embryonic stem cells that you have derived.

Now, this has got, you know, 50% chance that it has carried your knocked out allele. Now, you can cross, for example a brown animal with the other, which are supposedly heterozygous and you can get a homozygous animal which has got both alleles in which the exon that you wanted to be deleted is deleted and now you can study the phenotype.

So, this is the way you engineer, you delete a certain exon and look at the phenotype.

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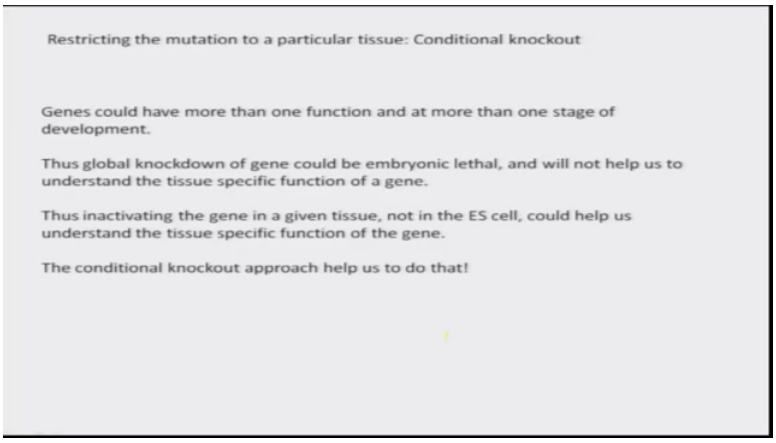

Now, when you do this, when you do such approach, when you want to delete, in this approach what we have discussed is that, this deletion happens in all the cells that animal carries, because you have deleted it in the embryonic stem cell itself. So, every tissue that is derived from embryonic stem cells will have the deletion. But, what is, the bottle neck is that, if the gene that you are studying has got more than one function, one of the function could be, for example the early embryonic development; the other function could be something very unique to, for example the brain function, neuronal function. So, if the gene has got a function in the embryonic development, if you have deleted that, then embryo is not going to survive.

So, you will not be able to understand the function of this particular gene in the neuron. So, what you should do in that case is, you should allow the gene to be present during embryonic development, but delete only when the embryo becomes an adult and then you have the neuron differentiated, only then you want to delete and then look at the function of that gene, right, in the adult. So, that is, this approach is called as, you know conditional knock out, meaning you want to delete the gene in a particular tissue, at a particular time point during development. So, that is called as conditional approach. It is very similar to the global knockout just now we described, except that you do not inactivate the gene in the embryonic stem cells. So, you sort of construct your, the target is constructed in such a way that it does not disrupt the function of the gene. Still it is able to code for the protein, but it has some

desired change which will use it to delete that gene in a given tissue, at a particular stage of a development. So, this is called as conditional knockout. How do you do this?

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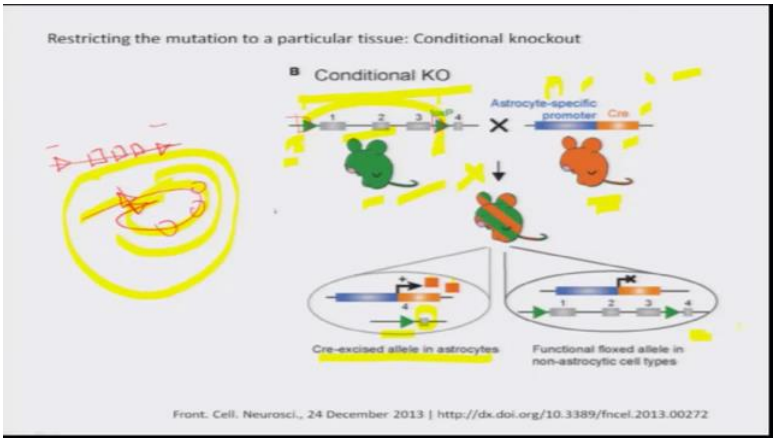

So, what you do in this case is that you create a construct, right which has a unique site called loxP site. These are a few bases of sequences, which are recognized by a recombinase called cre recombinase and this recombinase, if it is present, it is going to identify these loxP sites and promote what is called as site specific recombination. So, how does it happen? For example, in this allele what we are seeing is that we have introduced two loxP sites flanking exon 1, 2 and 3. So, what you are trying to do is, when loxP sites are present there, the gene is still able to function, because here between exon 3 and 4, this loxP is present in the intron, therefore it will be spliced out when the transcript is being made, still the gene is functional.

However if you, express the cre recombinase in that particular cell, wherein you have this engineered copy of the gene, then the cre recombinase would, you know, favour a recombination called as site specific recombination. So, what is that? So, you have this. You have the loxp sites and let us say you have the three exons and our loxP sites is there. So, what happens is that, you know, because the sites are directional it can form this kind of arrangement and your three exons are in between here and the recombinase would make a double stranded cut here and stitch them.

As a result, now what could happen is that you would have the DNA; this to this gets joined and all the intermediate region having all the three exons will be spliced out, ok. So, this way you are able to see that. So, that is what you are seeing here. So, you are going to cross an

individual animal in which you are able to put loxP on either side of exon 3 in the embryonic stem cells, selected the embryonic cells and you made the animal. Now, in the animal genome you have this. Now, you cross that with a transgene, you know, this different method that we discussed earlier, wherein you are expressing a cre recombinase. Now, the question is where the recombinase would express? I can use a promoter which drives the cre recombinase only in, for example astrocytes. These are supporting cells in the brain, for example or I can drive the cre recombinase coding region with a promoter of a protein normally expressed in muscle or heart or liver. So, it all depends on where I want the recombination to take place.

So, in this case now we have crossed the animal which expresses cre recombinase in astrocytes and crossed it with an animal which carries the loxP sites on either side of the three exons of a gene that you are interested in. As such this animal is normal, right; that is what we discussed that loxP that you put in such a way that it does not affect the expression of the gene. The moment you cross them what happens? In the astrocyte in this particular cell type, you have the cre recombinase, which favour this kind of a recombination leading to the elimination of all three exons. Now, the DNA from here to here will be joined, this is lost. So, that is what shown here.

So, you have had a condition, wherein in the astrocyte the cre recombinase is expressed and it has deleted all three exons 1, 2, 3, but exon 4 is present. That does not really help, because the gene cannot express. Whereas in other cells wherein you have no cre recombinase, this loxP sites is intact, it does not change the function of the gene and the gene is happy, the cells are happy. But, if you are seeing a phenotype in this animal that means that this protein is essential for the astrocyte function or if you want to likewise model it for liver, you can model it for muscle and so on. So, that is what you call as conditional knockout. You want to delete or disrupt a particular gene in a given tissue or in a given stage of development, depending on what question that you are asking. So, that is, you know, precisely the way people, remove the gene to understand the tissue specific function of the gene.

So, what I am showing is one example of a conditional knockout, wherein they are able to identify a function, neuron specific function of a protein, which otherwise was not appreciated much.

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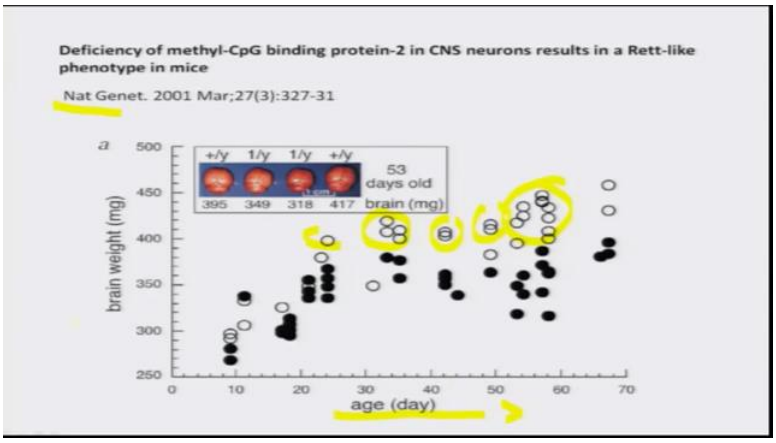

So, this protein called as a methyl-CpG binding protein-2, MeCpG-2 gene, if this gene is mutated, then the large number of individuals, you know, shows a mental retardation call Rett syndrome. This is one of the very common mental retardations and more often, this disease affected the female. You know, there are many patients that are children that are female, right, very few boys and one of the hypothesis was that that if you have this gene defect in, children that are XY, it could be embryonic lethal right? So, this was the hypothesis. So, what, and it was very surprising, because this is a methyl-CpG DNA binding protein, because it is a transcription factor and they never thought it could be such, having such a tissue specific function or neurological function. They thought it was a generic transcription factor involved in all the tissues, because expressed everywhere.

So, it was a surprise when people identified mutations in this particular gene and before that there were papers, wherein they have shown, if you knockout this gene, removed the entire copy, even the embryo do not survive, right? So, may be in these individuals who develop Rett syndrome, the mutations are such that it is a leaky protein, meaning the function is still intact, not to that extent that you see in normal, but still some function is left, therefore you are seeing, you know, they have survived during development, but later they show, you know, neurological, mental retardation and so on. So, they wanted to do model. So, you know, you create an embryonic knockout, and what happens, embryos do not survive. How would you study the function of the protein in the neuron?

So, this particular group, authors of this paper, you are interested in, you can go and read, they developed what is called as a conditional knockout, wherein they deleted the gene only

in the neurons, differentiated neurons, by using a cre recombinase. It is expressed only in the differentiated neuron and they were able to model and showed this animal, which had a deficiency of this protein only in the neuron, showed many of the phenotype that are seen in Rett syndrome. One of them being that the size of the brain is very very small as compared to the wild type. You can see that all the unfilled white circles are the, the tissue sample representing the brain of wild type animals. So, with age and if you look at the brain weight, you see that consistently the MeCp-2 deficient animals showed smaller brain and they have had many, you know, stereotype behaviour and other thing that, you know, typical of Rett syndrome and there are even now lead molecules people have identified using them, animal model, to cure possibly the Rett syndrome.

So, that is the power of the animal models, the approaches people have developed. In fact, for the knockout animal, the approach that they developed, this approach led to the Nobel Prize. You want to go and read who are the people who developed the technique and why this approach was considered to be a Nobel Prize award winning, you know, discovery or invention, whichever way you look at it.

So, that pretty much brings end to the third week of lectures, wherein we started looking into the various forms of the mutations, the so called loss of function mutations, gain of function mutations, discussed issues with regard to why certain mutations are more frequent in the population and how complex the gain of function mutations are and how do you model organisms to understand human genetic disorders. So, with that we end third week. The last week lectures, the week 4 lectures would, would be devoted to discuss how you identify the disease gene. So, you know only a disease that runs through a family. So, how will you identify which gene is defective. So, the gene discovery we are going to discuss, both for a monogenic form and the current approach to identify the genes responsible for so called polygenic disease or a complex disease. How do you know what are the genes that are risk factors? So, that would be the last set of topics that we will be , discussing in this course, in the coming week.