Eukaryotic Gene Expression: Basics and Benefits

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Lecture No. # 35

Transgenic Animals

Welcome to this lecture series on eukaryotic gene expression: Basics and benefits. Today, we are going to discuss about a very interesting topic called transgenic animals. This is the lecture number 35 in this series.

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Eukaryotic protein expression systems-II (lecture 31)
Protein expression in mammalian cells (non viral vectors)
Cell-free protein expression systems

Eukaryotic protein expression systems-III (lecture 32)
Protein expression in mammalian cells (viral vectors)

Human gene therapy (lecture 33)

DNA vaccines (lecture 34)

Transgenic animals (lecture 35)

The last four or five lectures we have primarily been discussing about various eukaryotic expression systems; we discussed about the use of viral vectors and non-viral vectors for expressing genes in mammalian cells in cultured. And then, we went about and discussed about how these non-viral and viral vector systems can be used for expressing genes in humans. A field called as human gene therapy – how it took off? What were the problems and prospects? These were discussed in detail. And, in the last class, we discussed about another very interesting area of research called DNA vaccines. And, how a very simple gene delivery and gene expression technique namely, injection of just a naked DNA plasma into skeletal muscle or skin leading to very inefficient gene

expression; but, although did not failed as a gene therapy technique, but found a new application in the form of evoking an immune response for a foreign antigen. And, how this is a very simple and versatile technique led to the development of a whole new field of research called DNA vaccines? And, how by at least two products are right now in the market based on this DNA vaccine technology?

Today, we are going to discuss about another very interesting area of research called transgenic animals. The main distinction between this lecture and the lectures I have listed above here from 31 to 34 is that in all the previous lectures, we focused our attention primarily on introducing genes into somatic cells of the body. And, especially when we were discussing about human gene therapy, I told you that so far, all the gene therapy experiments are basically confined to only somatic cells. There is no germline gene therapy.

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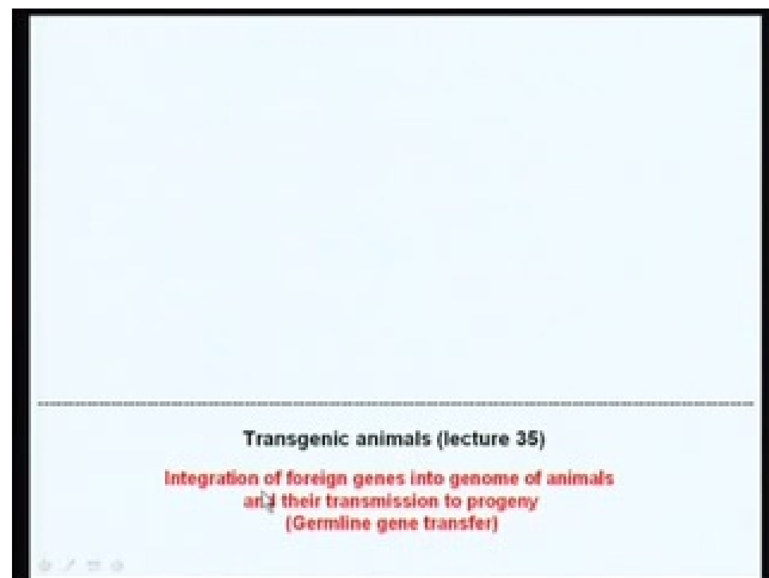

Today, we are going to discuss about introducing genes into germline of animals, and how transgenic animals can be generated by integration of insertion and integration of foreign genes into the genome of animals and their transmission to the progeny. This is the most important. So far, all the gene transfer techniques were discussed with reference to the DNA vaccines or human gene therapy. They are all performed only in somatic tissues of the body. For the first time, we are going to discuss about introducing genes to the germline, but this transgenic technology is primarily confined to only the non-human

animals namely, the mammals, which are not humans; no germline gene insertion or expression is permitted in humans till now. And, we will primarily now discuss about how genes are being inserted into animals, such as mice, domestic, animals and so on and so forth either for the purpose of understanding the function of genes or understanding the function of the regulatory sequences or for the benefit of mankind for expressing certain proteins, which are of economic importance, which are very useful to man and so on and so forth.

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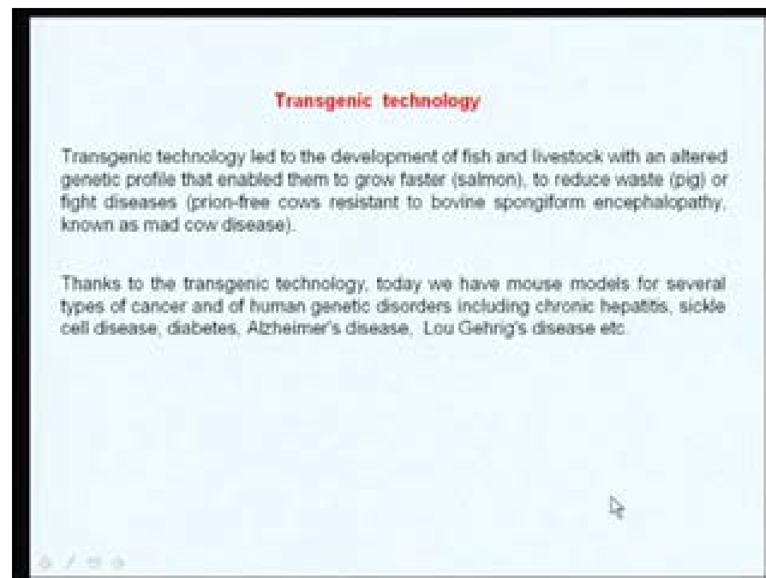

So, what is transgenic technology? Transgenic technology, as I said in the previous slide, is the introduction of genes into the germline of animals or integration of these genes into the chromosome of the animals, so that not only the gene is introduced and expressed in the animal in which we have introduced, but this gene is carried through the successive generations as well. So, the offspring generated by the transgenic animal also contains a transgene. So, this transgene is carried through from one generation to another. This transgenic technology is very important, because it led to the development of number of domestic animals and fish, birds, etcetera with an altered genetic profile that enabled them to either grow faster – these are all very useful traits to have.

For example, if you have fish, which can grow faster than the normal fish, that means, you do not have to grow them for much longer time you can cut down on the culture cost. So, by introducing growth hormone gene into fish for example, you can make this

fish grow much faster. So, you do not have to keep them in the aquarium for too long. Similarly, you can generate pigs with reduced waste or you can generate cows, which are prion-free, which are resistant to some of the mad cow disease or the bovine spongiform encephalopathy and so on and so forth. So, basically, by introducing appropriate genes into the animals or birds or fish, you can get the desired characteristic, so that those transgenic animals have a very useful trait that is beneficial to the them as well as to the humans. So, thanks to the transgenic technology.

Today, we have mouse models for several types of cancer and human genetic disorders including chronic hepatitis, sickle cell disease, diabetes, Alzheimer's and so on and so forth. So, not only we are trying to express genes into animals with the purpose of making a protein, that may have economic importance or that may be useful to the humans. But, by expressing certain genes or certain proteins into these animal models, we are able to generate animal models that are more appropriate for certain diseases, because many a times, we are not able to test a drug or we are not able to develop a vaccine for a particular disease mainly because we do not have appropriate animal models. So, unless we have right animal model and unless we test these drugs or vaccines in these appropriate animal models, we cannot go and test the drugs and vaccines into humans. So, some of the major problems and certain major diseases is to have lack of appropriate animal models.

So, by using transgenic technology, you can actually introduce the right kind of genes into these animals like mice or other animals and create better models for understanding the disease or for testing drugs and vaccines and so on and so forth. So, transgenic technology is useful not only for expressing proteins of economic importance, but also can be used for developing animal models for a number of diseases.

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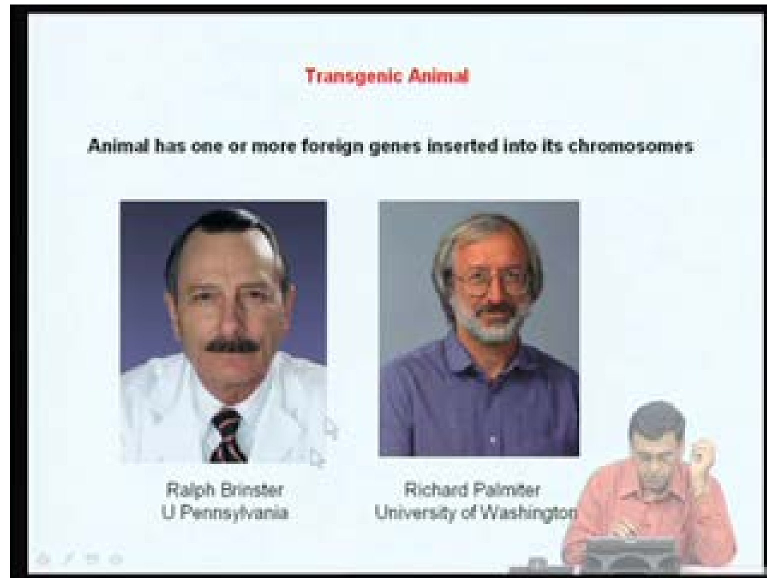

The credit for developing transgenic technology goes to two people; I have given the pictures here – Ralph Brinster in University of Pennsylvania, United States and Richard Palmiter, University of Washington in United States. So, these were the two people, who developed the transgenic mice technology or the transgenic technology or the first created transgenic mouse. So, by definition, transgenic animal should have one or more foreign genes inserted into the chromosomes, so that not only the gene is carried by the organism, but also by its future generations. So, let us see what exactly Brinster and Palmiter did, how did they developed this transgenic mice.

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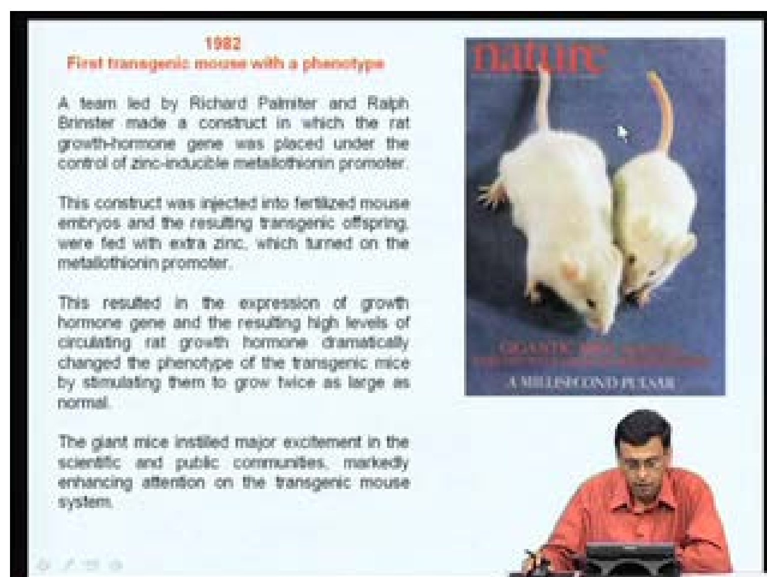

The landmark paper or the landmark research that happened in the area of transgenic technology was the paper, which appeared in the year 1982.

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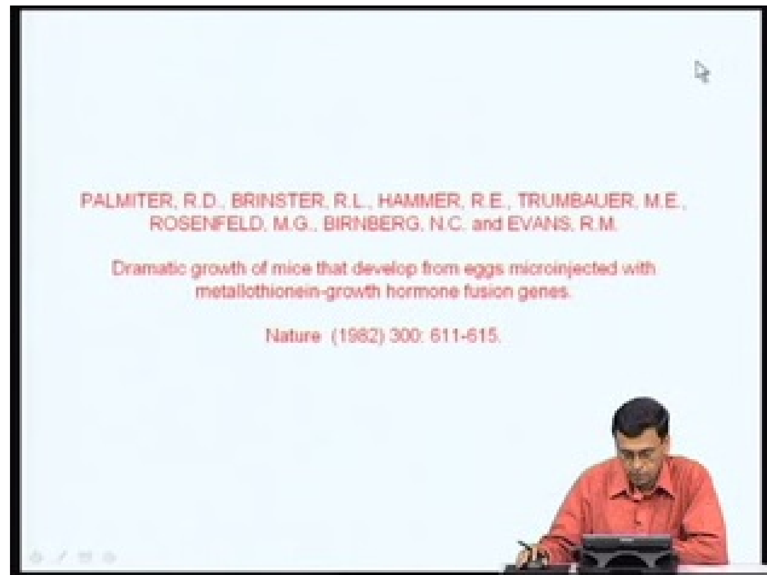

This paper appeared I think in Nature, 1982. And, what basically (Refer Slide Time: 07:21) this paper actually do, the attention of researchers across the globe primarily, because this was the first time a mouse was created by introducing a foreign gene into the mouse. And, the introduction of this foreign gene into this mouse led to development of a very stunning phenotype. For example, I am showing a picture here, which actually appeared in the cover page of nature of this particular issue in the 1982, where you can see, the one on the left, which is much bigger, is a transgenic mouse and the other one is a normal littermate born to the same mother. And, this mouse actually carries a transgene expressing rat growth hormone. So, when you express rat growth hormone, these mice grew much bigger than the normal littermates. So, for the first time, Brinster and Palmiter actually showed that by expressing a growth hormone gene in a mouse and by generating transgenic mouse, you can alter the phenotype of the mouse. And, not only that, this phenotypic trade can be passed on from one generation to another generation. So, a team led by Richard Palmiter and Ralph Brinster made a construct in which the rat growth hormone gene was placed under the control of a zinc inducible metallothionein promoter.

In our previous classes, we have discussed a number of examples of constitute promoters, invisible promoters and so on and so forth. And, how people are using both constitute as well as individual promoters for expressing genes in mammalian cells? So, what Brinster and Palmiter did is, they actually used inducible promoters called a metallothionein promoter. And, this promoter can be induced by adding metals like cadmium, zinc and so on and so forth. So, in the absence of these metals, the expression of metallothionein is very low. But, now, in the presence of these metals, metallothionein promoter is turned on. So, took the rat growth hormone gene and placed it on the control of a metallothionein promoter. And, this entire construct was actually introduced into fertilized mouse embryos, And, these mouse embryos into which this transgenic construct was introduced, was then put into a faster muscle.

We will discuss how exactly the transgenic mice are made in a couple of minutes. But, just for the time being, be aware that this transgenic construct, which contains the growth hormone gene under the control of a metallothionein promoter was introduced into the fertilized mouse, oocyte on mouse embryos. And, the offspring, which are formed from these embryos, when they were fed with zinc, they turned on the metallothionein promoter. And therefore, the growth hormone was expressed. And, this resulted in high levels of circulating growth hormone and dramatically changed the phenotype of transgenic mice by stimulating to grow twice as large as the normal. That is what is the picture shown here. So, by introducing a transgene under the control of a metallothionein promoter into the germline of mice and by feeding these mice with zinc, they demonstrated that this metallothionein promoter can be turned on by the zinc. And, this in turn, turns on the human growth-hormone gene. And, as a result, high levels of human growth hormone are produced in the circulation. And, that result in the very dramatic growth of these mice compared to the normal. So, these giant mice instilled major excitement in the scientific and public communities, markedly enhancing the attention on the transgenic mouse system. So, the 1982 is the landmark in the area of transgenic technology, because it demonstrated for the first time. You can actually manipulate the germline of animals, and you can introduce genes and express them, and you can get a desired phenotype.

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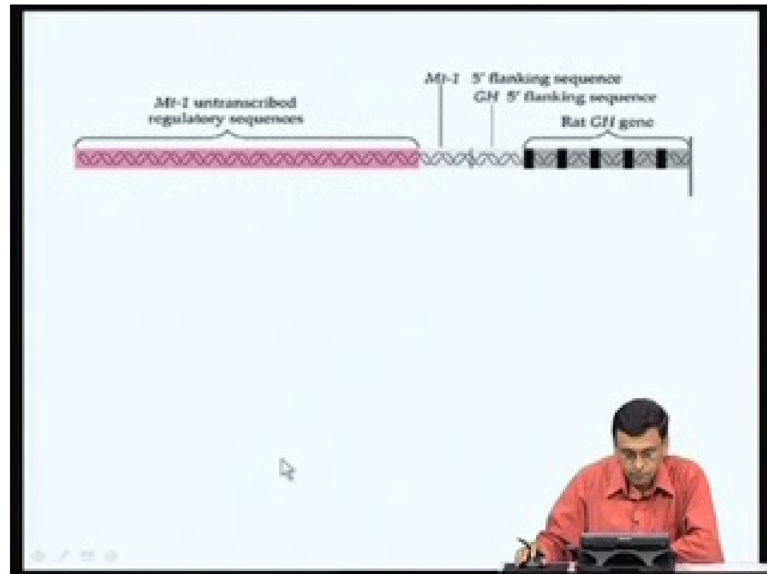

So, this is basically what they did – they took the promoter region of the mouse metallothionein promoter; and using restriction enzymes and the usual genetic manipulation techniques, cloned the rat growth-hormone gene downstream of the metallothionein promoter and put the appropriate 3 prime regulatory sequences for polyrelation and so on and so forth; and, introduced this construct into the germline of the mouse.

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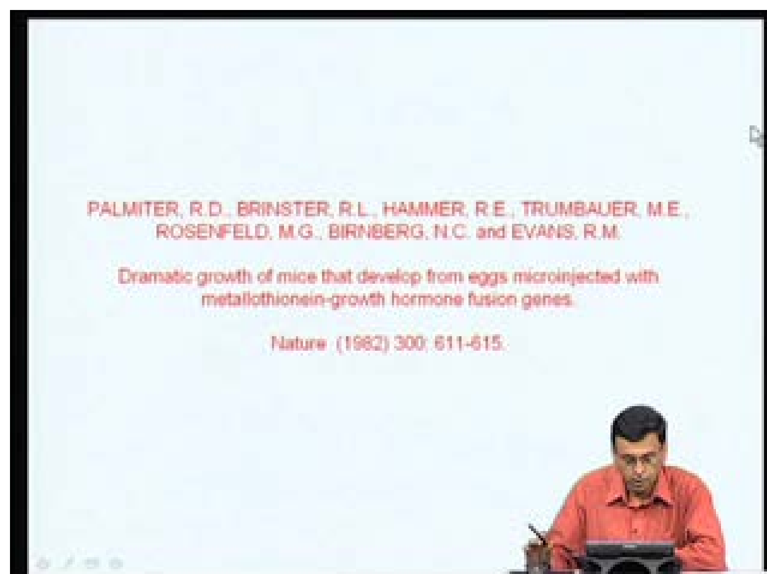

So, this is the landmark paper published in the year 1992 in the journal nature, which is a dramatic growth of mice that develop from eggs microinjected with metallothionein-growth hormone fusion genes by Brinster, Palmiter and Ron Evans.

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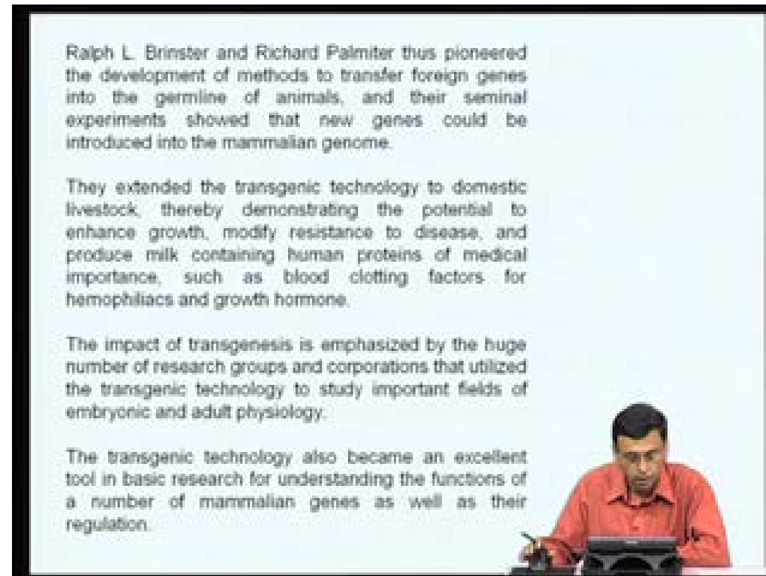

Ralph Brinster and Palmiter thus pioneered the development of methods to transfer foreign gene into germline of animals, and their seminal experiments showed that new genes could be introduced into the mammalian genome. So, this is the first successful demonstration of introducing genes into germline of mammals and expressing them leading to a very dramatic phenotype. This technology, which was originally developed in transgenic mice, was later extended to other domestic animals like cattle, goats and so on and so forth. And, this led to the demonstration of potential of this technology for the enhancement of growth; you can make pigs grow bigger, you can make cattle give more milk and so on and so forth; or, you can modify resistance to a particular disease; or, you can produce milk containing human proteins of medical importance, such as clotting factors and so on and so forth, indicating that it became a very powerful technology. So, by extending this transgenic technology, which was originally developed in mice to other domestic animals, it became very clear that you can actually use this farm animals for making proteins of which are useful to you.

So far, we have been discussed about expressing proteins or putting genes into e cells or equalized cells or mammalian cells; and then, making these cells, converting these cells

into bio reactors for making proteins of our interest. But, now, we are discussing about introducing genes into the germline of farm animals. The farm animals now become bio reactors. So, you have now cattle-producing growth hormone, cattle-producing medically important proteins like factor 8, factor 9 and so on and so forth. So, by using this transgenic technology, it became possible to convert animals into bio reactors. So, the impact of transgenesis is emphasized by the huge number of research groups and corporations that utilize the transgenic technology to study important fields of embryonic as well as adult physiology. The transgenic technology also became an excellent tool in basic research for understanding the functions of a number of mammalian genes as well as their regulation.

So, what I am going to do next few slides is, give some very exciting examples of how transgenic technology was used and what exciting research was done and what was the exciting outcome that came out of this.

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Transgenic mice are often generated to :

1. characterize the ability of a promoter to direct tissue-specific gene expression
   - e.g. a promoter can be attached to a reporter gene such as LacZ or GFP

2. examine the effects of overexpressing and misexpressing endogenous or foreign genes at specific times and locations in the animals

So, the transgenic mice are usually generated to characterize the ability of a promoter to direct tissue-specific expression or inducible expression. I have given example of a tissue-specific expression.

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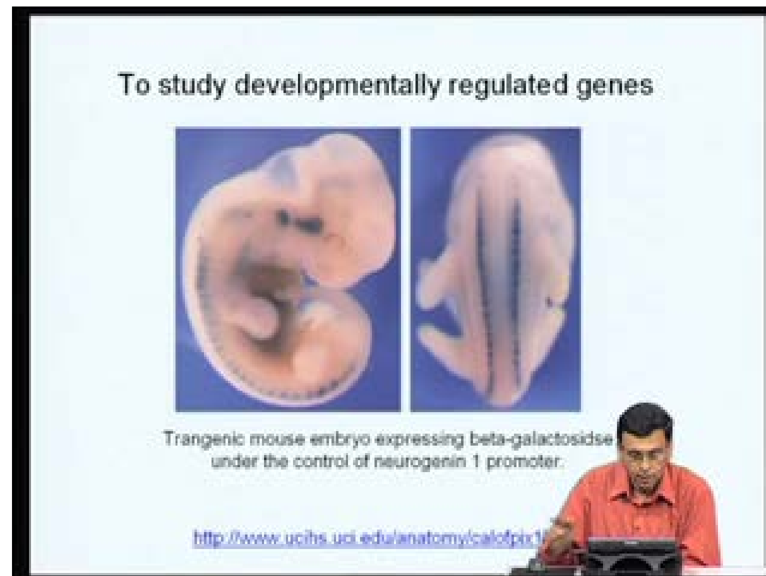

For example, here is a study that was published by this group, where they have actually used the promoter region of a gene called neurogenin 1, which is actually expressed in very specific regions of the nervous system. And, if you now put a beta-galactosidse gene under the control of this neurogenin promoter; and, when you make a transgenic mice; and, when you look at the expression, when we now look for beta-galactosidse expression in the transgenic mice, wherever the neurogenin promoter is expressed in these embryos, in those regions, you can detect beta-galactosidse expression. And, you can see, here the beta-galactosidse is expressed wherever the neurogenin promoter is active under this particular stage of embryonic development. So, the transgenic technology became a very useful tool for mapping or for identifying spatial and temporal expression of very important promoters.

If you want to know, for example, where is the hox promoter or a homeobox promoter is expressed; or, when a particular homoerotic gene is expressed; or, where are the homoerotic gene is expressed, you can use this kind of approach, where you take the promoter region, put a reporter gene and make this kind of transgenic convergence and see what happens. So, you can understand both the developmental regulation as well as… Similarly, if you can put a tissue-specific promoter and put a reporter gene and see a question, whether this gene gets expressed to only that particular tissue? So, the transgenic technology became an excellent tool to understand tissue-specific and developmental-specific expression of genes by using reporter genes linked to their

promoters. So, we can use (Refer Slide Time: 15:51) the transgenic technology to understand the ability of a promoter to direct tissue-specific expression. Like what I said, a promoter can be attached to a reporter gene such as LacZ or GFP. The other important reason why people make transgenic mice is to examine the effects of overexpressing and misexpressing endogenous or foreign genes at specific time and locations in the animals.

For example, the study, where I showed, where I actually expressed human growth hormone into kind of metallothionein promoter; what I actually showed that when you overexpressed growth hormone, it results in a gigantic phenotype; it enhances the weight of the animal. So, like that, you can ask the question what happens if we express an oncogene? What happens if I overexpress a tumor suppressor gene? Or, what happens if I express a growth factor? You can ask any number of questions and see what happens when you express. Or, suppose a gene is getting expressed only in one particular tissue and you ask the question, what happens if I express in another tissue of an animal? Will it have any dramatic effects? So, a huge number of questions could be asked using this transgenic technology. So, not only you can use this transgenic technology for understanding these functions of the promoters, where they are expressed and when they are expressed, you can also use transgenic technology to understand functions of many of these genes; what these genes normally do?

This is one example (Refer Slide Time: 17:09) I showed, where by expressing a reporter gene as the control of neurogenin promoter, you can exactly identify during development, where exactly this promoter is turned on in animals.

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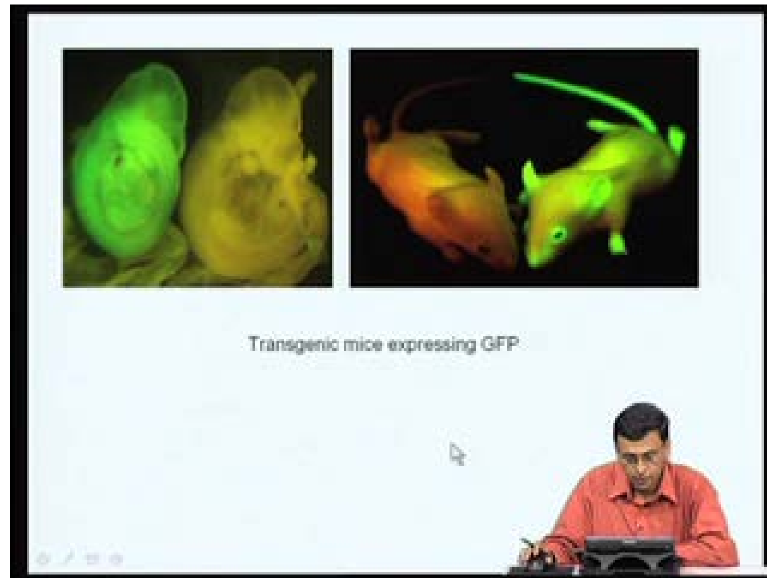

Similarly, here is another example, where using another reporter gene called the green fluorescent protein, the gene coding for green fluorescent protein, you can now clone it into a constitute promoter. And, when you now make a transgenic mouse of that, if you now shine a UV light on that, you can see the entire embryo or the entire mouse, fluorescent green. So, this became very powerful to ask a number of questions in animal systems.

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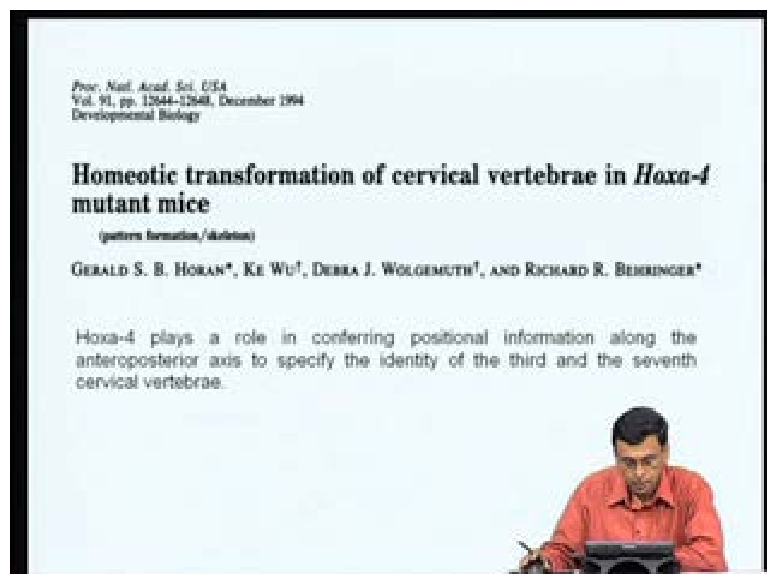

Similarly, I think I gave many examples while we studied about homeotic genes and how gene expression plays an important role in development, about how homeotic genes play a very important role. And, we discussed a number of examples wherein when you express homeotic genes in certain regions, it can create havoc. When you misexpress or overexpress, it can have a lot of developmental abnormalities. Here as example, homeotic transformation of cervical vertebrae in Hoxa-4 mutant mice; so, using this kind of… By expressing a particular Hox gene in mouse, they got a particular phenotype and this led to the new knowledge that this particular Hox gene has a particular role in that particular event.

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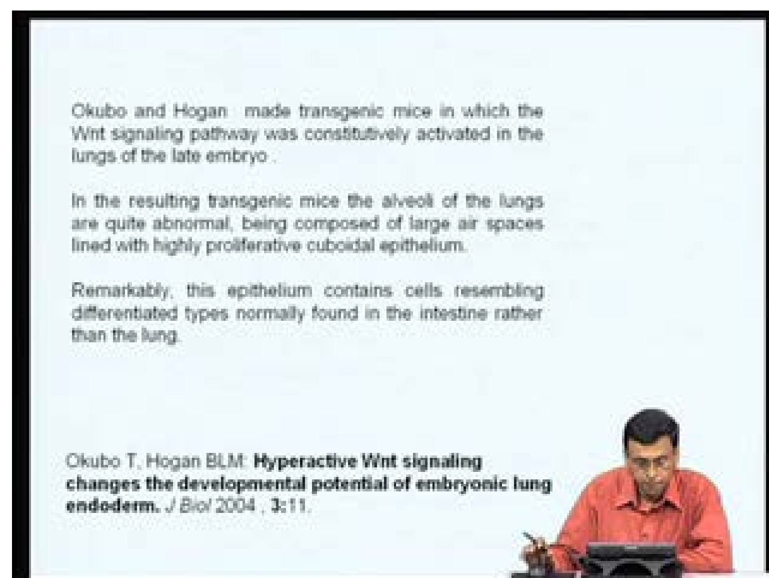

Similarly, for example, Okuba and Hogan; somewhere around 2004, this was the paper published; wanted to understand what is the significance of this Wnt signaling pathway during development. So, what they did is, they made transgenic mice in which the Wnt signaling pathway was constitutively activated in the lungs of the late embryo. So, Wnt signaling as… If you remember when we discussed about some of the genes important in embryonic development, we discussed extensively about Wnt signaling; and, how if you medal with the Wnt signaling can lead to colonic cancer and so on and so forth. So, Wnt is normally expressed in the intestine.

Now, these people ask the question, what happens when I express this Wnt signaling pathway in lungs? So, they used the lungs-specific promoter and expressed these Wnt

signaling proteins in the lungs and they found that the resulting transgenic mice, the alveoli of the lungs are quite abnormal, being composed of large air spaces lined with highly proliferative cuboidal epithelium. Now, cuboidal epithelium is a very characteristic of the intestinal villi. So, epithelium, which is normally found in intestinal villi, now, we start seeing in the lungs. So, this epithelium contains cells resembling different times; normally found in intestine rather than lung very clearly telling that this want signaling pathway plays a very important role in the development of the intestinal epithelium. And, if you now express this Wnt signal area, activate this Wnt, signaling pathway in the lung, the lung will now start developing cells, which are normally present in the intestine. So, you can understand a function of number of these genes by doing these kinds of transgenic experiments.

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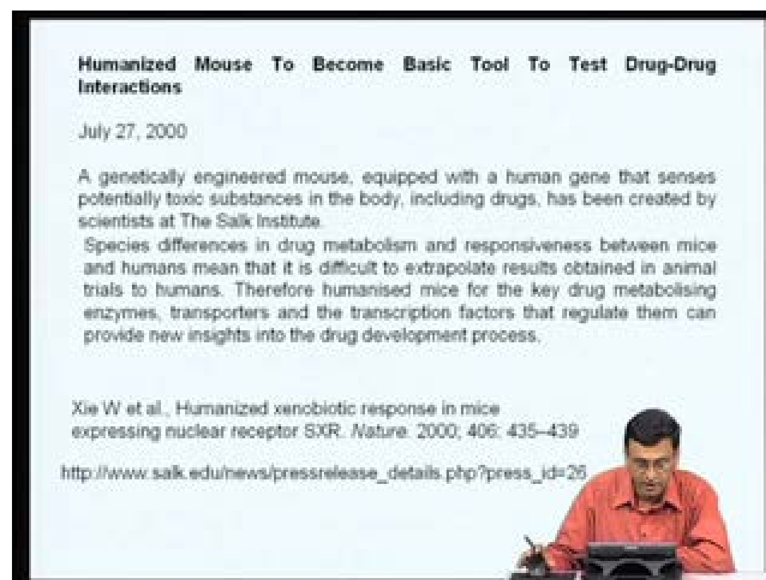

Similarly, another very interesting paper that came sometime in 2000 by Ron Evans Velscope at Salk Institute humanized mouse to become a basic tool for testing drug-drug interactions. What did they do? They developed a genetically engineered mouse, which contained a human gene that is involved in toxification of detoxification of certain drugs. And usually, when you want to develop a new drug, the one thing that you want to see is that how well our body detoxifies these drugs. Now, the ability of… There are proteins called SecretomeP 450, which play a very important role in detoxification of these drugs.

And, the way the mouse SecretomeP 450 system works is quite different from the way the humans SecretomeP 450 system works. So, many times, when we want to test the ability of our body to metabolize a drug or detoxify a drug, when we have to first use animal models, many of times, the way the mouse P 450 system detoxifies the drug, does not exactly reflect the way the human P 450 system detoxifies the drugs. So, the pharmacokinetics of these drugs often varies between mouse and humans. So, what did they do? They actually expressed the human P 450 in a mouse. And, this now demonstrated that this kind of a transgenic mice expression in human P 450 exactly mimics how the drug will be metabolized in a human system. So, very interesting; so, you can actually use the humanized xenobiotic response in mice expression in nuclear receptor SXR and so on and so forth.

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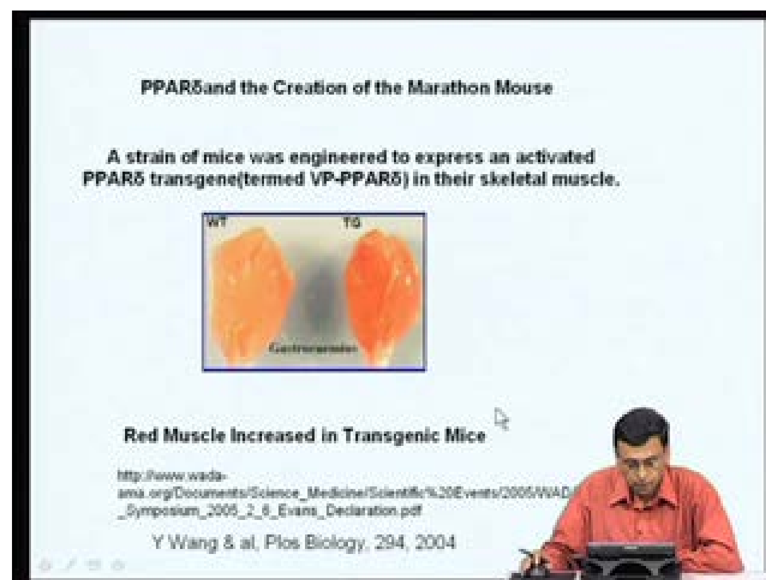

Similarly, another very interesting observation again by the same group, Ron Evans group demonstrated what is called as creation of the marathon mouse. What did they do? They developed a strain of mouse, which expressed a nuclear receptor called PPAR gamma in this skeletal muscle. Now, PPAR gamma is a nuclear receptor, peroxisome proliferator-activated receptor. And, they asked the question, what happens if you constitutively activate this nuclear receptor in the skeletal muscle of the mouse? So, the generated transgenic mouse in which this particular nuclear receptor is expressed constitutively in the skeletal muscle; and, this is what happened, the red muscle – this is the normal mouse, this is how the gastronomous mouse muscle looks like the normal

mouse, but the entire muscle turn red. So, the red muscle increased in these transgenic mice. And, these mice were able to exercise much better. So, if we take these mice and put it on treadmill, they exercise nonstop; their ability to work on the treadmill was much better than compared to the non-transgenic mice.

Now, what does it tell you? This clearly tells you that this particular nuclear receptor plays a very important role in this kind of exercise-induced tolerance and changes the muscle physiology and muscle structure. So, people now ask the questions, can you now develop drugs, which can constitutively activate PPAR gamma and delta? And, can you now produce better athletes? Who can run much better than those, where the PPAR gamma is not constitutively activated in the skeletal muscle? So, by using these kinds of mice models and expressing these genes or overexpressing these genes in specific regions, you can try to understand what is the physiological function of these nuclear receptors and these transcription factors and so on and so forth.

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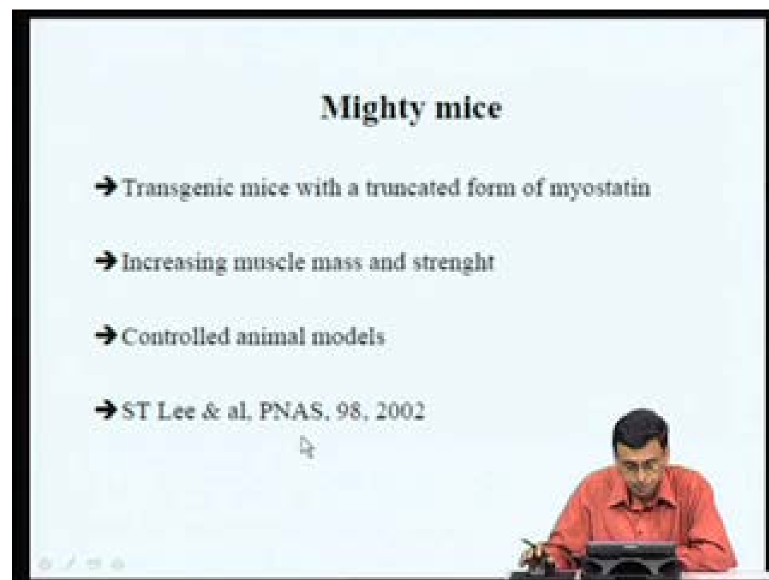

There are number of examples. Again, there is an example of paper published in 2002 about generation of what is called as mighty mice. Transgenic mice with a truncated form of myostatin; they had increasing muscle mass and strength and so on and so forth. So, sky was the limit. Following the Brinster's and Palmiter's experiments that you can actually introduce genes with germline of mouse, a number of research groups started looking or using this kind of transgenic technology for either understanding how

promoters enhances work or to understand what is the physiological function of key genes and key transcription factors and so on and so forth.

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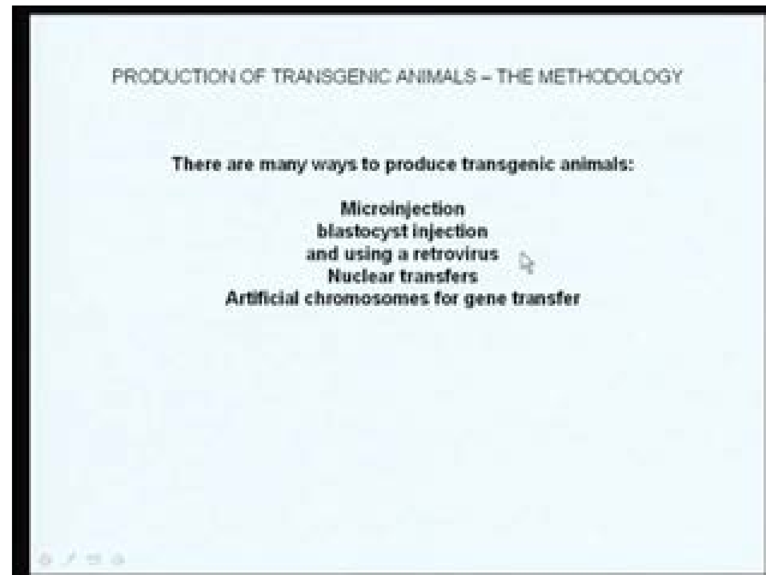

So, having explained to you how the transgenic technology became very important… And I just gave a few examples, very exciting and interesting examples, but the literature has number of such examples one can go and look up – basically tell you that the generation of transgenic mice technology by Brinster and Palmiter opened up the flood gates for introducing genes into germline of mice as well as other farm animals for a number of problems both for understanding basic aspects of gene function and promoter function as well as for some very important human applications. So, let us now spend some time to understand, how you exactly produce the transgenic animals or a transgenic mouse?

There are many ways of producing these transgenic mice, but what Brinster and Palmiter basically did was to use a technique called as microinjection of genes into fertilized eggs. There are other things, where you can actually introduce genes into the blastocysts; you can take embryonic stem cells from the mouse and introduce the genes into the embryonic germcells; select those embryonic stem cells, which are taken of the gene and put these ES cells, back to the blastocysts. So, you can also generate transgenic mice technology; you can also inject a retrovirus into the blastocysts. So, the retrovirus goes

and infects the embryonic stem cells. And therefore, the gene gets integrated and can be going to germline and you can generate transgenic mice.

There are other technologies like nuclear transfers, artificial chromosomes for gene transfer using even sperm as gene transfer vehicles. But, we will not spend too much time on this. I am going to discuss only two of these techniques for the want of time, how microinjection is a very popular technique, widely used technique for generating transgenic mice as well as blastocysts injection can be used for generating transgenic mice and transgenic animals. So, the first step in the generation of transgenic mice, is you have to make a construct. Whether you want to study the function of a gene or whether you want to study the function of a promoter, you have to make a transgenic construct.

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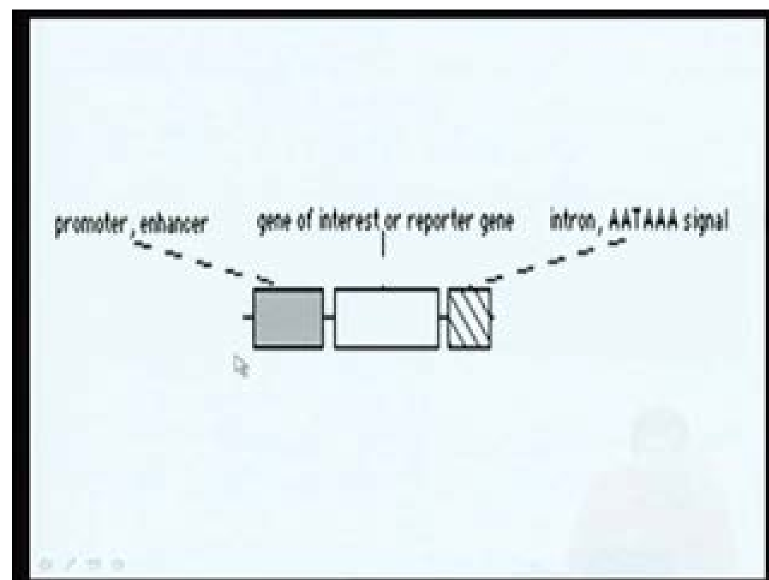

So, typically, a transgenic construct should consist of a promoter or enhancer sequence of our interest. This can be a constitutive promoter; this can be a tissue-specific promoter; or, this can be a developmental regulator promoter and so on and so forth. Depending upon your choice, it should contain your gene of your interest; your gene can be a transcript code for a transcription factor; or, it can be a reporter gene and depending upon what you want to do. Whether you want to study the function of a promoter, then you put a reporter gene. Or, if you want to express a gene of your interest, then you choose a well-characterized promoter and put your gene of your interest, which you want

to examine. You should also have the appropriate 3 prime regulator regions, so that the RNA gets properly polyrelated and so on and so forth. So, the first step in the generation of transgenic animals is to make a transgenic construct or a vector, which contains your gene of interest with an appropriate promoter and 3 prime regulatory sequences.

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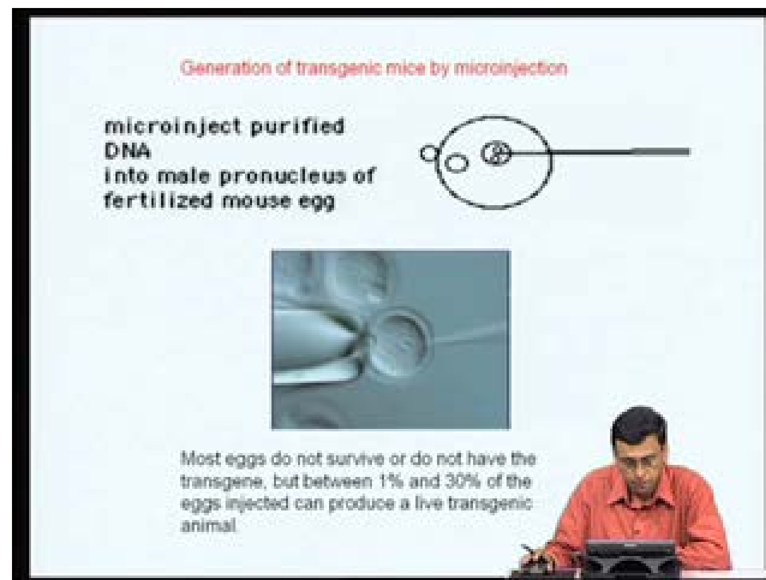

Once you made this transgenic construct, then you have to introduce this transgene into the nucleus of a fertilized egg. So, what you do? You do what is called as microinjection. So, the DNA of your interest is then introduced in the male pronucleus of a fertilized egg. So, basically, what you do, you take fertilized mouse fertilized egg, so that once the mouse, whose egg is fertilized by the sperm, you will have both the female pronucleus as well as the male pronucleus. And, you hold this fertilized egg as shown in this figure with a holding pipette with a very mild vacuum. And, using a holding pipette, you hold the fertilized egg. And then, through a very fine injection needle, you introduce this DNA of your interest into the male pronucleus of the fertilized egg.

You can see here, there is a nucleus here. And, through this (Refer Slide Time: 28:00) needle, you can actually introduce very tiny amounts of gene of your interest into the male pronucleus of the fertilized egg. When you do this, most of the eggs do not survive, because you are basically poking into the egg and sometimes it may be damaging and so and so forth. But, about 1 to 30 percent of these injected eggs actually survive. So, this depends on how good a technician you are. So, if you can do it well, then the efficiency

of transgenesis will depend upon how well you can do this microinjection without damaging the eggs. So, once we introduce this gene into the eggs, you then take these injected eggs, culture them for some time, and make sure that they are viable; and then, take this viable ones. And, once it is going to a two cell or a four cell stage, you put them back into what is called the foster mother.

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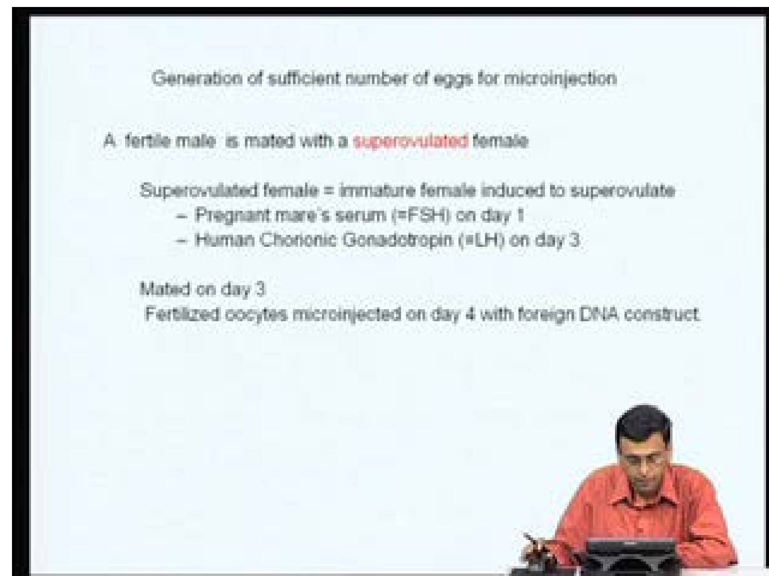

So, the first step – if you want to now inject DNA into the eggs, first you have to generate sufficient number of eggs. Normally, there are number of eggs, which are produced by a normal mouse, which is not sufficient, because as I said, many eggs get damaged during the microinjection experiment; many do not survive the procedure. So, you need to inject large number of these eggs in order to do transgenesis. So, that means, you need to have large number of this mouse eggs. So, you do what is called as a superovulation; that means, you make this mice lay more eggs, so that by killing less number of mice, you can get more number of eggs.

So, what you do is you generate what is called as superovulated female; that means, you take a female mouse and inject them with a pregnant mare's serum – pregnant horse's serum, which basically contains follicle stimulating hormone, which stimulates the growth of ovarian follicles; and then, you inject human chorionic gonadotropin on day 3, which now results in the ovulation. So, you increase the number of follicle's maturation by injecting FSH and then you make the mice ovulate, so that you get more number of

eggs per mouse. And then, you collect; then, you make this on day 4. So, on day 1, you give FSH; day 3, you get HCG; and, on day 3, you actually make them with a fertile male, so that the sperm will now go and then fertilize the egg and you get fertilized eggs. So, then, you can dissect on day 4; and then, you can collect all the fertilized eggs; and then, inject DNA.

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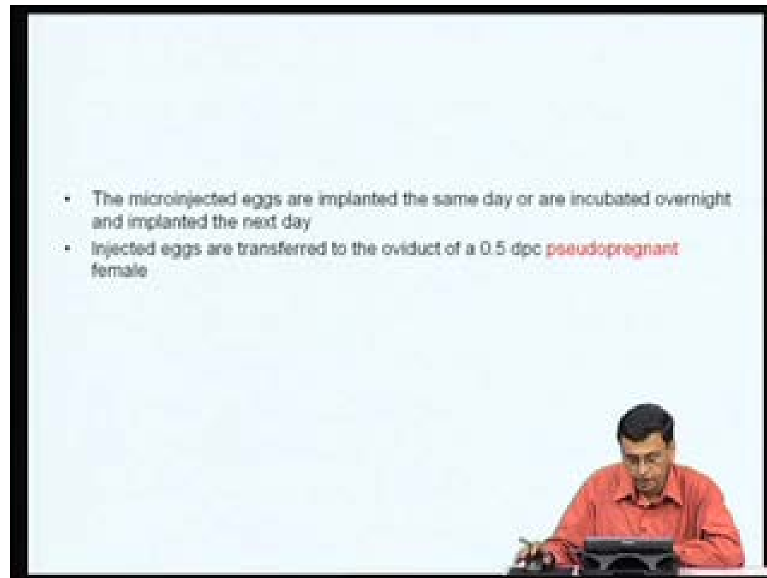

And, these microinjected eggs are then implanted either on the same day or are incubated in CO 2 incubator overnight and then implant on the next day. And, this is the time you make sure that only those eggs, which look healthy or which are not damaged or segregated, those are implemented into the uterus of a pseudopregnant female. The injected eggs are transferred into the oviduct of a 0.5 days post coitum pseudopregnant female.

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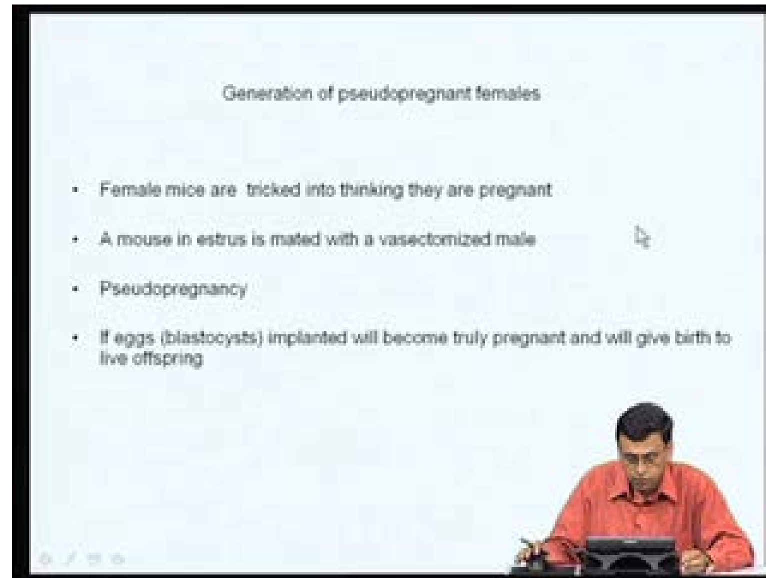

Now, what is a pseudopregnant female? Again, a pseudopregnant females are actually generated by mating a normal fertile female with a vasectomized male. So, basically, the female mice are tricked into thinking that they are pregnant. So, when these two mate, because it is vasectomized, the sperms are not delivered, but the uterus now becomes ready to receive the embryos, which are now implanted after the injected embryos. So, you generate what is called as the pseudopregnancy. And therefore, when you implant into such pseudopregnant females the embryos, then they go on implant in the uterus and you generate offspring. So, there are three major events for generating transgenic mice: one is you need to prepare a construct of your interest; and then, you have to inject this construct into the fertilized egg, preference should be to the male pronucleus of the fertilized egg; and then, once you have injected, you take these microinjected eggs and put them into the oviduct of pseudopregnant female mice, so that these eggs go through the oviduct into the uterus and get implanted, and you will get the offspring. So, these are the three major events that you have to do for generating transgenic mice. So, this is one major method of generating transgenic mice using what is called microinjection technique.

The other popular method of generating transgenic mice especially after the advent of the embryonic stem cells, is instead of doing all these things, once we have embryonic stem cells in culture, you directly introduce gene of your interest in the embryonic stem cells, because we know here cells are pluripotent and they can generate all three germ layers.

Therefore, you can introduce gene to the embryonic stem cells rather than into fertilized egg.

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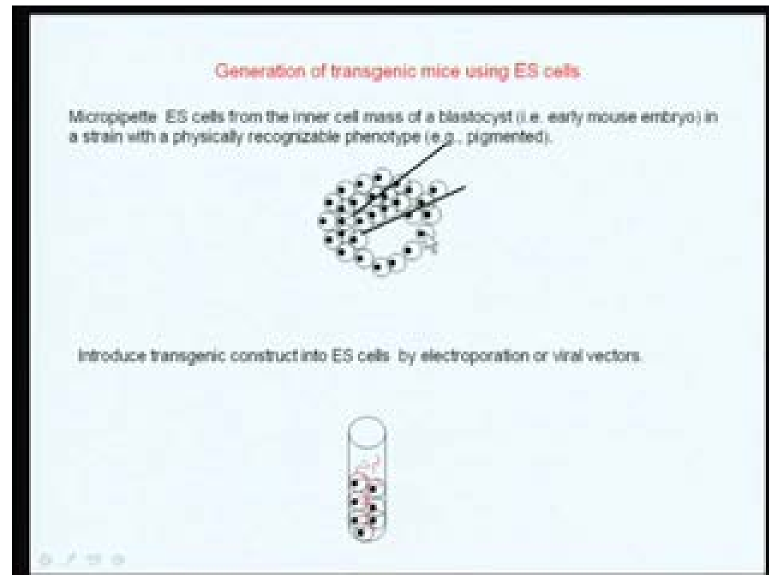

So, we have embryonic stem cells; you isolate either from the inner cell mass of a blastocyst; or, if you have ES cells in culture, you take these ES cells, introduce transgene into the ES cells either by what is called as an electroporation or you can use viral vectors like lenitvirus or retrovirus and so on and so forth.

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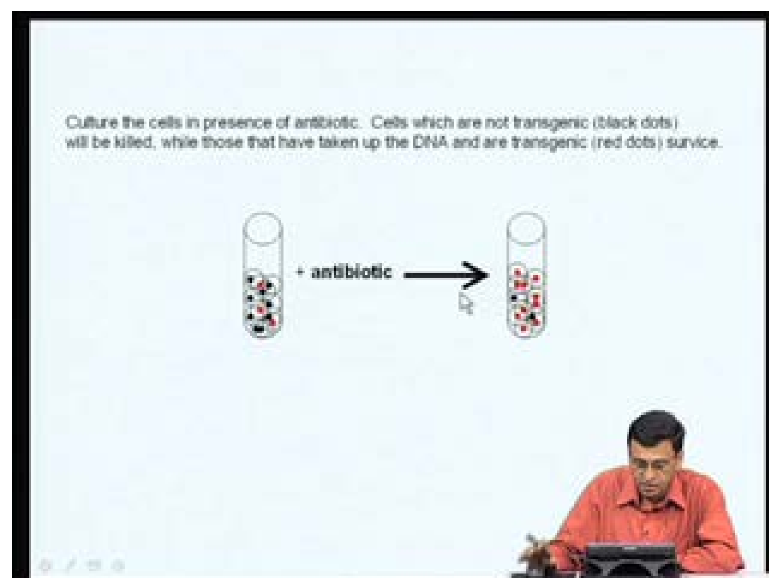

And then, you select the embryonic stem cells, which have taken up the DNA. Usually, the transgenic construct mate should also contain a gene coding for an antibiotic resistance marker. Therefore, you can actually select only those ES cells, which have taken up transgene construct. And, those ES cells, which contain transgenic construct is now introduced back into the blastocyst.

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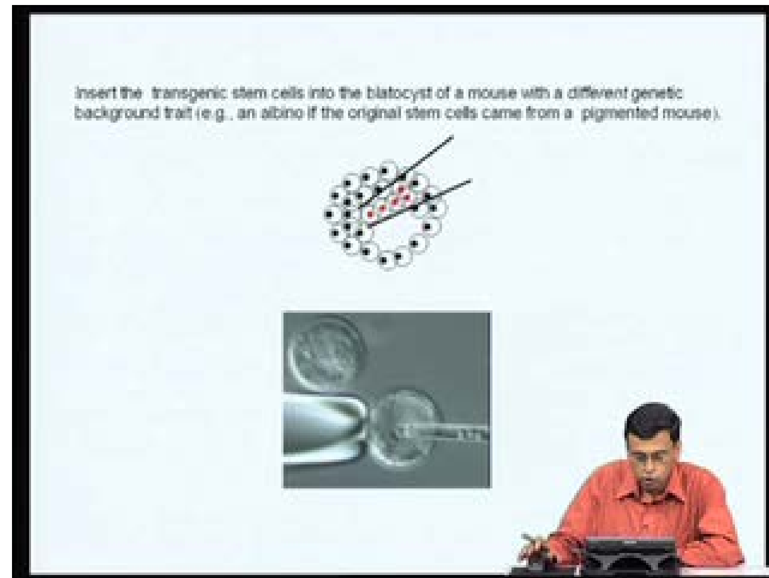

So, in the case of ES cells, in the case of the microinjection technique, you are introducing DNA into the male pronucleus of the fertilized egg; whereas, in this case, you are first introducing your transgene embryonic stem cells. And, the embryonic stem cells is then introduced to the blastocyl of the blastocyst, so that ES cells now mix with the endogenous ES cells and repopulate, and you get a transgenic mouse.

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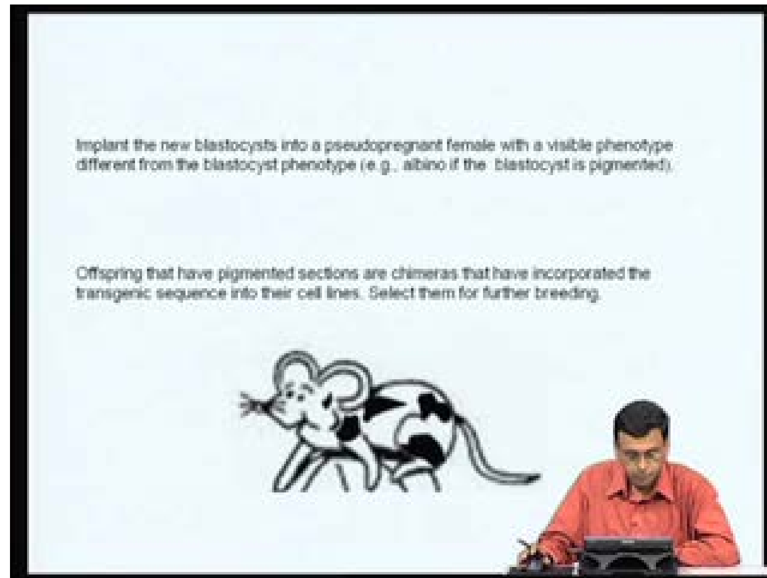

So, implant the blastocyst into pseudopregnant female. So, in the case of the microinjection, you are implanting the injected fertilized eggs. Maybe day 2, two cell embryos and so on and so forth; whereas, in the case of the embryonic stem cells, you are actually implanting the embryos containing the ES cells into the pseudopregnant female. So, the offspring generated, because the embryo contains both normal ES cells as well as ES cells, which contains transgene of interest, they become chimeric; that means, they are populated by both population: nontransgenic as well as transgenic ES cells. Therefore, they become chimeras.

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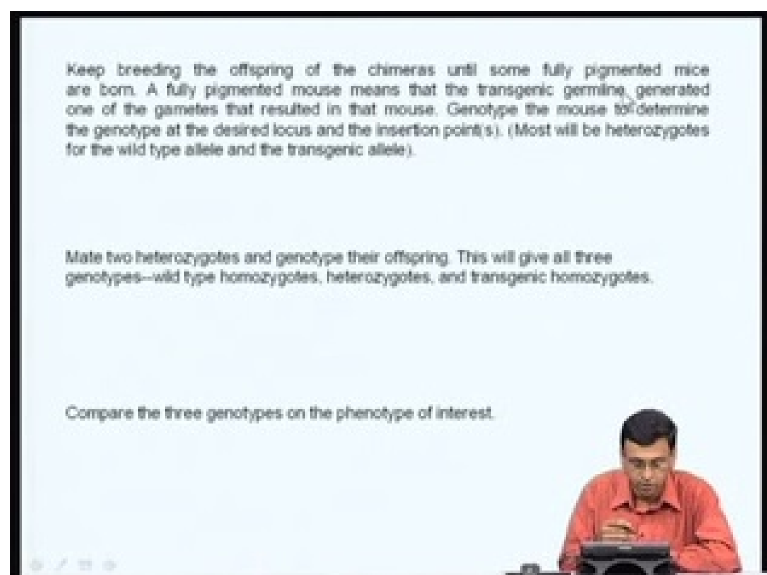

And then, you bring them further and then segregate them by successive breeding; you can generate homozygotes, heterozygotes, as well as transgenic homozygotes. So, by successful breeding, you can generate both homozygous, that is, mice which are homozygous for transgene; mice, which are heterozygous as well as the nontransgenic ones.

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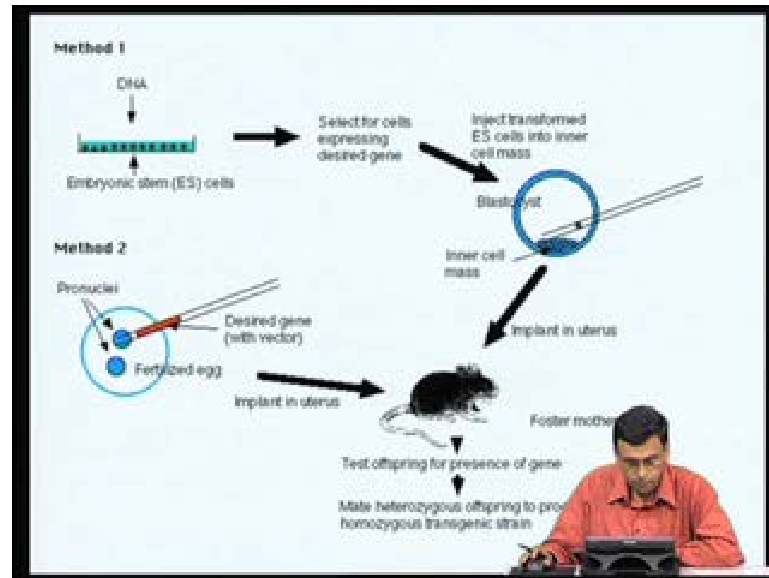

So, this is the summary of the two very popular methods of generating the transgenic mice. In one case, you inject DNA into the male pronucleus of the fertilized egg and then you implant it into the oviduct of a pseudopregnant female, and then you screen the offsprings for the presence of transgene and do your experiments. In another case, you introduce DNA into the embryonic stem cells; select those embryonic stem cells, which are expressing your gene; introduce them into the blastocyst cavity, so that along with the nontransgenic ES cells, these ES cells, both go and populate, and you get a chimeric mouse. And then, by successive breeding, you can either get a homozygote or a heterozygote.

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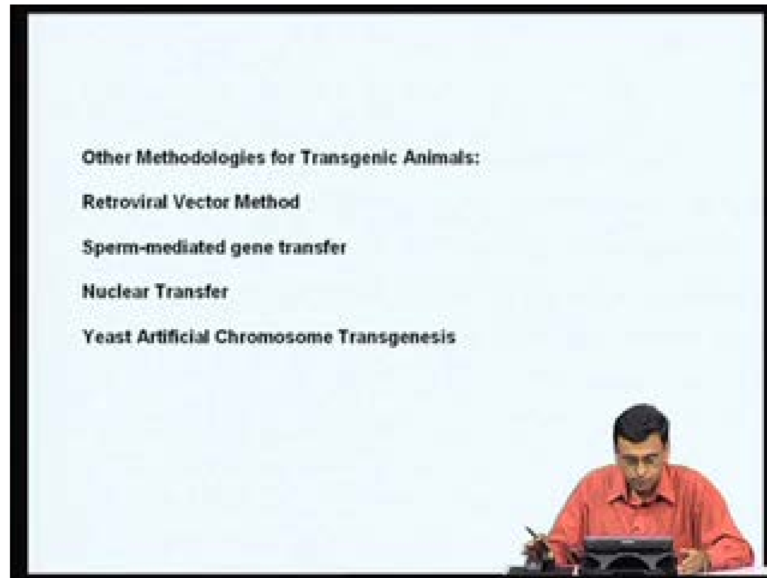

As I said, there are also other methodologies of generating; instead of electroporation, you can also introduce genes by retroviral methods; you can either use a typical retrovirus or a lentivirus for introducing transfect in the ES cells. You can also do what is called as sperm-mediated gene transfer, because sperm have the ability to penetrate genes, penetrate into egg and fertilize.

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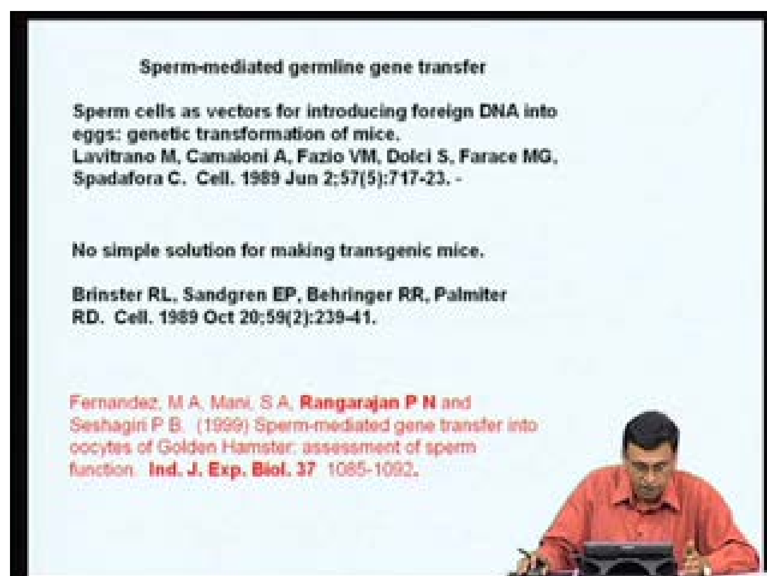

This was the classic paper, which was published in Cell in the year 1989, where Italian group led by a Lavitrano et al, they actually demonstrated that instead of doing

microinjection, you can actually use sperms as gene-transfer vehicles. So, what they did is that if you simple incubate sperm with the DNA of your interest, somehow the DNA gets into the sperm. And, when you now take this sperm and fertilize the eggs along with the sperm, the DNA also goes inside and you can do transgenic mice. This again creates a lot of excitements in the field saying that it is very simple method of transgenic mice, but many people could not reproduce the experiments. And in fact, Brinster came up with this paper saying that there is no simple solution for transgenic mice. So, somehow the sperm-mediate transfer did not become a very efficient technique of generating transgenic mice. In fact, our own group here at the Indian institute of Science actually used this kind of a sperm-mediated gene transfer to actually demonstrate that Hamster sperm can in fact, if you now add DNA, let it will able DNA to have Hamster sperm, the DNA actually goes in such Hamster sperm indicating that sperm somehow has the ability to bind to this DNA; and, they probably can be used the gene-transfer vehicles. But, somehow the sperm-mediated gene transfer did not become a very versatile technique for generating transgenic mice.

Like that, you can also do (Refer Slide Time: 37:04) what is called as a nuclear transfer. Like what we discussed in the previous classes, you can take a somatic cell and a continuous origin of your interest to the somatic cells; and then, replace the nucleus of a oocyte and put the deployed nucleus for a somatic cell. And, we do this nuclear transfer and then introduce them into pregnant mice and then generate offspring. So, of all the methods of generating transgenic mice, the ones which became very popular are the microinjection and the use of ES cells for generating transgenic mice. These are the two most popular methods of generating transgenic mice.

When you generate transgenic mice, the gene that you have introduced, the gene into the germline do not get expressed all the time. There are many problems in this transgenic technology. Some of them, for example, it can be multiple insertions. You have no control over the no of copies that get integrated. Many a times, the gene gets inserted in multiple copies in a head to tail fashion. And, in such cases, you are not really mimicking the exact physiological regulation. And, many a times, you have overexpression and you are making too much of protein; and, if that protein is a very important protein or a transcription pattern, it can have very deleterious effects.

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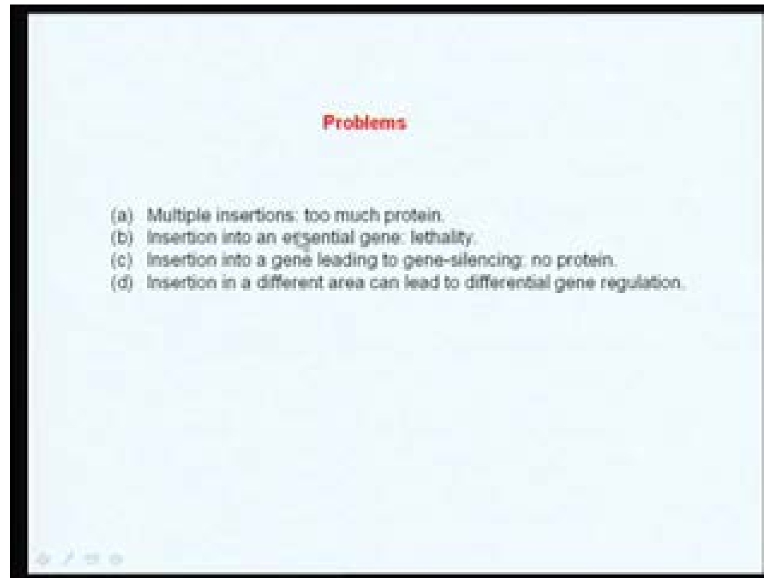

So, multiple insertions leading to too much of protein can post problems; or, the transgene may go and insert into an essential gene. Again, we see this was another problem when we discussed about gene therapy, where we used retroviral vectors. When the retroviral vector went and integrated into the genome, it inactivated, it activated an oncogene; and, as a result, it led to cancer. The same way, the transgene goes and randomly integrate somewhere in the genome; in the process, it may go and insert into an essential gene; and therefore, it can go lethal and you will not get an offspring.

Similarly, the insertion of random integration (( )) and goes into many problems. It can silence a gene or it can activate a gene or it can lead to differential regulation. Especially if you want to study a promoter for example, when you introduce, make a transgenic mouse, the gene goes and integrates in a region in the chromosome, which is quite different from the natural place, where the promoters is active. For example, if it goes and integrates a heterochromatic region, the promoter may not function at all, because you need to know that you need to go and integrate the euchromatic region. So, the context in which the transgenic promoter is functioning is not exactly the same as the native promoter. So, many a times, if you want to understand this function of a promoter, that may not exactly reflect the actual promoter functioning vivo, because it is being expressed from a different chromosome location. So, there are problems in this transgenic technology. But, by enlarge, I showed you many examples that transgenic

technology has been successfully used to understand the function of a number of promoters as well as for expressing the number of genes of interest.

Let us spend some time now to understand what the applications of this transgenic technology are. I already highlighted some of the applications. And, most of the basic research was actually done using mouse as a model system. The entire transgenic technology was developed for generating transgenic mice; and, by introducing promoters or by introducing genes of your interest, a number of interesting results have been applied. But, what happened in the years to come, after 1982, in Brinster and Palmiter paper, the transgenic technology was extended not only for mice, but also for the domesticated animals like cattle, goat, pig, birds and so on and so forth. So, what were the applications of using the transgenic technology in these other animals?

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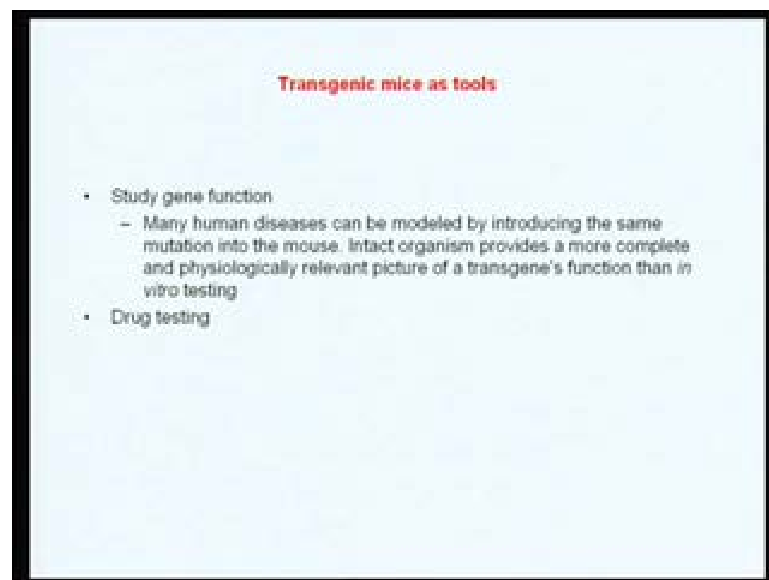

As I mentioned, in the case of the mouse, the transgenic mice became an excellent tools for understanding gene function. And, I gave you few examples earlier, how you can study the function of novel genes by making transgenic mice. Many human diseases can be modeled by introducing the same mutation into the mouse. And, intact organism provides a much more complete and physiological relevant picture of a transgene's function than in vitro testing. So, many a times, when you want to understand the function of a gene or when you want to study the effect of a mutation in a particular gene, many times when you do these things in cell lines, you will not actually get the

same picture that happens in the normal physiological situation. So, studying these mutations in animal models on the exact tissues, can give it a more better reflection of what is happening in the normal physiological situation than testing them in cell lines. So, the transgenic animal models became an excellent system to understand mutations of certain genes and how do these mutations affect gene function in an animal context.

Similarly, drug testing – I already explained; gave one example how using a human-specific before 50 and expressing a human before 50 gene. And, in a most genome 50, you can develop a better appropriate model and use it for drug testing.

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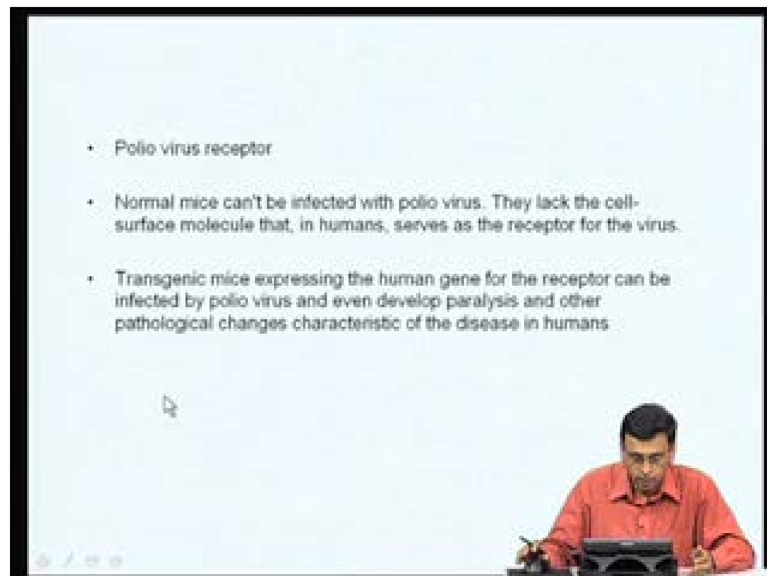

There is another example, which again tells you, how transgenic technology can be used. Polio virus receptor – for example, the mice cannot be infected with the polio virus, because for the virus to enter our cells it has to bind to a specific cell surface receptor. Normal mice cannot be infected with the polio virus, because they lack the cell surface molecule that in human surface as a receptor for the virus. So, mice cannot be infected with the polio virus. So, what do you do? You generate transgenic mice expressing the human gene for the receptor that can be infected with the polio virus. And, when you do that, these mice cannot only be infected with the polio virus, but it also develops the same kind of paralysis and all other disease symptoms that is normally seen in the humans. So, by expressing the gene coding for a polio virus receptor, you can now generate a mouse model, which exactly mimics a disease that happens in the human. It

can now be infected with the polio virus; it even develops the same kind of paralysis that happens in the humans. Now, you can actually study lot of things by using this kind of disease models. This is just one example to tell how transgenic mice have been very useful in understanding certain biological aspects.

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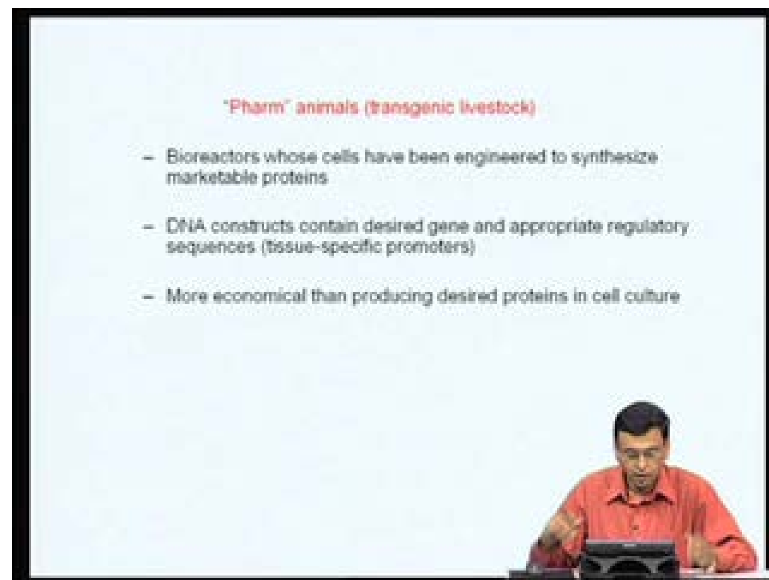

Now, coming to farm animals; instead of introducing genes to the fertilized egg of mouse, you now introduced fertilized eggs of cattle or goat or sheep and generate transgenic animals, farm animals. And, when you do this, if you for example, introduced a gene for expressing factor 8 or if you can use a gene expressing factor 9 or growth hormone, now you have a transgenic animal or transgenic cattle or a transgenic goat that is expressing this recombinant protein. So, you do not have to express these genes in mammalians cells and culture or you do not have to express the genes in yeast or equalized cells; you can express animals. And, these animals will now start producing protein of your interest. So, we can actually convert animals into bioreactors, whose cells have been engineered to synthesize marketable proteins; that is, you can sell these proteins and make money out of them. So, people usually use what are called as tissue-specific promoters for expressing these kinds of genes; and, it turns out, making these kinds of recombinant proteins in these kinds of farm animals, which is much more economical than producing these proteins in cell culture. And, this is much more economical than mammalian cell culture, because mammalian cell culture is very expensive.

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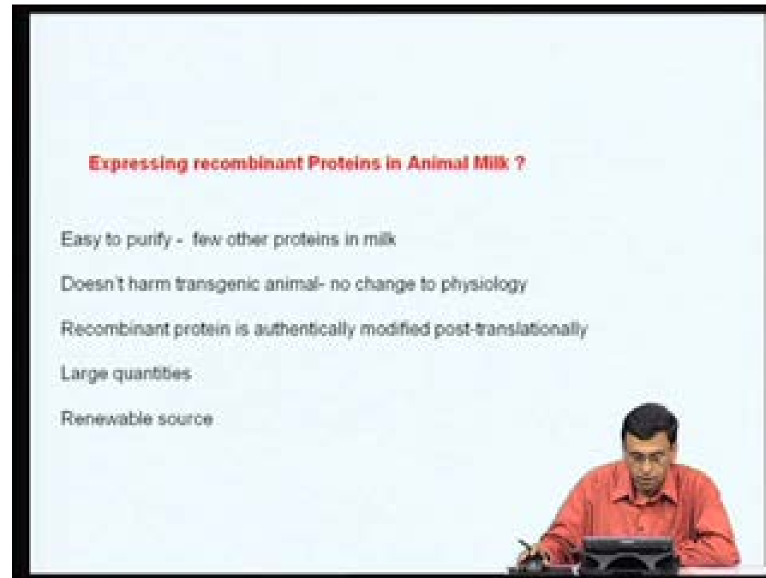

And, a very popular method of making this recombinant proteins and transgenic farm animals is actually by expressing these recombinant proteins in the animal milk. Why is that? This is because milk is a byproduct; it is very easy to purify, because there are very few other proteins in the milk; there are few proteins in the milk. So, you need to have less downsteam processing, less number of protein purification steps to purify your recombinant protein; whereas, if we express the same protein in animal systems or a mammalians cells, because there are so many other proteins, the purification protocols are much more rigorous. You require lot more steps of protein purification by expressing them in mammalians cells than expressing the proteins in milk. It does not harm the transgenic animals, because this is just a byproduct and the gene is expressed only in the mammary gland. Therefore, it is not going to harm the animal.

The recombinant protein is authentically modified post-transitionally, because you are expressing in the mammalian system. You can make large quantities, because there are very powerful promoters, which are expressed in the mammary gland. So, you can use mammary gland-specific promoters; like for example, casein is one of the most permanent proteins present in the milk. Therefore, you can use the promoter coding for the casein promoter and put your transgene, so that the gene of interest can be produced in large amounts in the mammary gland. And, it is a renewable source, because the animals can be propagated; and, not only the animal, the entire offspring of the animal will generate revenue for you.

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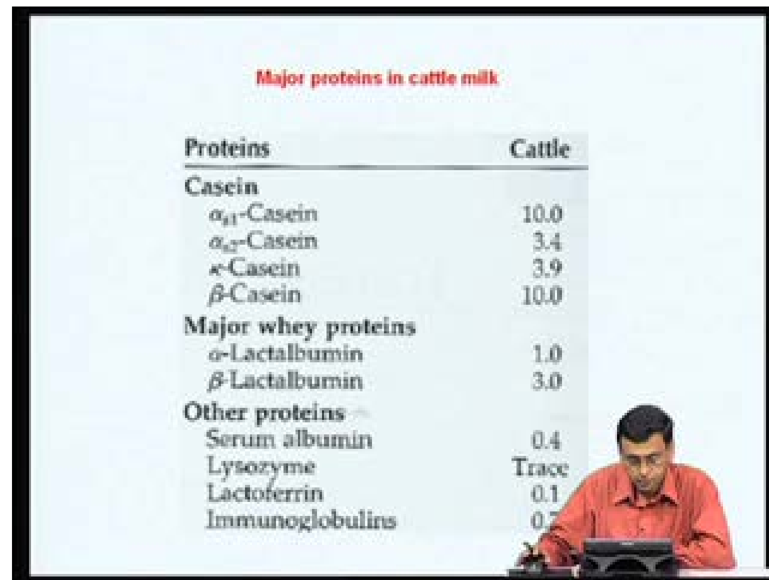

Major proteins in cattle milk

Proteins	Cattle
Casein	
α_{s1}-Casein	10.0
α_{s2}-Casein	3.4
κ-Casein	3.9
β-Casein	10.0
Major whey proteins	
α-Lactalbumin	1.0
β-Lactalbumin	3.0
Other proteins	
Serum albumin	0.4
Lysozyme	Trace
Lactoferrin	0.1
Immunoglobulins	[illegible]

These are just some of the major proteins, which are present in the milk. And, you can see casein, certain V proteins like lactalbumin and other proteins like serum albumin, lysozyme, lactoferrin, immunoglobulins and so on and so forth. So, you use any one of these mammary gland-specific promoters, especially some of the most permanent proteins like casein for expressing the transgene of your interest, so that the protein can be produced in large amounts in the mammary gland.

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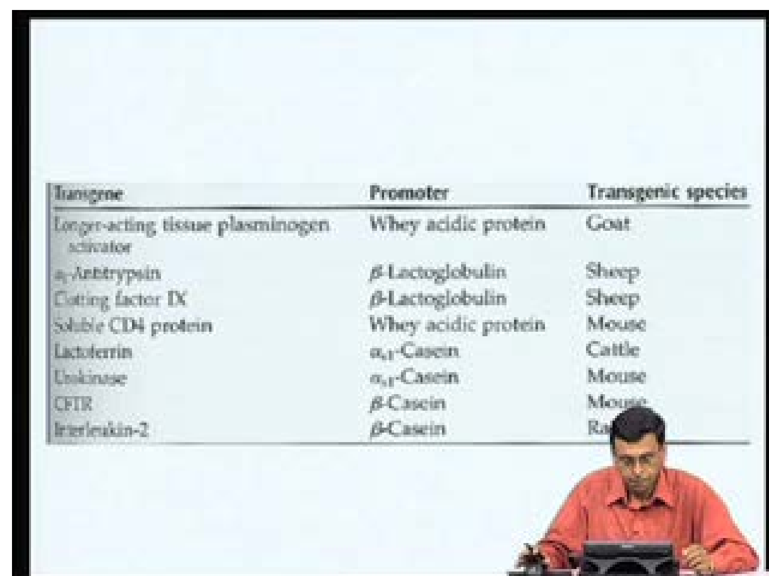

Transgene	Promoter	Transgenic species
Longer-acting tissue plasminogen activator	Whey acidic protein	Goat
α_1-Antitrypsin	β-Lactoglobulin	Sheep
Clotting factor IX	β-Lactoglobulin	Sheep
Soluble CD4 protein	Whey acidic protein	Mouse
Lactoferrin	α_{s1}-Casein	Cattle
Urokinase	α_{s1}-Casein	Mouse
CFTR	β-Casein	Mouse
Interleukin-2	β-Casein	Ra

So, this is just some of the examples I have listed here, where specific mammary gland-specific promoters have been used for making economically important proteins like for example, the whey acidic protein promoter has been used for expressing tissue plasminogen activator, which dissolves in blood clots; and, transgenic goats have been made. Similarly, beta-lactoglobulin promoter have been used for expressing alpha 1-antitrypsin, which can actually be used for what is called as pulmonary emphysema a disease that affects lungs. Same promoter has been used for expressing clotting factor 8, 9 and transgenic sheep have been produced. Similarly, you can read up a few more; using this mammary gland-specific promoters, very useful proteins like lactoferrin, urokinase, the cystic fibrosis transmembrane conductance regulator, interleukin-2 – all these genes have been expressed in a number of animals including mouse, sheep, cattle, rabbit, goats and so on and so forth.

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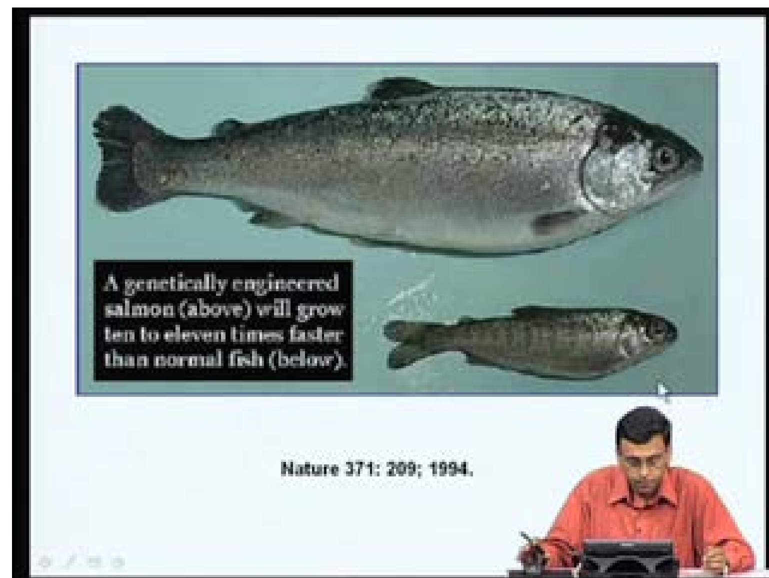

And, transgenic fishes are also been generated; here is an example. If you now express growth hormone in the salmon fish, these salmon grow much faster. And, this is a non-transgenic fish; and, it is a transgenic fish. You can see, the fish grows eleven times faster than the normal fish. So, it is actually published in Nature in 1994. Of course, this does not happen in all the fish species, but certain fish species can be made to grow faster by expressing growth hormone in the transgenic fish.

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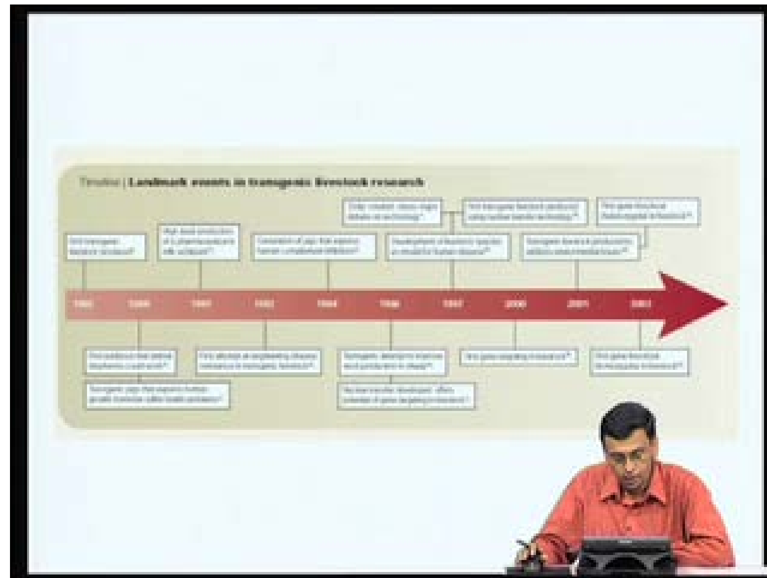

This just summarizes a number of events that have taken place following the development of transgenic technology by Brinster and Palmiter ever since the development of the transgenic mouse in the year 1982 with a visible phenotype. The first transgenic farm animal was produced in 1985. And, ever since, a number of development have taken place; I will not into details; but, this transgenic technology has made a very important difference leading to a number of very interesting events. And, one go through this time chart to see what are the land mark events that actually took place in transgenic livestock research.

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This is another example. Here is a researcher, who is trying to again generate new kind of transgenic animals. What she found is that the triglycerides found in the milk of echidna, a primitive monotreme, defers from those found in the milk of other mammals. In that, they have a fatty acid distribution similar to that found in the vegetable oils; or, the animal fat is different from the oils, which are present in the plants. And, most of the vegetarians actually use only vegetable oils; they do not use the oil fat. And, what she is now trying is, that can you actually clone and characterize the substate specificity of the echidna triglyceride biosynthetic enzymes with a view of making vegetarian milk. For example, can you now express these kinds of genes in the animals, so that you can now get milk, which actually contains these triglycerides, which are normally present in the plants rather than the triglycerides, which are normally present in the animals? Again, this I will publish in some of the papers; one actually go through and then read up little bit more. These are all just examples to see what kind of genes you can express and how you can generate revenue out of very interesting ideas and making useful proteins or expressing useful genes in transgenic animals.

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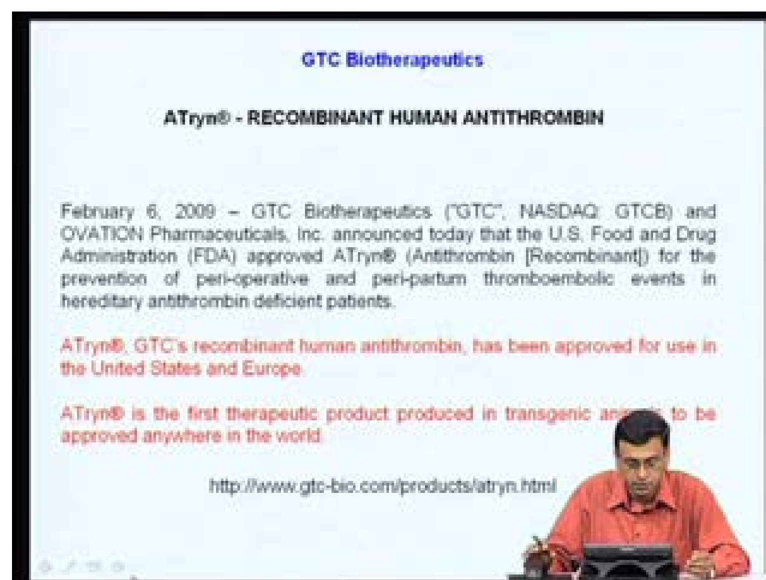

Since we are talking about this course on gene expression: basics and benefits, wherever possible, I have been telling you what products or processes that came out of these kinds of basic research; here is another example. Company called GTC Biotherapeutics actually produced the first recombinant human antithrombin, produced using transgenic technology. So, GTC Biotherapeutics in February 2009, announced that the United

States Food and Drug Administration approved antithrombin for the prevention of peri-operative and peri-partam thromboembolic events in hereditary antithrombin defective patients. So, it is a company called GTC Biotherapeutics, which actually developed the first recombinant human antithrombin. So, in February 2009, this company announced that the United States Food and Drug Administration actually approved what they call as A T R Y N – ATryn, which is nothing but antithrombin recombinant for the prevention of peri-operative and peri-partam thromboembolic events in hereditary antithrombin deficient patients. And, this is the GTC's recombinant human antithrombin, which has been approved for use in the United States. And, this is the first therapeutic product produced in transgenic animals to be approved anywhere in the world. So, thanks to the efforts of (( )) Brinster and Palmiter, which started way back in 1982.

Today, we actually have a product, which have been approved for human use produced in a transgenic animal. So, many such recombinant therapeutics likely to come out. So, transgenic farm animals can be used as bioreactors for making proteins. And, these proteins go through clinical trials and can get approvals from regulatory agencies, can actually be used in humans for certain therapeutics purposes. So, one can actually go to the website of gtc-bio to look and see what all the other products that are likely to come out of this company.

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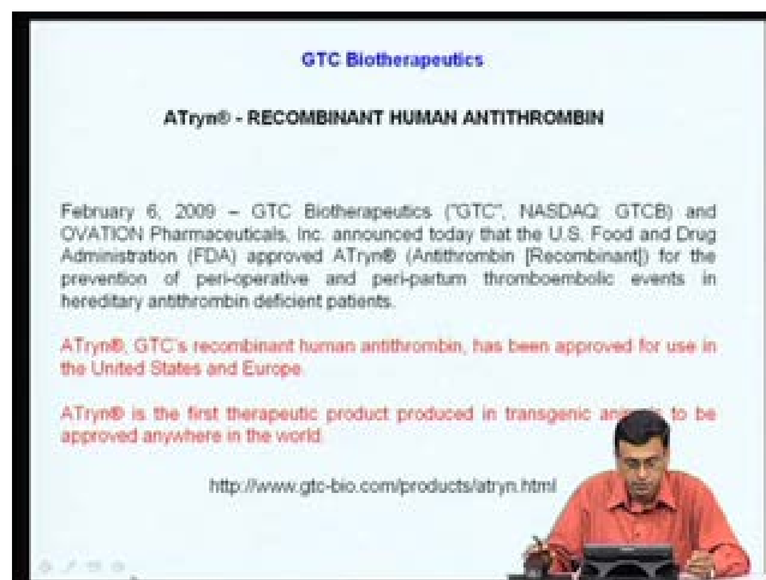

In fact, the company says that they are in the process of producing a number of useful proteins in transgenic animals, for example, coagulation factors like factors 8 and factor 9 and so on and so forth in collaboration with one other company called LFB Biotechnologies, using the transgenic production platform. They are establishing the transgenic rabbit production system for the production of recombinant factor 7a for the treatment of patients with hemophilia. They are also trying to establish a transgenic goat production system for the production of certain monoclonal antibodies for treatment of specific cancers. So, indicating that, companies have now come forward to make use of this transgenic technology. Transgenic farm animals are being now produced, which can express many therapeutic proteins. And, some of these proteins have either got regulatory approvals or in the process of getting regulatory approvals for use in humans.

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There are many other companies, which are using this transgenic biotechnology for making human proteins. There is some company called Revivicor, Alexion Pharmaceuticals, Sygen International, BresaGen. One can actually go to the websites of these companies and see what kind of exciting research is going on in these companies, and how many more recombinant therapeutic proteins are likely to come out of these transgenic animals.

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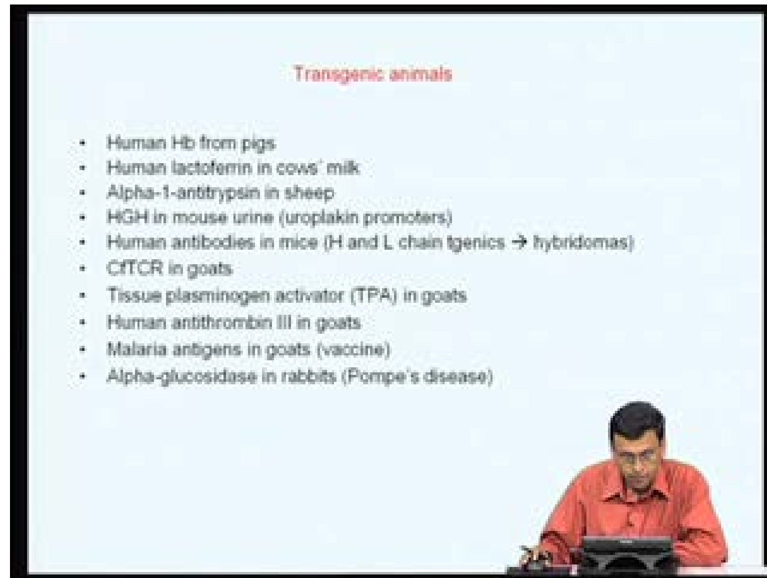

This is just a list to show that what all the proteins that are being produced in transgenic animals: human hemoglobin from pigs; human lactoferrin from cows' milk; alpha-1-antitrypsin being produced in sheep; human growth hormone in mouse urine using uroplakin promoters; human antibodies in mice; tissue plasminogen activator; antithrombin; malaria antigens are being expressed in goats for making malaria vaccines; alpha–glucosidase – these are like Pompe's diseases and so and so forth. So, a number of useful proteins are likely to be expressed or being expressed in this transgenic farm animals. And, many of them are likely to become products after getting regulatory approvals.

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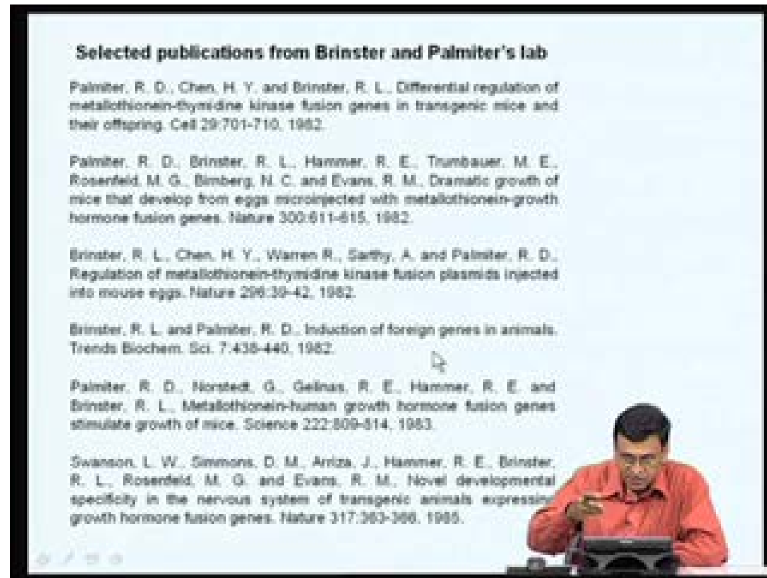

So, I have just listed some very interesting and important publications that actually came in the 1980s, once Brinster and Palmiter discovered this transgenic technology. One can go through some of these publications to see what was the excitement that was generated in the early 1980s when these two pioneers generated this transgenic technology.

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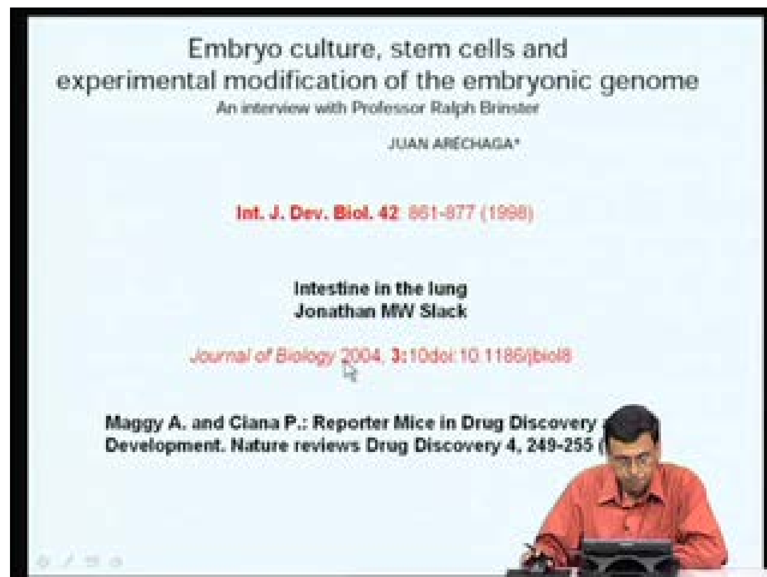

It was also a very nice interview with Ralph Brinster, which was published in this International Journal of Development Biology. And, he explains how exactly he developed this transgenic technology; and, what all the interesting excitement he went

through; how he began his experiments ultimately culminating the development of that growth hormone expressing transgenic mice. There is another interesting review called intestine in the lung. As he just explained how by expressing the Wnt genes in the lung, you can actually transform the lung cells into intestine cells. This is the nice review in the Journal of Biology. There is again a very nice review in Nature Drug Discovery about using Reporter Mice in Drug Discovery and Development. And, one can study this review to understand how transgenic animals are being used for drug testing by expressing things like P450 genes and so on and so forth. So, I think I will stop here.

And, in the next class, we will discuss about generating what is called as a knockout mice. So far, we discussed about expressing genes, overexpressing genes and generating transgenic mice. In the next class, we will discuss how we can knockout a gene; how you can delete the function of a gene; and, how this technology was developed; and, where we are heading; what is the basic as well as benefits that came out this knockout technology.

Thank you.