**Eukaryotic Gene Expression**

**Prof. P N Rangarajan.**

**Department of Biochemistry**

**Indian Institute of Science, Bangalore**

**Lecture No. # 38**

**Regulation of Eukaryotic Gene Expression by Small RNA's**

**(RNA interference, RNAi)**

Welcome, to this Lectures series on eukaryotic Gene Expression basics and benefits. Today, we are going to discuss about a very fascinating and, very interesting area of regulation of Gene Expression eukaryotes. That is about regulation of eukaryotic Gene Expression by small RNAs, a phenomenon known as RNA interference or RNAI.

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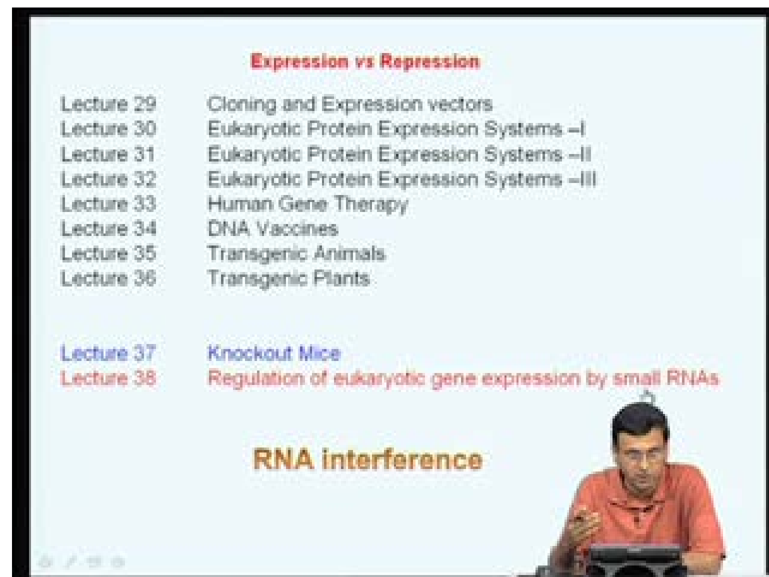


Now, the last few Lectures especially from lecture 29 to 36, we focused our attention primarily on expressing genes like, we started with basic cloning and Expression vectors. And then we looked at some of the basic Expression systems in prokaryotes, and then we moved to expressing genes in to yeast and insect cells. Then we discussed about viral and non Gene non viral Gene delivery techniques, for expressing genes and mammalian cells.

Then we looked at some of the applications of expressing genes, in vivo we discussed extensively about human Gene therapy. How by expressing genes we can cure human diseases or at least we are attempting to cure, human diseases which still have not succeeded. Then we moved on to another very interesting area called as DNA vaccines, how we expressing antigen genes, can lead to evoking in immune response leading to DNA vaccination. Then we discussed about transgenic animals transgenic plants. And in the last class instead of discussing about, expressing various genes in various systems we discussed about knocking out genes.

So, we discussed about KNOCKOUT mice KNOCKOUT rats, and so on and so forth. Where, technology was developed to specifically KNOCKDOWN or KNOCKOUT the Expression of a particular gene. So, that the protein cannot be made and how this Knockout technology has been very useful, in understanding Gene function. And why this technology of developing Knockout ultimately got the Noble prize.Today we are going to discuss, about another very interesting area of again repressing the Expression of genes and eukaryotes. Namely regulation of eukaryotic Gene Expression by small RNAs. And I am going to discuss about or introduced to a very fascinating area of research known as RNA interference or RNAI.

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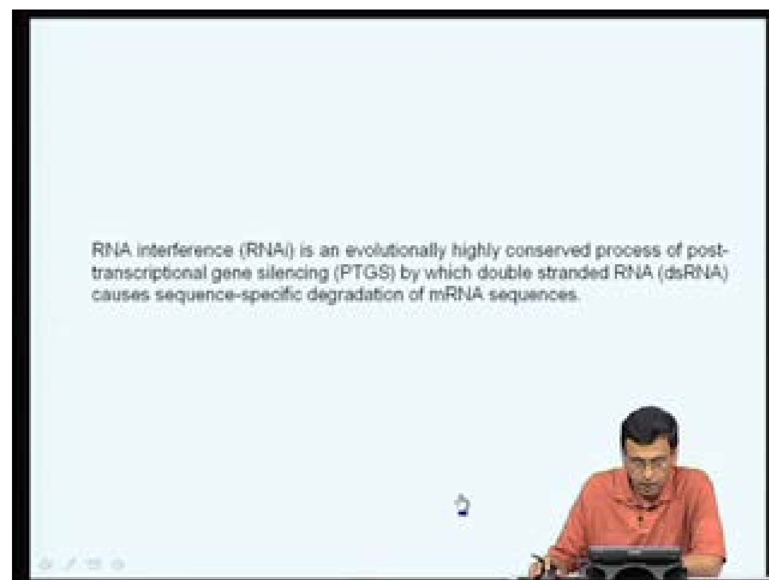


What is RNA interference, let us first define RNA interference and then try to understand what RNA interference is. RNA interference or RNAI as it is popularly known is an

evolutionally highly conserved process of post transcriptional genes silencing. Remember this is important; it is a highly conserved process of post transcriptional genes silencing .That is this silencing happens, after transcription by which double stranded RNA causes, sequence specific degradation of mRNA sequences. Remember, RNA interference is a post transcriptional event. It involves formation of double stranded RNA and once double stranded RNA is, formed inside eukaryotic cells that somehow silences the homologous genes sequences.

So, the Expression of the corresponding genes with as, homology to the double stranded RNA gets abolished and leading to loss of Expression of that particular gene. You will be able understand better once we explain, what is RNAI then the definition will become much more clearer.

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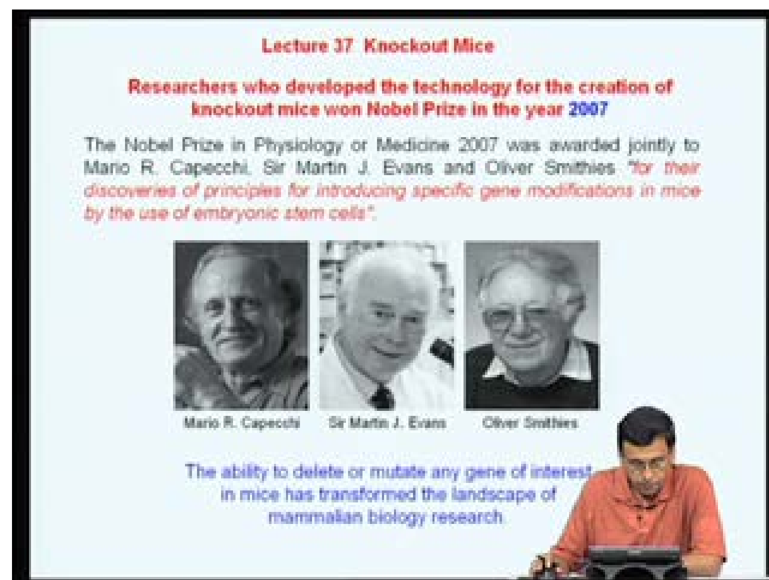


As I, said in the last class. I had discussed about Knockout mice that is the technology for developing. The technology for knocking out genes specifically, in the germ line leading to creation of what are called Knockout mice was, one of a important landmarks. In the area of, the eukaryotic Gene Expression and because of its importance. The people who discovered this Knockout technology were actually, awarded a Noble Prize in the year 2007.

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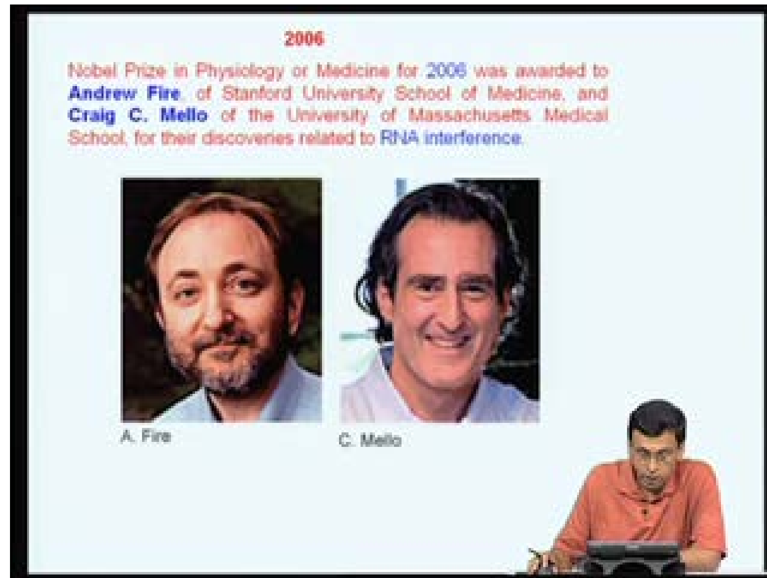


Now, today again we are going to discuss about another Noble Prize winning work. The Noble Prize for discovering RNA interference was, awarded in the year 2006 for Andrew fire and Craig Mello. So, RNA interference is a very, very important discovery. And since it has been awarded a Noble prize that means, it as really had profound implications, in the area of biology and medicines. So, let us now try to understand, what is this Noble Prize winning work of Andrew fire and Craig Mello. And what exactly how exactly, RNA interference has changed research in biology and medicine.

So, as is important it always important to understand the history of, some of these important developments that took place in the, area of biology and medicine. So, I am going to briefly tell you about what is the history of RNAI how is this RNAI was discovered. Now, at least we can trace back to the year nineteen ninety. When the first concept of something that is, repression of Gene Expression takes place when you put double stranded RNA inside the cells.

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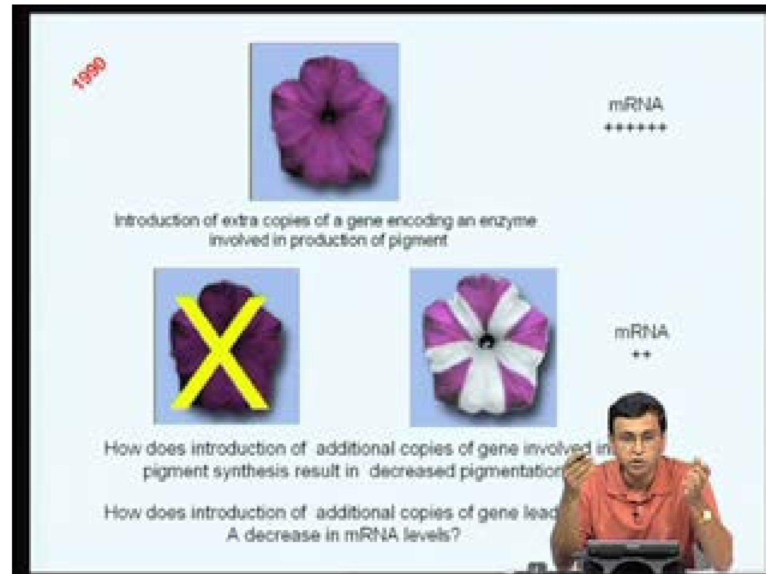


This initial idea came sometime in the year nineteen ninety. This was actually, came from plant biology. Researchers working in the area of plant biology, they found that if you take plants like petunia which produce beautiful colors. For example, here is a petunia flower. When they express the Gene that actually, course for an enzyme involved in this production of this pigment they actually expected. That they should get a plant with, flowers which are much more, more dark purple in color. So, this is a purple color flower when you express a Gene that is responsible for, producing this particular purple pigment.

When you over express, the Gene you should actually get much more dark purple color flower. But instead, what they actually got is a flower which is paler and there are a actually, many regions of this flower where there was no pigmentation at all. So, this was totally unexpected. So, instead of getting a flower with a, dark purple more dark purple color you got a flower which is paler. And In fact, there was a certain way variegated kind of a pattern, in which certain area flowers did not have color at all. So, this was very puzzling. So, how introduction of, additional copies of a Gene does involved in, pigments synthesis result in decreased pigmentation. This was not expected what they expected is this, but, what actually happened was this.

When they, actually looked at the RNA. That is levels of RNA that is actually present for this particular Gene in both. The plant where they are introduced extra copies as well as

the normal plant surprisingly they found. That if this is the level of m RNA for that particular pigment producing Gene. In the normal flower, when you introduce additional copies of this Gene instead of finding much more RNA, they actually found less RNA. So, this was against the dogma, you introduced extra copies of a Gene and expresses it more you should actually see much more RNA. But you ended up seeing less RNA and as a result you got less pigmentation, rather than more pigmentation.

So, again the question is how does, introduction of additional copies of a Gene lead to decrease in lessen RNA levels. Because all in previous Lectures if have not talk talking only about expressing genes, we want to make more protein. So, we expressed how we can introduce genes in to plants how we can introduce into genes into yeast insect cells. So, that we can make much more of protein. But here now I am saying here is, a situation where I put extra copies of a gene, but instead of finding more RNA and more protein I am finding less RNA and less protein. So, this against what we have been discussing. So, let us now try to understand why is happened, why instead of getting a darker pigment and more messenger RNA why ended up getting less RNA and a paler pigment color.

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This phenomenon which was first discovered in plants, as well as a Neuro spora which is a fungus at that time people did not understand what exactly is going. Because it beat beats the normal conundrum of the biology. So, they simply called it as post transcriptional Gene silencing PTGS. That is after the RNA is formed, some of the RNA

is getting degraded. This phenomenon which was originally found in plants, like petunia was later observed in the fungus Neuro spora crass as and it was, called either quelling or PTGS post transcriptional Gene silencing.

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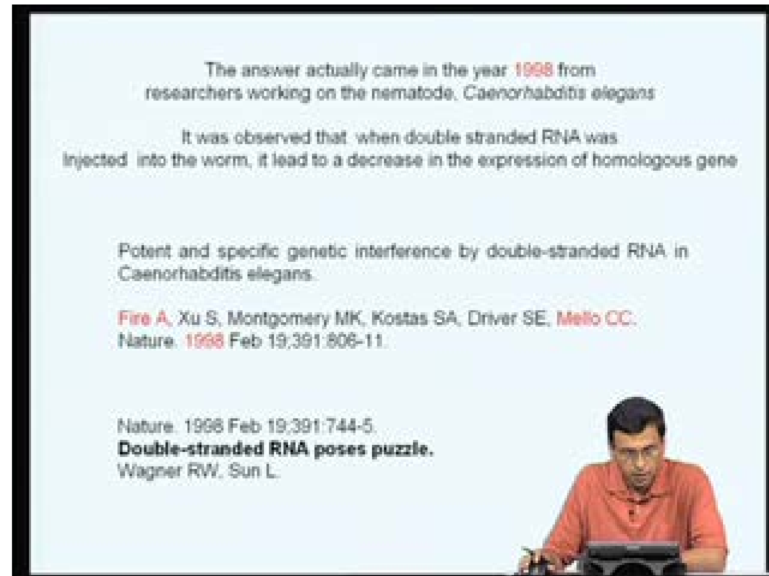


These things were happening sometime around nineteen ninety. For almost eight years people did not understand what exactly was going on. All though people started describing this phenomenon in number of physiological situations in variety of species the answer actually came in the, nineteen ninety eight. By researchers working on a nematode called caenorhabditis elegans. So, C elegans is a very, transparent nematode it is a wonderful organism to study development of biology. So, people who are actually using this as an model system, model organism for understanding embryonic development.

And when they were working with this, they unexpectedly stumbled upon a very interesting observation that actually, opened up an entirely new area and did let to the phenomenon of RNA interference. Now, what was this they observed? It was observed that, when the double stranded RNA was injected to the worm it lead to a decrease in the Expression of the homologous Gene. This was the observation that Andrew fire and Craig Mello, with the landmark paper was published in nineteen ninety eight. Potent and specific genetic interference by double stranded RNA in caenorhabditis elegans and,

there was a news and views nature on this, particular research article saying double stranded RNA poses puzzle.

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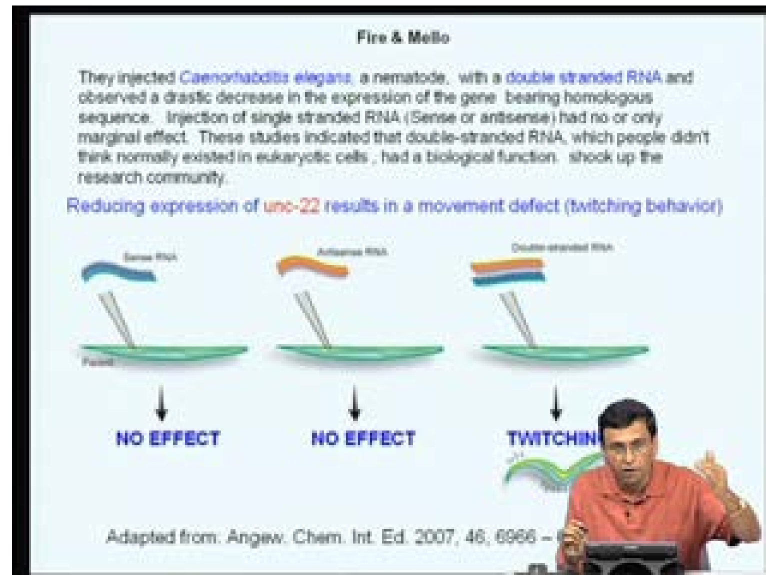


Because this was nobody, could explain this unusual phenomenon. Lets now, see what exactly this authors did what is the key experiment and the key result of this, particular paper, this is what fire and Mello reported in this paper. When they inject a caenorhabditis elegans which is a nematode with a double stranded RNA. And observed they observed a drastic decrease in the, Expression of a Gene where in homologous sequence. Now, injection of single stranded RNA either, the sense strand or the antisense strand had no or only marginal effect.

These studies indicated that double stranded RNA, but not single stranded RNA. Which people did not think normally existed in eukaryotic cells had a biological function. This shook up the entire research community. So, I am just I am just schematically show you what exactly, is this experiment. This is the experiment here is a caenorhabditis elegans worm, which are transparent worm you can just take a Micro injection pipet. And inject directly into cells of your interest DNA RNA or any protein or any solution. So, you can study a number of biological phenomena and study various regulators of Gene expression.

So, when the injected the sense RNA into this worm, for a particular Gene. The Gene they have chosen they called as unc-22 and it turns also in earlier studies, that if you

reduce the Expression of this unc-22 by other approaches. It resulted in a movement defect. So, as long as you have normal levels of unc-22 rather worm was normal. But when you reduce the Expression of unc-22 the worm started twitching. It is called as a twitching behavior. So, it starts twitching. So, the levels of unc two determines whether the worm moves normally or whether worm will twitch.

So, what they did when they took the sense RNA, for this unc-22 and they injected into the worm nothing happened. When they took the Anti-sensed RNA it had very little effect or a very marginal effect or almost practically there has a no effect. Very interestingly, when you combine these two and injected in a double stranded RNA you started to, doing a twitching effect. And they had evidence to show. That when you injected double stranded RNA it actually, lead to a decrease in the unc-22 messenger RNA and lead to a decreased levels of unc-22 protein.

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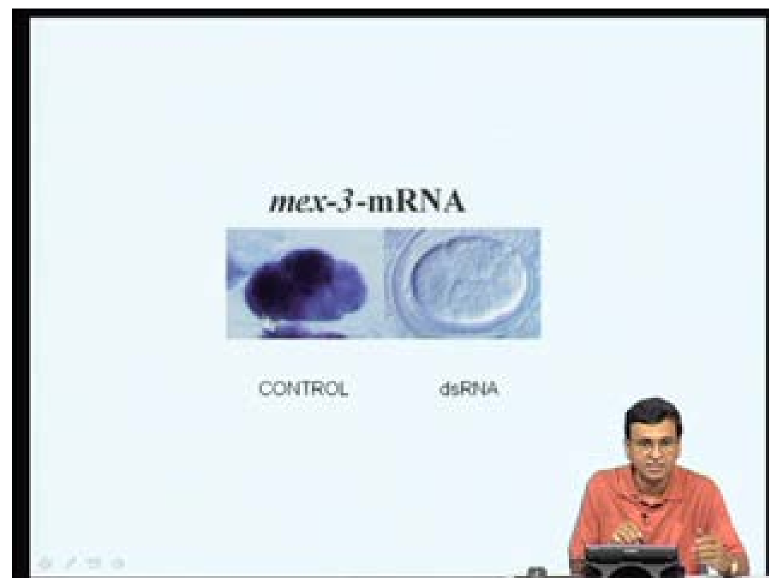


And soon these observations were followed up with number of other genes in C elegans. Here is for example, another example another mRNA called mex-3. Again in a normal animal you can see this is the levels of mex-3 as the dark blue color indicates the levels of the messenger RNA for this particular gene. Now, if you inject this double stranded RNA for this particular Gene, you absolutely seen a blue color seeing that there is no Expression of the gene. So, what was initially observed with the unc-22 was later found to be true for a number of other genes not only in C elegans. But variety of others

systems like, drosophila trypanosomes mammalian cells and so on mammalian cells was not difficult drosophila and,

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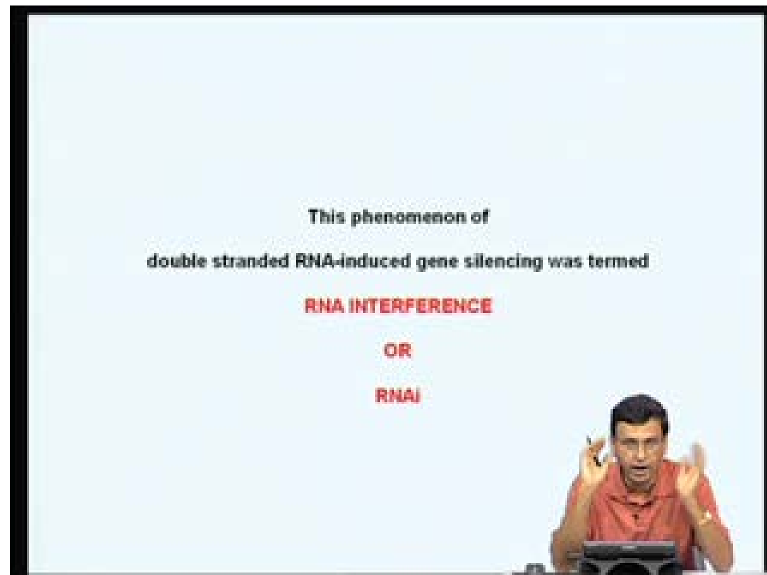


So, on and so forth. So, this phenomenon of double stranded RNA induce Gene silencing was termed as RNA interference or RNAI. So, when you inject double stranded d r RNA. Which is homologous to a particular Gene in to eukaryotic cells it results in the loss of Expression of that particular Gene, this was the phenomenon.

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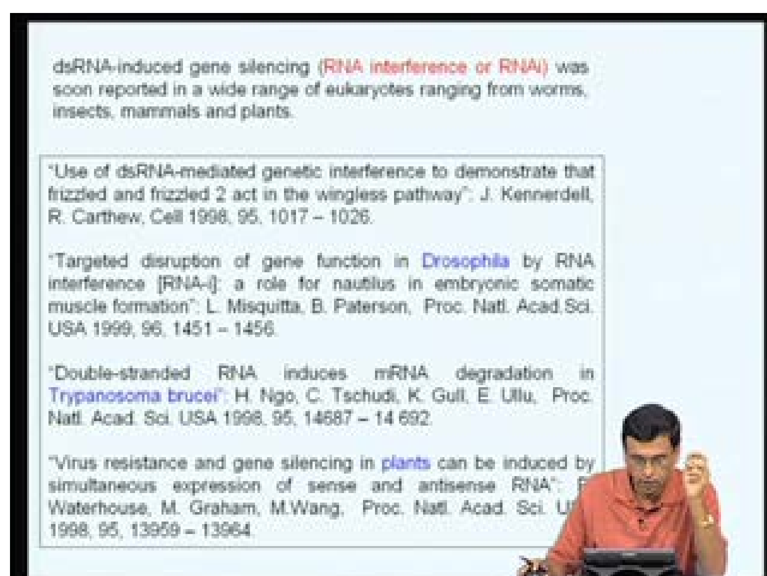

So, the double stranded RNA induced genes silencing or RNA interference or RNAI was soon reported for a wide range of eukaryotes ranging from, worms insects mammals and plants. Here some of the papers like, use of double stranded RNA mediated genetic interference to demonstrate that frizzled and frizzled two acted in a wingless pathway. Nineteen ninety eight targeted disruption of Gene function in drosophila by RNA interference a role for, nautilus in embryonic somatic muscle formation. Double stranded RNA introduces mRNA degradation in Trypanosome brucei. virus resistance and genes silencing in plants can be induced by simultaneous Expression of sense and antisense RNA.

So, this initial observation they report by Andrew Craig and, Mello that Andrew fire and Craig Mello. That very introduced double stranded RNA in to cells it can result in shutting of loss of Expression of the genes lead to a flurry of papers. And people started demonstrating that yes it is true not only for, a particular Gene in a particular in the organism, but in a variety of other organisms as well.

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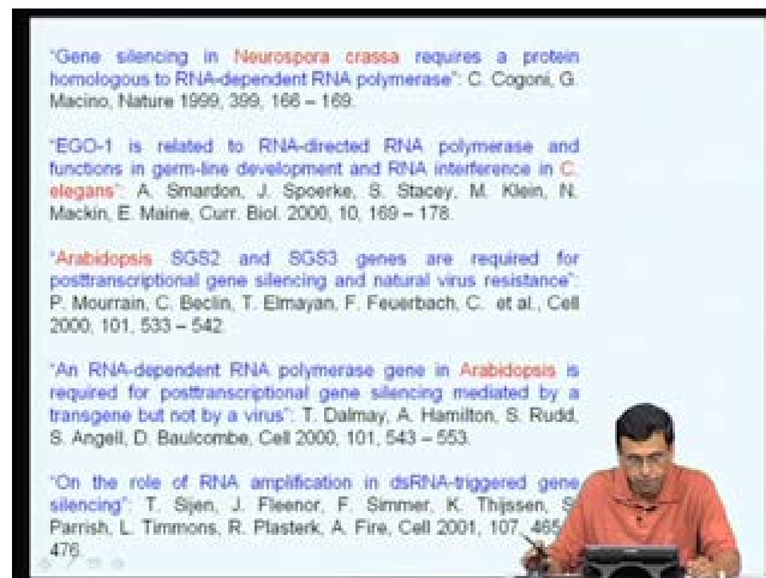


There are some more papers, genes silencing in Neurospora crassa requires a protein homologous to RNA depend on a polymerase, I think I should come to this.

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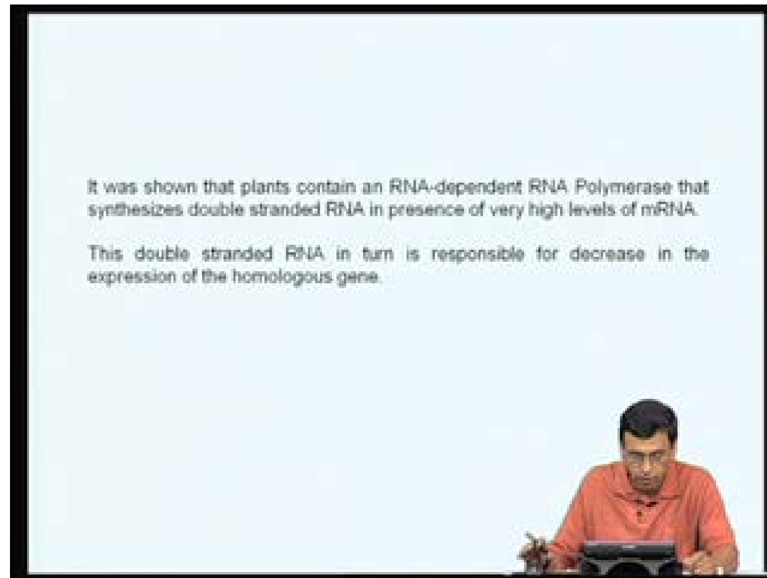


So, people you remember this phenomenon of, over expressing a Gene leading to loss of Expression was first adhered in plants. Petunia I gave an example of petunia as an example. So, once Andrew fire and Craig Mello reported that, where you inject double stranded RNA it leads to loss of Expression of a Gene. The plant biology started re looking their initial observations, which they were in nineteen nineties. And they soon discovered that the plants actually, contain an enzyme called RNA dependent RNA polymerases that synthesizes double stranded RNA in presence of, very high levels of RNA. This double stranded RNA in turn is responsible for the decrease in the Expression of the homologous gene.

So, the observation they made in nineteen nineties. So, what happened in petunia when you expressed, over express this high levels of this pigment producing Gene means of getting darker pigment we actually got a paler flower. Now, can be explained because when you put extra copies of a Gene. And when you produce, large amounts of this RNA instead of leading to higher amounts of protein, it actually synthesize lead to the synthesize of a double stranded RNA. And this double stranded RNA in turn inhibited Gene Expression that is why you had less of the protein.

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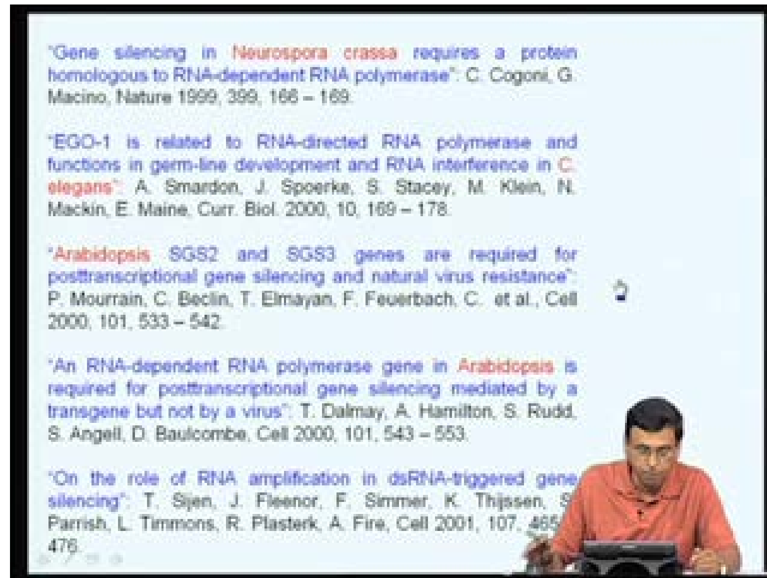


In fact, a flurry of papers came out came in both Neurospora as well as plants. Reporting that kind of a RNA polymerase, capable of synthesizing double stranded RNA is present. Like Gene silencing Neurospora crassa requires, a protein homologous to RNA dependent RNA polymerase. EGO-1 is related to RNA directed RNA polymerase and right functions in germ line development of RNA interference in C elegans. Neurospora C elegans arabidopsis say SGS 2 and S 3 Gene are required, for post transcriptional Gene silencing and natural virus resistance.

Again Arabidopsis in RNA dependent RNA polymerase Gene is required for, post transcriptional Gene silencing mediated by a Transgene. But not by a virus and on the role of RNA amplification in double, stranded RNA triggering Gene silencing. So, you can see a variety of organisms soon found to have this RNA dependent RNA polymerase. Which is capable of synthesize in double stranded RNA when large amounts of RNA synthesized, in the cells of this these organisms.

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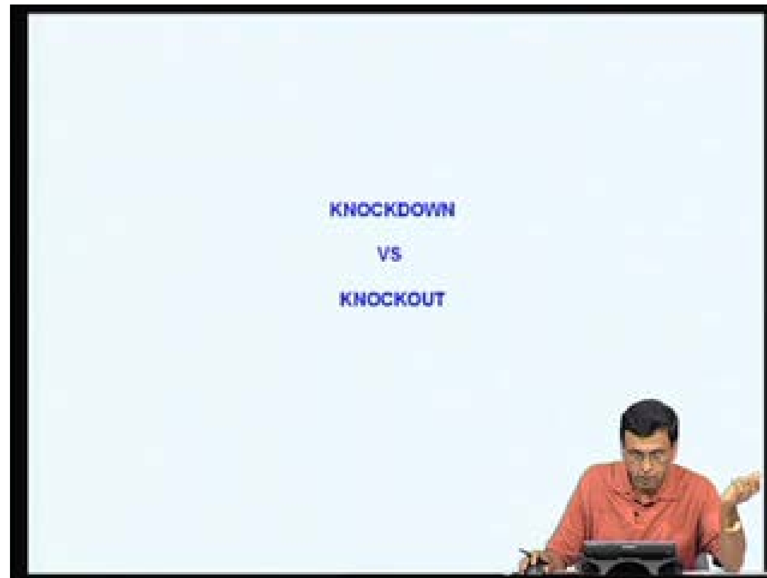


So, this observation that, when you over express when you make too much of RNA of a particular Gene. That can actually lead have a negative effect on Gene Expression it can actually result in repression of, Gene Expression was soon known as KNOCKDOWN. So, the repression of the decrease in the expressed, of a Gene as a result of introduction of double stranded RNA or as a result of RNA interference is known as KNOCKDOWN. As approach to KNOCKOUT which was studied in the previous class KNOCKOUT is primarily because of, loss of a Gene or a deletion of a particular Gene that is what we studied in the last class.

Whereas KNOCKDOWN the Gene is very much there, but due to a post transcriptional mechanism, there is a decrease in the levels of RNA leading to decrease level of protein. So, this is called as a KNOCKDOWN versus Gene deletion decreased Expression is called as KNOCKOUT. So, RNA interference results in KNOCKDOWN not KNOCKOUT.

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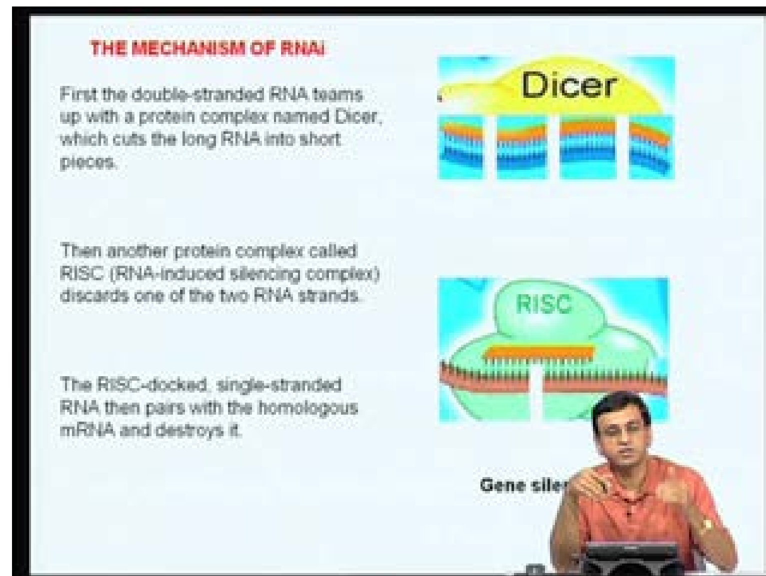


Now, lot of effort then went on to understand how does, introduction of double stranded RNA inter cells can result in decrease in Gene Expression. Took at a long story short, enormous amount of effort was spent in understanding the mechanism. I am just trying to simplify the whole thing and giving a very, schematic representation of what was the final outcome. And what we understand at present, the mechanism by which when you introduced double stranded RNA inside. The cells how does it lead to how does it result in the decrease, in Gene Expression and less amount of a protein.

Now, what happens first the double stranded RNA teams up with a protein complex called as dicer. Which cuts the long RNA in to short pieces? This is the first thing that happens. So, whenever large amounts of double stranded DNA or RNA is introduced inside the cell. This double stranded RNA is recognized, by a multi protein complex called as dicer. Remember it is not a single protein it is a multi protein complex known as dicer. This dicer recognizes double stranded RNA, and chute cuts this double stranded RNA strength that you get smaller RNAs.

Then another protein complex called as a RISC. Which is as acronym for RNA induced silencing complex RISC is RNA induced silencing complex. This RISC now, takes up this double stranded RNA discards one of the two strands and takes up only, one this what happens. So, the dicer choose the double stranded RNA in to small pieces. And then RISC takes one of this RNAs, it is of the two it will only takes one of the RNAs.

And this RNA then, the RISC docked single stranded RNA then pairs with the messenger RNA which is present inside the cell.

By homologous base pairing and once the RISC in the RISC complex when this RNAs, small RNA pairs with a m RNA that results in the cleavage of the messenger RNA leading to RNA degradation and Gene silencing. So, this is finally, the mechanism that was discovered the mechanism by which, introduction of double stranded RNA inside the cells leads to m RNA degradation. And therefore, results in the loss of protein or decrease in the levels of the protein.

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The same thing I have shown it in a very schematic manner here. So, when you have double stranded RNA inside the cells. This double stranded RNA is recognized by this dicer complex which is then cuts them into small RNAs called small interfering RNAs. These small RNA produced by dicer RNAs, siRNAs or small interfering RNAs and Then one of the two strands of this siRNA is now loaded on the RISC complex; RNA induced silencing complex. And this when it hybridizes messenger which is present inside the cell the messenger RNA is cleaved. And therefore, the messenger RNA is degraded and therefore, the protein cannot be produced.

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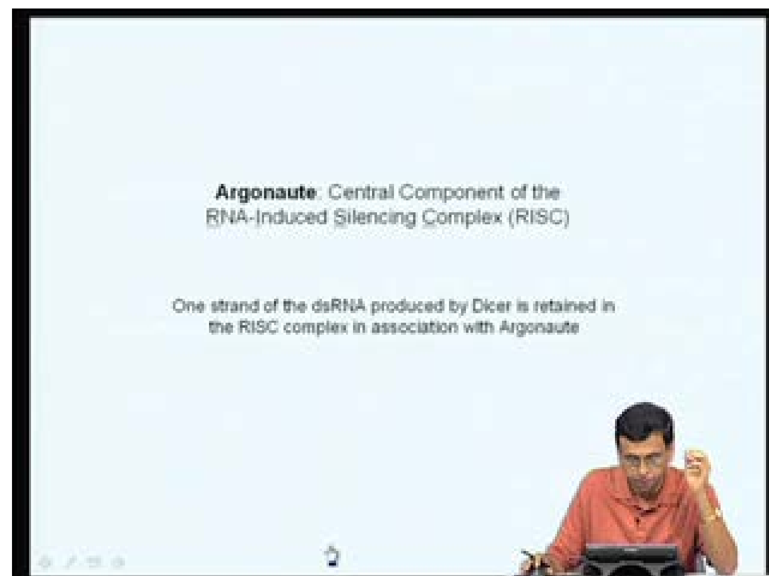

So, this is the mechanism by which Gene silencing is brought about by RNA interference pathway. Now, as I said the dicer is a multi protein complex, where lot of effort as gone in to understanding the key components of this dicer complex. And one of the key components of this dicer is called as Argonaute which is the central component of this RISC or RNA induced silencing complex. And once strand of the double stranded RNA that is produced by dicer remember. The r double stranded RNA is get shopped by dicer in to smaller pieces. And one of the strands that is produced by double stranded is retained and the RISC complex and association with Aragonite. And the strand is what that is loaded on the RISC complex therefore, it can pair with messenger RNA and then degrade the messenger RNA.

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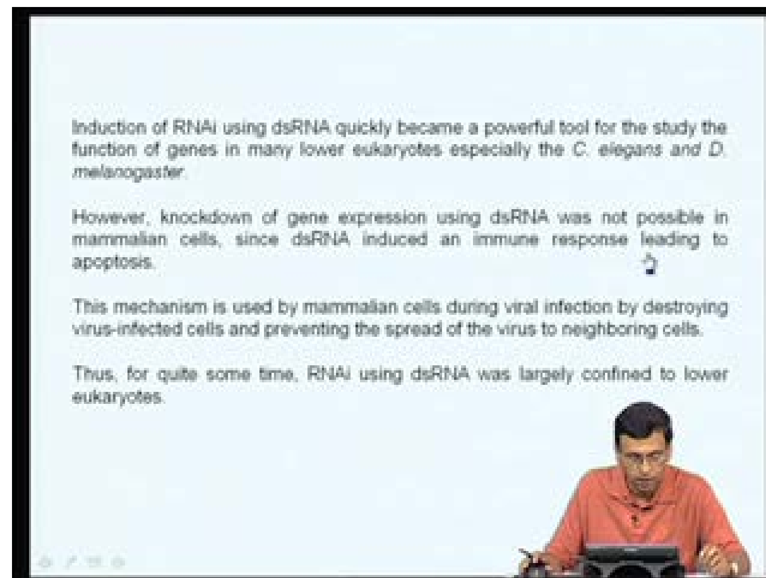


So, induction of RNAI using double stranded RNA quickly became a very, powerful tool for the study of function of genes in many lower eukaryotes especially C. elegans and drosophila melanogaster. So, you can see it became very, very powerful tool. Because we want to understand a particular function of a particular, Gene all that you have to use make double stranded RNA for the Gene. And inject in to this worm or you can even just make a plasmid construct expressing double stranded RNA. And you take these bacteria, and simply feed this worm actually feeds on bacteria.

So, if the worm just eats this bacteria, which are making this RNA then also Gene can silenced. So, they can either inject the double stranded RNA into these worms or you can

just feed them, that results in loss of Expression of the particular gene. So, instead of doing KNOCKOUTs, making the KNOCKOUT constructs, and then doing all kinds of gimmick to introduce the genes. And then KNOCKDOWN the Gene Expression Here was a very simple method by which you can KNOCKDOWN the Expression of a particular Gene by simply injecting double stranded RNA into these organisms.

However, this kind of a knockdown using RNAI was not possible in mammalian cells. Because there was a huge problem in mammalian cells, when you introduce this double stranded RNA the double stranded RNA actually induces, what is called as a immune response leading to apoptosis. Now, this is a kind of a immune mechanisms that eukaryotes have a award because whenever, the eukaryotic cells are infected by viruses. These viruses as part of their life cycle actually, produce double stranded RNA and whenever double stranded RNA is made inside the cells these, double stranded RNA act towards what is called as an interferon pathway leading to apoptosis.

So, that this virus infected cells are destroyed and thereby, preventing the spread of the virus, to the neighboring cells. So, this is a kind of a protective mechanism that eukaryotic cells are revolved to recognize virus infected cells. Because it is only the virus infected cells, that is double stranded RNA is made. And therefore, whenever double stranded RNA is made inside the cell recognizes that a virus as infected. And therefore, immediately activates, this dicer mediated mechanism and cleaves the viral RNA double stranded RNA. So, that the virus cannot multiply and therefore spread of the virus infection to the remaining cells is abolished.

So, because of this problem this kind of a double, stranded RNA injection or a introduction of double stranded mammalians, is also not possible to do RNA interference in mammalian cells. So, the mechanism of destruction of double stranded RNA is used by mammalian cells during viral infection by destroying virus infected cells. And preventing the spread of the virus to neighboring cells thus for quite some time. RNAI or RNA interference using double stranded RNA was largely confined only to lower eukaryotes.

So, you quite study Gene functions, or you can do RNA interference only in lower eukaryotes like insects and worms. But you could not do this double stranded RNA induced Gene silencing in higher eukaryotes.

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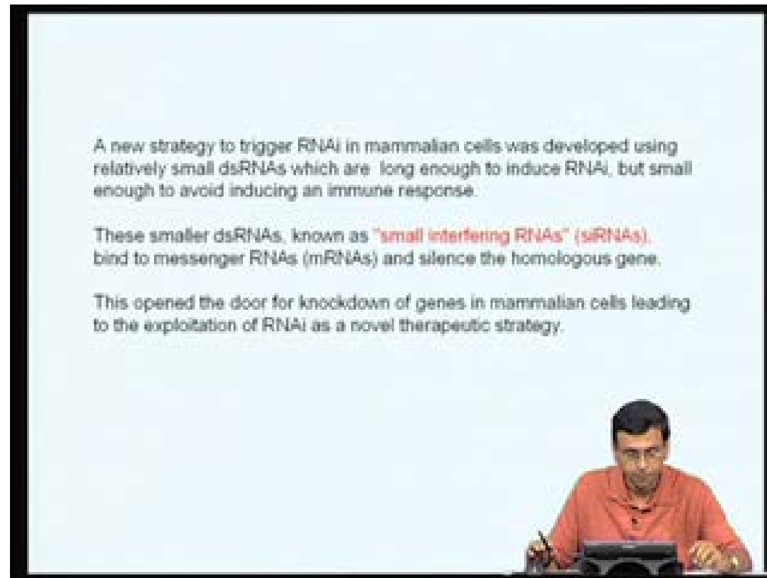


Then another interesting observation came. Then a new strategy was developed by school and his co workers, what they actually showed is that you can trigger RNA interference, in mammalian cells by using small double stranded RNAs instead of long double stranded RNAs. So, what the school and his co workers actually, demonstrate is that instead of using long double stranded RNAs if you use a RNAs of about twenty to twenty-two base pairs. Then you can trigger RNA interference and mammalian cells and these, small RNA strength do not induce the anti viral or the immune mechanism, leading to destruction of the virus of the cells and does not trigger upper process.

So, the key for the use of RNA interference in mammalian cells came from the observation that, do not use long double stranded RNAs. But use short double stranded RNAs which are about twenty to twenty-five base per sense. And now you can trigger interference in the high eukaryotes or mammalian cell also. So, these small double stranded RNAs where ultimately known as small interfering RNAs or siRNAs. So, this is the origin of the siRNAs. So, the discovery is that large double stranded RNAs induce apoptosis or activate the double stranded mediate degradation of the RNAs and results, in destruction of these cells.

And therefore, RNA interference cannot be done in mammalian cells, but if you use small RNAs or what are known as the small interference RNAs, they can actually bind to messenger RNAs and silence the homologous gene. So, this was the origin of this

terminology siRNAs or small interfering RNAs. This actually open the door for knockdown of genes and mammalian cells leading to exploitation of RNAi both are basic research as well as therapeutic too us. Now, try to understand some of these important experiments in the eukaryotes.

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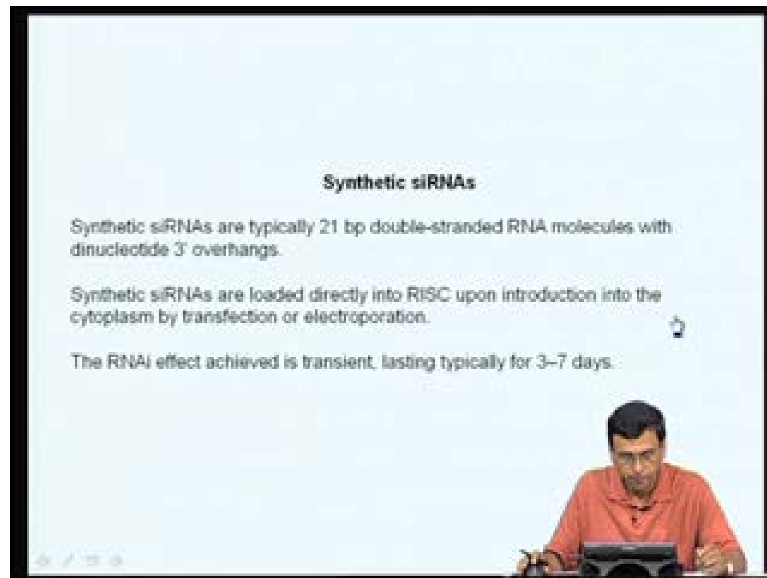


So, synthetic siRNAs are typically 21 bases per double stranded RNA molecules with di nucleotide 3 prime overhangs. When introduce this short 22 and 21 bases per double stranded RNA with two bases per overhangs or the 3 primates. These synthetic RNAs are directly loaded on to the RISC complex and upon introduction to the cytoplasm by transfect ion or electro oration and then it leads to RNAI. So, you can degrade specific messenger RNAs or you can silence specific genes in eukaryotic higher eukaryotic cells, by introducing small double stranded RNAs known as siRNAs which are about 21 base per cell length with it two base per three prime overhangs.

And when you introduce these double stranded short double stranded RNAs or s I RNAs, into mammalian cells by either electro oration or by using Transfect ion techniques this is these RNAs are directly loaded over this complex. And once strand is then base pad with messenger RNA leading to degradation of that particular messenger RNA. Therefore, instead of using long RNAs if you use short RNAs. RNA interference can be demonstrated in mammalian cells as well.

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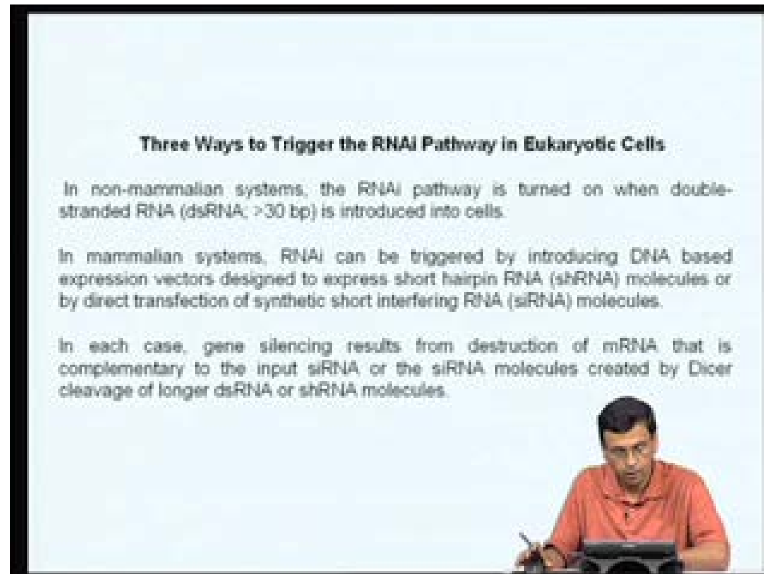


So, if you want to now do RNA interference, if you want to KNOCKDOWN the Expression of a particular Gene in mammalian cells by RNA interference, you can do it in three different ways. Number one, in non mammalian systems the RNA path way is turned on when double stranded RNA is introduced in to the cells. This as been very useful especially, people working in the area of insects. Such as silk worms and drosophila because, you can simply KNOCKDOWN the Expression of a particular Gene. In these insects by simply feeding or by simply injecting the double, stranded RNA in to this lower eukaryotes like worm cell insects.

So, you can simply here size does not matter, you can inject as well as the long double stranded RNAs. Because it does not activate the immune system there, it can directly be digested by the dicer into smaller fragments. And then loaded on the RISC complex leading to silencing of that particular Gene this is one method; however, if you want to do the RNA interference in mammalian systems, there are two other methods by which you can do the RNA interference. In one case in mammalian systems RNAI can be triggered, by introducing DNA based Expression vectors; designed to express short hairpin RNA or shRNA molecules or by direct transfection of synthetic short into si RNA molecules.

So, by introducing plasmid that actually, can make what is called as a shRNA which is then cleaved in to generating sIRNA or by directly expressing sIRNAs. You can actually

induce RNAI or RNA Interference mammalian cells. Either you express this plasmids express in this what is called as a short hairpin RNA, which hare then convert to a sIRNA inside. A mammalian cells or you can directly inject sIRNA molecules in both this cases, genes silencing results from the destruction of the messenger RNA that is complemented to the input s I RNA or the s I RNA molecules is created by the dicer cleavage of long RNA double stranded RNAs and or shRNA molecules.

So, this is the mechanism by which you can do RNA interference, in mammalian or non mammalian cells.

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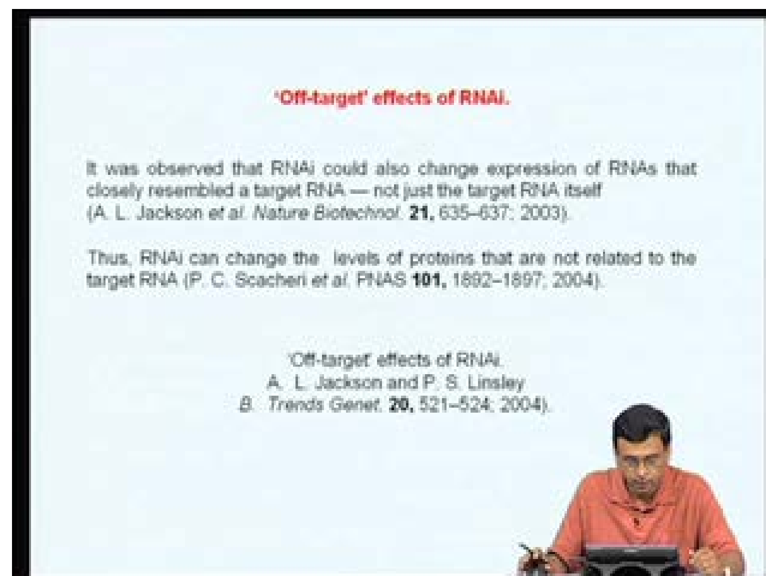


Now, so a this triggered a whole bunch of experiments because, people want to know under. Especially in the era human and mouse genome was been sequence people wanted to understand the function of a number of genes. And the best method of understanding many of the function of many of these genes is to KNOCKDOWN the Expression of these genes using sIRNAs. So, if you want to understand for example, what is the function of a particular protein in a particular signal transactional, pathway all that you have to do is make a short 21 base per sIRNA for that particular Gene.

And transfer them in to cell lines KNOCKDOWN the Expression and then see what happens those particular, signal transactional pathway so it is just one example. So, but the problem came which soon realize that, when you introduce this sha 21 base per RNAs, sIRNAs into the cells many at times we had what is called it as off target effects.

That means, in addition to specifically inhabiting that particular Gene which is complemented with the sIRNA. The sIRNA also, had nonspecific effects and it nonspecifically repress the effects of Expression of a number of other genes as well.

So, these are known as off target effects of RNAI. So, it was observed that RNAI could also change Expression of RNAs, that closely resembles the target RNA and not just the target RNA itself. So, the exact man for a while died. So, soon it became very clear that the RNAI can change the levels of proteins, that are ore related to the target RNA . So, the euphoria that was there initially, is saying that sIRNA can be used for KNOCKINOUT Expression of genes kind of died out, when people realized that. The effect that you are getting the ultimate effect that you are getting for example, if you take or make or do a take Micro or a experiment if you take them.

Genes which are getting which are, expressed in a normal cell and genes which are getting expressed in a sIRNA transected cell. And see what are the changes a number of suppose a number of genes, getting up regulated down regulated. It is not because, your sIRNA specifically KNOCKDOWN particular gene. But also, hybrids number of other genes and therefore, the effect is nonspecific and not actually, specific for that particular target gene.

Now, how do you overcome this is a very nice review on trends in genetics, of target effects of RNAI. That actually, talks extinct fully about this off target effects of RNAI. So, how to avoid this off target effects the RNA ; that means, you want your siRNA to KNOCKDOWN the Expression of only your Gene of interest. But not other related genes how do you do that.

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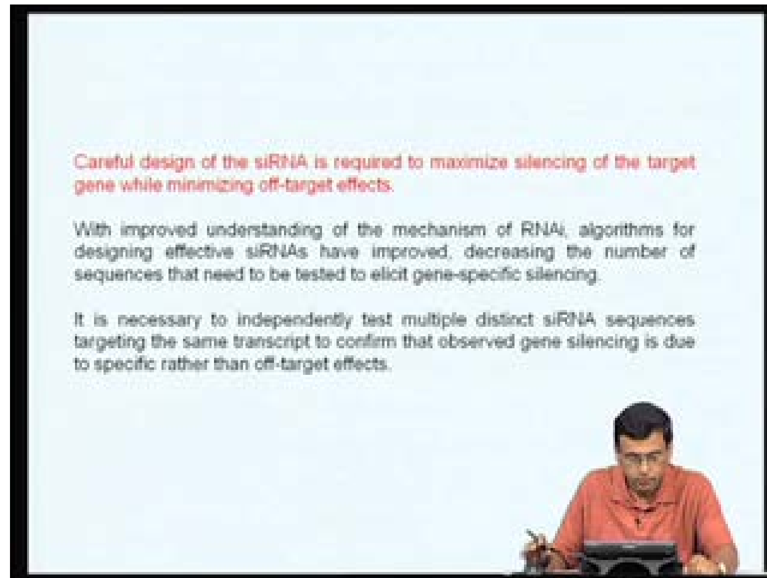


So, people started developing algorithms. So, if you want to know make a twenty-two base pair RNAI, sIRNA for a particular Gene you make sure these, twenty-one base pair RNA does not hybridized to any other Gene in the mouse genome or human genome. So, powerful algorithms were developed to make sure that s I RNA the sequence that you have chosen is highly specific, for only your target Gene. And it does not go and hybridize related Gene and therefore, KNOCKDOWN. So, you have to score for those regions of the Gene which do not share any homology with rest of the sequence, in the human genome.

Therefore, when this set of segment of RNA hybridizes, it can only hybridize to your Gene of your interest and it does not provide any off target effects. So, carful reason of s I RNA is required to maximize the silencing of the target Gene and minimize the off target effect. With improved understanding of the mechanism of RNAI algorithms for designing a effect outwards sRNA is were developed decreasing the number of sequences that need to be tested, for a distinct Gene silencing. So, it became clear for example, if you if you want to show that if I KNOCKDOWN the Expression of this particular, Gene by using s I RNA. And if I am getting this particular phenotypic effect people said you cannot just show.

This effect with one sIRNA you at least design sIRNA for three or four different regions of this Gene. And show that you get a same effect. Then only we believe the result. So,

just by developing one sIRNA for one particular, for a long Gene and showing that it results in a particular, phenotypic effect was not accepted. So, you to designs at least two or three different sIRNAs in compassing different regions of the Gene and if there all have the same effect. Then you start believing that yes this effect t is actually very specific and it is likely the effect, due to the down regulation of the particular target gene.

So, it was necessary to independently test multiple distinct sIRNA sequences targeting. The same transcript to conform that the observed Gene silencing is due to specific rather than a perfect target effects. Today there are actually, companies which are made sIRNA for almost all the genes of mouse or humans or other mammals. You can just buy sIRNA suppose I want to KNOCKDOWN the Expression of a particular Gene. I can just go to a company like hygiene or the thermion and there are. So, many companies now you can just go then order sIRNA for the particular Gene. They will simply ship you three or four best sIRNA s. And they guarantee you that if you introduce this sIRNA to the cells you get more than eighty percent mark down of that particular gene.

Otherwise they will give the money back. So, lot of algorithms were developed lot of work has gone on to make sure that the sIRNA that are designed will not have any off target effects. And they exactly KNOCKDOWN you target Gene and therefore, you will get very significant KNOCKDOWN of your interest.

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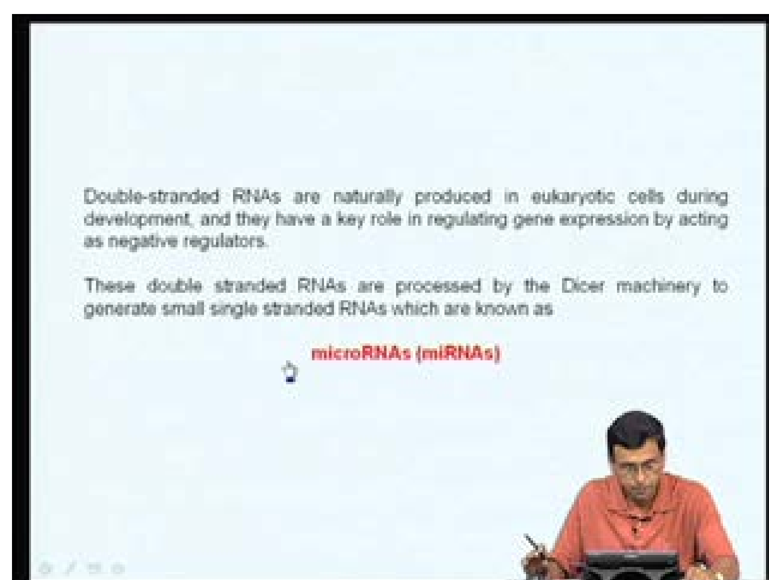

Now, so for we discussed about introduction of a exogenous double stranded RNA. That is when you introduce exogenous double standard RNA, how it gets broken down to small twenty-one base pair RNA. And then, how it hybridize to messenger RNA by the rest and then results in the knock down of the Gene expression.But then the question came do such small RNAs are the naturally present in the eukaryotic, cells are the naturally made. So, double- standard RNA are actually, found to be naturally produced in eukaryotic cells during development. And there were found to have key role in regulating Gene Expression by acting as negative regulators of Gene expression.

So, you can see initially, the entire RNA interference was studied using RNAs which were, introduce into the cell from the exogenous source. You here the introduce double standard RNA into the cell or you either introduce, small double standard RNA into the cell. And that inhabited the target Gene expression. But soon it became clear, inside a naturally many such small double standard RNAs are actually produced. And these double RNAs that are, produced naturally can have very important regulatory functions.

So, these double standard RNAs which are processed by dicer machinery to generate small single standard RNs are, known as Micro RNAs or mIRNAs. So, I am introducing a new terminology here. So, for we discussed about sIRNA s, which are small interfering RNA s which are actually, generated by the cleavage of exogenously introduced double standard RNA. But here I am telling you that small double standard RNA also generated endogenously. And these endogenously generated RNAs which are capable of inhibiting Gene Expression are known as mIRNAs or Micro RNAs.

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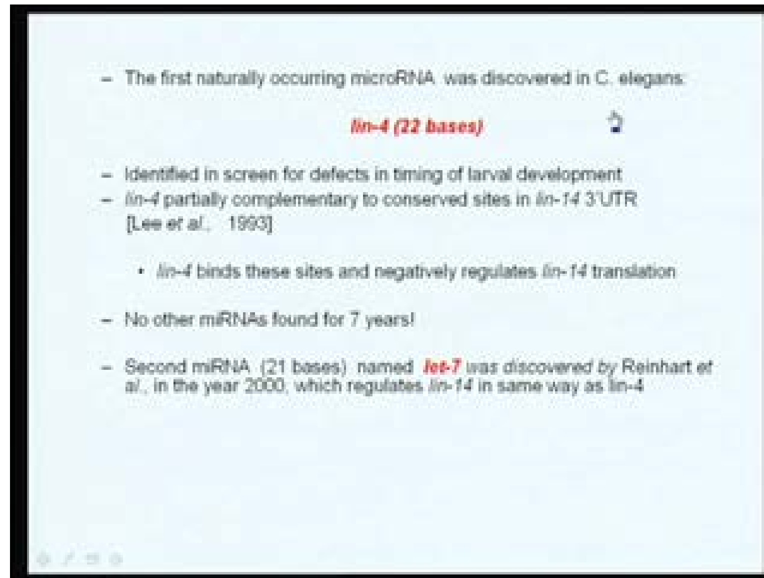


So, I have sIRNS and now, mIRNS. The first naturally, occurring mIRNA or Micro RNA was again discovered in the worm C elegans. And this mIRNA was known as lin-4which is about 22 base pair slot. This was actually identified in a screen for defects in the time of timing of larval development. And line-4was partially complementary, to the conserved sites in a very important regulatory Gene known as lin-14 3 prime and translate region of this lin-14 RNA. Therefore, when this lin-4 Micro RNA goes in the hybridize to the lin-14 un translated, region it is in the KNOCKDOWN leading three reduced Expression of their particular Gene. Line-4actually, binds to this side and negative regulates lin-4 translation.

So, translation is inhibited and therefore, proteins not made. So, the first naturally occurring Micro RNA lin-4was actually discovered in the year nineteen ninety three. And for almost 7 years no other Micro RNA was discovered. It took almost 7 years to discuss the second Micro RNA again in C elegans this is about 21 bases long. This was named let-7 and was discovered by Reinhart at, in the year 2000 which also regulates lin-14 RNA in the same way as lin-4. So, this small RNAs of twenty-one, twenty base space are also occurring naturally. And they also simply have a important role in the regulation of embryonic, development in C elegans.

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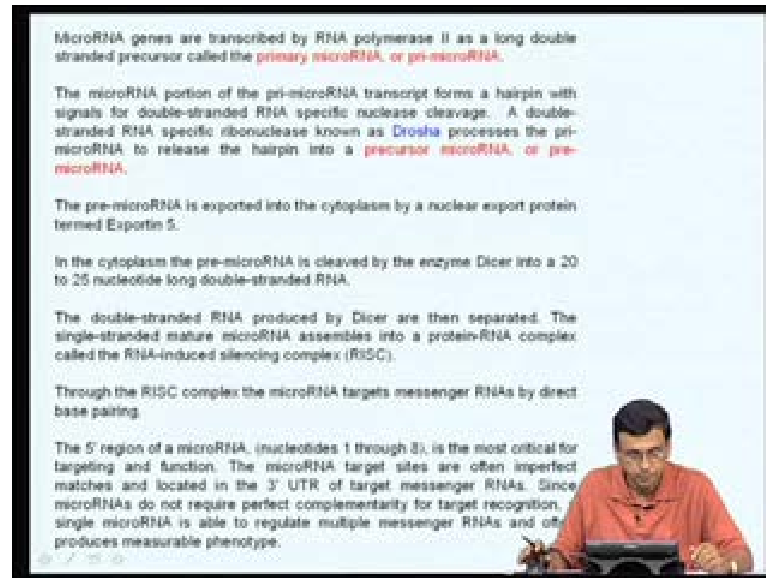


So, Micro RNA genes were discovered a number of other organisms including drosophila mammals plants and. So, on and. So, forth and these Micro RNA they are genes which actually, core for Micro RNA. And this Micro RNA genes are actually transcribed RNA transcribed RNA polymerize two as long double standard. RNA precursor called primary Micro RNA or prime Micro RNA.So, just like you have protein coding genes, that is you have genes which code for messenger RNA which then code for proteins. There are actually genes in the eukaryotic genomes which actually synthesize precursors for Micro RNAs.

These precursors are called primary Micro RNA or prime Micro RNA, which are a single RNA which folds into number of hairpin structures leading to a long precursors of Micro RNA. The Micro RNA portion of this primary Micro RNA transcribed forms a hairpin which signal for double standard RNA specific nucleus cleavage. So, when you have this long RNA with number of stem root structures this specific hair pin structures are actually, recognized by a specific double standard RNA cleaving enzyme. Actually, it is a RNA three type of enzyme this enzyme is known as Drodha a double standard RNA has specific ribonucleus known as Drosha. Actually processes primary Micro RNA to release this hairpin into a precursors Micro RNA or pre Micro RNA.

So, you have a Gene Micro RNA Gene from the, from that you get the prime Micro RNA or a primary Micro RNA. And in this primary Micro RNA the Micro RNA regions

or cleaved by Drosha to generate what is called as a precursors Micro RNA or pre Micro RNAs. These pre Micro RNA then exposed in the site of cytoplasm, by a nuclear export protein known as exporting 5. And once in the cytoplasm these pre Micro RNA is cleaved by, the enzyme dicer which we discussed earlier into a 22 to 25 base pay long double stranded RNA. So, the hairpin structures are removed and you now get a 22 base pair a 22 25 base pair double stranded RNA.

These double stranded RNA produced by dicer or then separated the single stranded matured Micro RNA assembles, into a protein RNA complex due to the risk complex which we discussed earlier. And this law this complex trough the RISC complex the Micro RNA targets the messenger RNAs by direct the base paring leading to knock down of the Expression. The 5 prime region of a Micro RNA especially, nuclear zed 1 to 8 is the most critical for targeting and function. The Micro RNA target sites or often imperfect matches and locate in the three prime un trans, radiation in the region of target messenger RNAs. And since the Micro RNAs do not require perfect complementary for target recognition.

A single Micro RNA is able to regulate multiple messenger RNAs and offer produce measurable phenotypes. So, unlike the sIRNA which are highly sequence specific because we are actually introducing cells many times, this Micro RNA need not have an exact complimentary (( )) it can even go on hybridize with one or two mismatch basis. And therefore, it can have a very drastic phenotype a measurable phenotype. So, a single Micro RNA can go and inhabit the Expression of a number of RNA molecules. So, whatever I showed here in the form of a text I have shown it actually in the form of a cartoon here.

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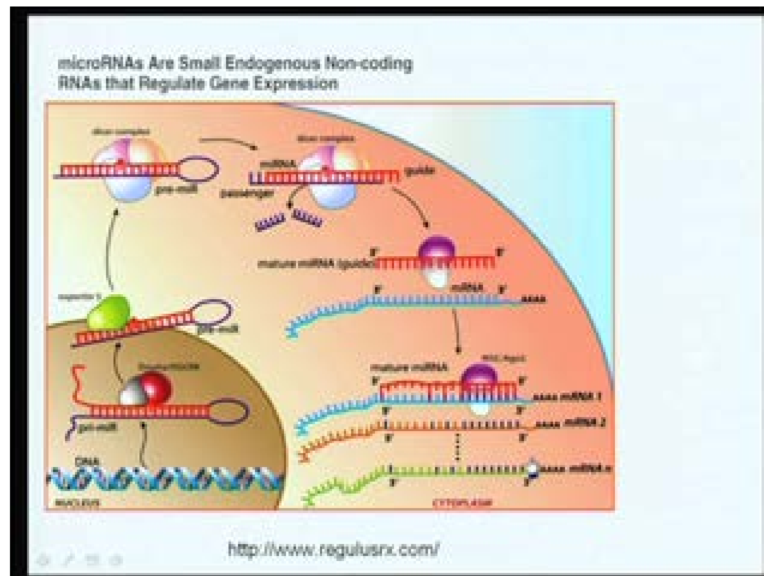


So, there are actually, genes inside the eukaryotic cells which actually code for which are actually responsible generation of Micro RNAs naturally in eukaryotic cells. These genes are actually transferred by RNA polymerizes too and you get what is called as a pri Micro RNAs or primary Micro RNAs. And these, primary Micro RNA are recognized by an enzyme called as Drosha which is a RNA. And it cleaves to generate what is called as precursors Micro RNAs. And these precursors Micro RNAs are, then exported in cytoplasm by a protein called exporting five.

And once the precursors Micro RNAs are in the in the cytoplasm it is recognized by the dicer complex and the dicer complex cleaves. The hairpin generating a generating the double standard RNA and one of the strands and then, hybrids to the mRNA in the risk complex leading to degradation of the specific messenger RNA,

so, the mechanism of mIRNA and sIRNA actually, more or less similar whereas, sIRNAs are actually, generated from exogenous source whereas, in the mIRNAs are generated in naturally inside the cells.

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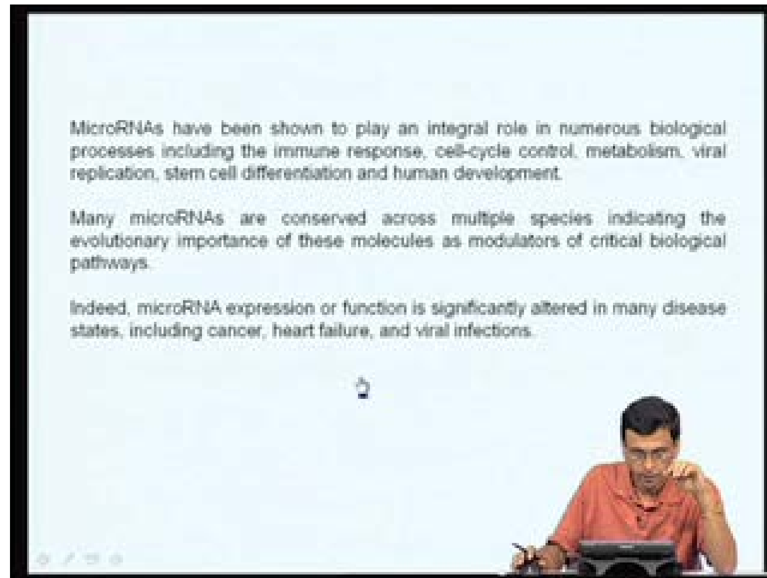


Micro RNAs have been shown to play integral role in numerous biological processes, including immune response cell cycle control metabolism, viral replication stem cell differentiation, and human development. You name a, biological processes Micro RNA seems to playing important role in this all the processes. Many Micro RNAs are conserved across multiple species indicating the evolutionary importance of these, molecules as modulators of critical Gene functions. Micro RNA Expression or function has was shown to be significantly altered in many diseases states, including cancer heart failure, and viral infections.

So, it soon became clear that these Micro RNAs which nobody heard of this Micro RNAs in the early nineteen nineties. But once people realized that these small Micro RNAs are actually made normally, natural inside the cells and you have important regulatory functions it opened up an entirely new area of research in biology in medicine.

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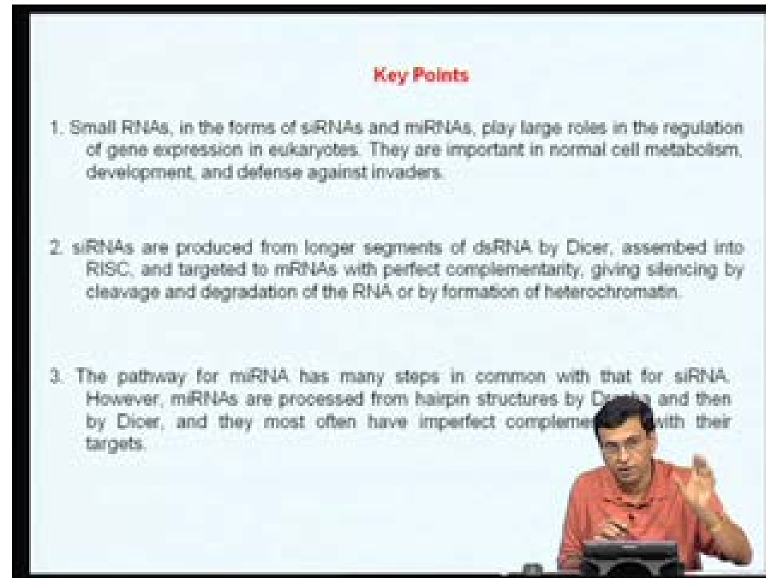


So, I just summarize some of the Key points that I have discussed. So, for and then will proceed further what I have discussed. So, for is that small RNA in the form of a sIRNAs and the mIRNAs platy large rolls in the, regulation of Gene Expression eukaryotes. They are very important for normal cell metabolism, development and defense against inverter such has viruses. The sIRNAs are small inferring RNAs are produced, for longer segments of double standard RNA by Dicer, assembled in to RNA into cell complex or RISC, and targeted to messenger RNAs with perfect complementarily, giving silencing by cleavage and degradation of the RNA or by formation of a heterochromatin.

We will discuss about this heterochromatin later it is little bit more complicated. The path way for Micro RNA has many steps is common with that of a siRNA because both are generated by the Dicer mechanism and risk mechanism. But miRNA is produced form a endogenous source siRNA produced from heterogeneous source, miRNAs are processed from hairpin structures Drosha and then by Dicer, and the most of I have imperfect complimentarily with their targets.

This is another important difference between siRNA are often have a perfect complimented to their targets. Is another important difference between siRNAs often have a perfect complementarily with their targets, miRNAs have often have complimentarily with their target sequences.

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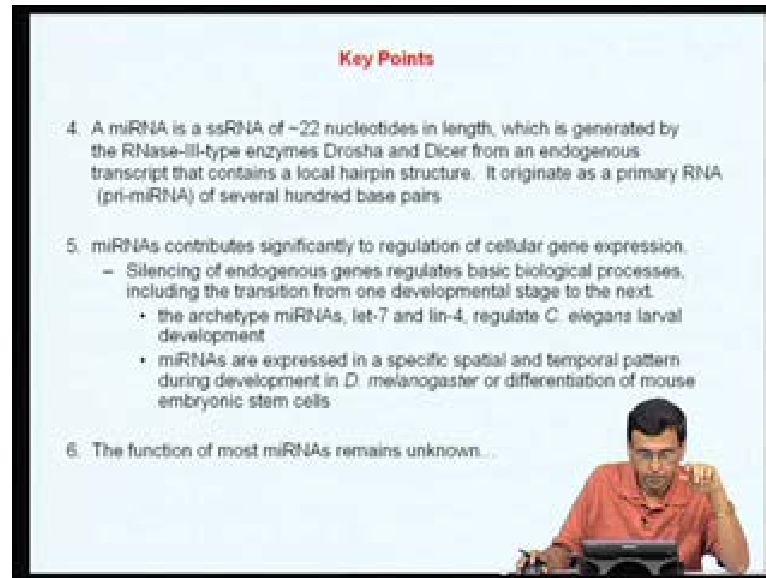


The miRNA is the single standard RNA of about 22 nuclearoids in length, it is generated by the RNAs type three type enzyme Drosha and Dicer from a endogenous transcribed. That contains a local hairpin structure, it originates primary RNA of several hundred base pairs. So, have a long primary RNA from this these hairpin structures are cleaved a single primary RNA can, actually can give rise to number of precursor messenger Micro RNAs. So, a single Micro RNA Gene many, Micro RNA can arise from a single primary Micro RNA Gene. Micro RNAs again contribute significantly, to the regulation of cell or Gene Expression silencing of the endogenous genes regulates basic biological process, including transition from one development stages.

To next many of this Micro RNA are actually, expressed in a development stage specific manner. Some of the Micro RNAs which are original discovered like the lin-7 and lin-4 regulates C elegans larval development. And miRNAs s specific spacial and temporal pattern during development, in D melanogaster and differentiation of mouse embryonic stem cells. And In fact, many of the Micro RNAs their function is still remains unknown. Say a entirely new area of research opened up with the discovery of this RNA interference mechanism. And that is why it became very important discovery, and Andrew fire and Craig Mello got the Nobel Prize for his work.

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miRNA vs siRNA

- miRNA: microRNA.
  - Encoded by endogenous genes.
  - Hairpin precursors - pre-miRNAs
    - The pre-miRNAs are hairpins with imperfect complementarity in their stems and frequent bulges, mismatches and G:U wobble base pairings.
  - Recognize multiple targets.
- siRNA: short-interfering RNA.
  - Mostly exogenous origin.
  - dsRNA precursors
  - May be target specific
- Discovered in different ways
- Similar biogenesis
- Share common pathway components and outcomes

So, what are the major differences between miRNA and siRNA, miRNAs are nothing, but Micro RNAs. They are encoded by endogenous genes they are hairpin precursors of their hairpin precursor are called pre Micro RNAs. These pre Micro RNAs with imperfect complementarily in their stems and frequent bulges, as well as mismatches and wobble bases pairs. They can ultimately recognize multiple targets. Whereas, siRNA is short, interfering RNA are small interfering RNAs.

They are mostly exogenous origin, they are double standard precursors they, may be target specific. siRNAs are of often target specific miRNAs may have multiple targets. So, siRNA and miRNA are differ discovered in different ways. One is discovered through an exogenous source and the other was endogenous source. But the biogenesis more or less similar both involved dicer and RISC complexes, for their function and they share common path way components and they have common outcomes. Both of them inhabit degrade RNAs and inhabit Gene expression.

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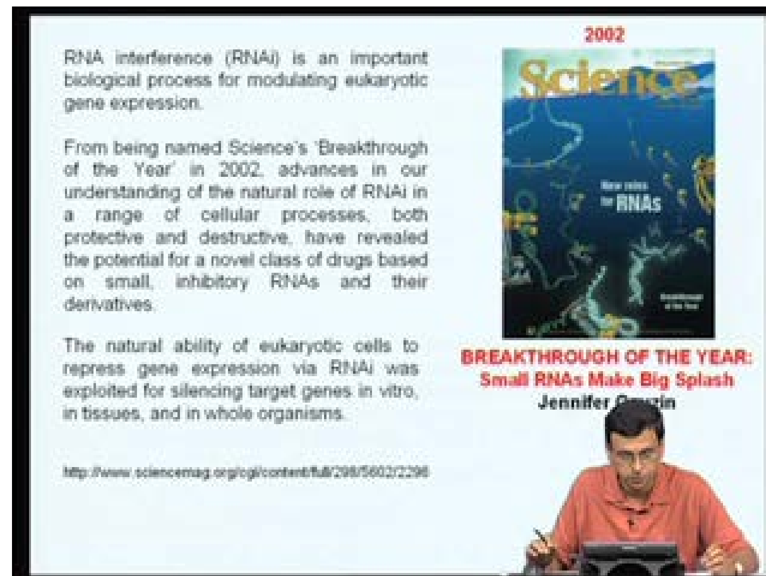


So, you can see this discovery of RNA interference, opened up entirely new area of research. And no doubt this RNA interference was recognized is a very important biological process, for modulating eukaryotic Gene Expression. And this actually, named as BRAKE THROUGH OF THE YEAR in thousand two by this prestigious science magazine. So, small RNAs became very important for regulation of Gene Expression as well as for, understanding Gene function as well as for, potential therapeutic agents for a number of diseases.

So, from being named as science Breakthrough of the year 2002, advances in our understanding of the natural role of RNAI in the range of cellar processor, both protective and destructive, have reveal the potential for a normal class of drugs based on small, inability RNA. And derivatives we will discuss some of the potential applications of RNA as we go along. The natural ability eukaryotic cells to repress Gene Expression where RNAI was exploited for silencing targeting Gene genes in vitro, in tissues as well as in whole organisms.

So, this area opened up an entirely whole range of, research areas for understanding Gene functions, as well as number of cellar processes. So, let us now, spend some time to understand what are benefits of this, RNA interference. So, for we discussed how RNAI you discovered how siRNAIs, were discovered how miRNAIs, were discovered what are the differences between the two? And how the discovery of this siRNAIs, mi

RNAIs change the entire our biological research, and became very important tools for inhabiting Gene Expression and for understanding the roles of a number of genes.

Now, let us see whatever the benefits of RNAI. Now, people now started talking about what is called as RNAI therapy. Now, in our earlier Lectures we have discussed what is called is a Gene therapy.

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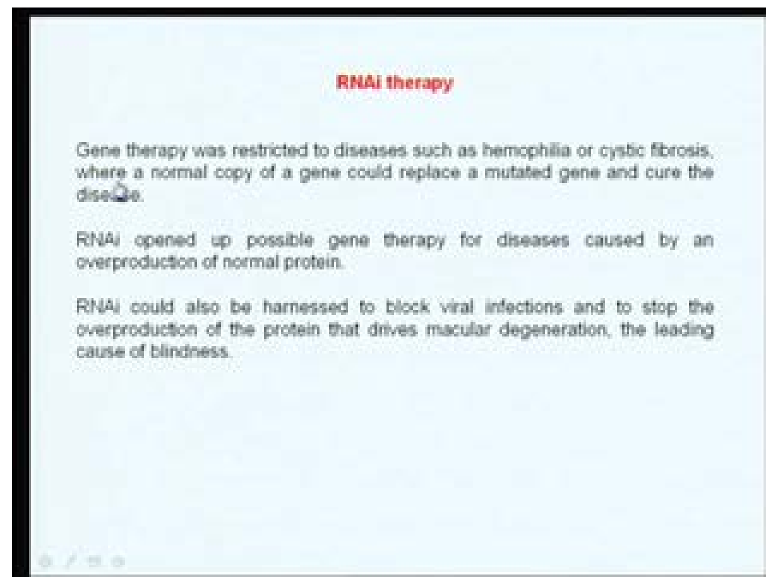


Now, Gene therapy was restricted to diseases such as hemophilia or cystic fibrosis, where a normal copy of a Gene could replace a mutant Gene and cure the disease. This is what we discovered in our Lectures on Gene therapy. Where, if this is a mutant copy of a Gene if you are normal functional protein is not made, you introduce the Gene into the humans. And see whether you can make the normal protein and cure a disease. But the discovery of RNAI interference actually, opened up the possible Gene therapy for diseases caused by over production of a normal protein. Now, at a Gene therapy we actually discussed about a missing protein or a mutant protein.

So, in such cases if we express a normal protein you cured the disease. But there are also many disorders; where over Expression of a normal protein can lead to a disorder. Many Neuro genic do generate disorders are actually cause by over Expression of a mutant protein. So, if he can somehow block the over Expression of this mutant protein, you can cure a disease. And RNAI interference can became an excellent tool for doing these kinds of or for preventing repression of the Expression of these mutant proteins. So,

RNAI could be harness to block viral infections, to stop the over production of a protein that drives diseases like macular degeneration which is a leading cause of blindness. We will discuss some of this things in detail.

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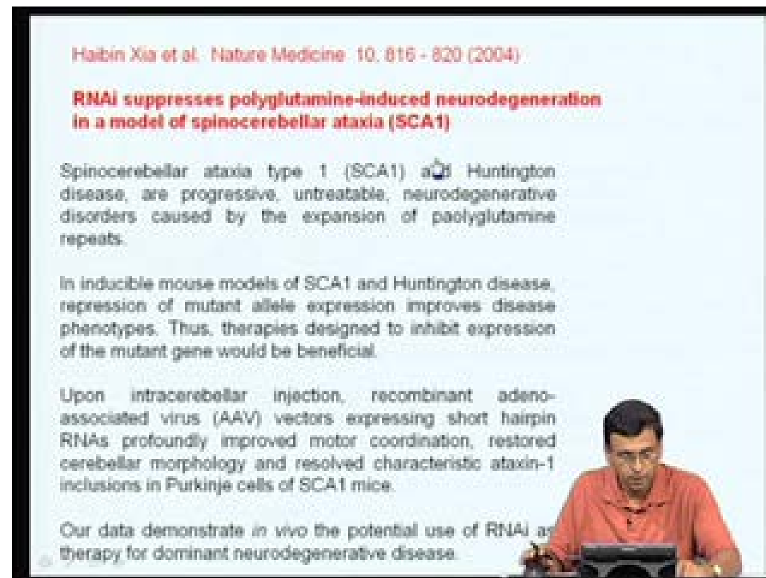


I am giving going to give some four or five examples now just tell what are the applications of RNAI here, is a paper that appeared in 2000 4in nature medicine talks about RNAI suppresses polyglutamine-induced. Neuro degeneration in a model of Spinocerebellar ataxia, which is a very important Neuro degeneration disorder. Now, (SCA1) are the cyro Spinocerebellar ataxia type one as well as other Neuro Neuro degenerated disease, called Huntington disease they are progressive, untreatable Neuro degenerated disorders cause by repeat of polyglutamine repeats.

I will not go to the details, but what is called as AVN polyglutamine repeat expansion. That actually leads to RISC Neuro generated degenerated disease. So, people ask the question can we prevent Expression of the polyglutamine containing proteins by RNA interference. In fact, in inducible mouse models of SCA1) when one Huntington disease repression of the mutant allele Expression improves disease phenotypes thus, therapy is designed to inhabitance of the Expression of the mutant Gene will be beneficial. So, people found out if he can express the, depress the repress or inhibit the Expression of this mutant proteins, which have huge polyglutamine repaired you can actually cure the disease.

So, what people have done is upon intercerebellar, injection recombinant adeno-associated viruses, vectors expressing the shRNAs which, can ultimately lead to generation of siRNAs was found to improve motor coordination restores a cerebral morphology. And resolve the characteristic attacks in inclusions and purkinje cells of s C a one mice. So, by inhabiting the accumulation of this mutant proteins by injecting siRNAs, complementary to this Gene coding for the polyglutamine repeat contain protein you can actually demonstrate clinical beneficial results clinical, significant results indicating that siRNAs can be used for potential therapeutic applications.

So, these data demonstrate that RNAI can be used as a potential therapeutic tool for number of neurodegenerated diseases.

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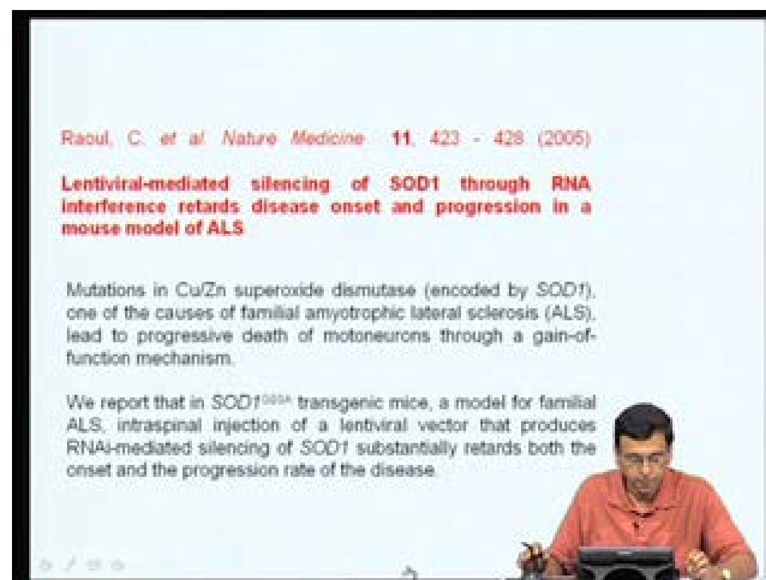


There is another a paper that said, lent viral mediated silencing of SOD1 through RNA interference retards disease on said and progression a mouse model of ALS. Now, mutations in the superoxide dismutase coded by the SOD1 Gene is one of the major causes of familial amyolotrophic lateral sclerosis, which is very complicated Neuro degenerated disorder, leading to progressive death of Moto Neuro ns through gain of function mechanism. And in this paper they show in a, SOD1 transgenic mice a model for the familial ALS, intera-spinal injection of a lent viral vector producing RNA. Immediate silencing of SOD1 results in the decrease in SOD1 protein leading to retardation of both onset and progressive of the disease.

There are again other examples here inhabitation of respiratory viruses by nasally administrated siRNA. In one case they express siRNA by adenoid associated virus. And their case they expresses siRNA lent virus here you can simply nasally administration siRNA. And show it has a therapeutic benefit again certain viral infections.

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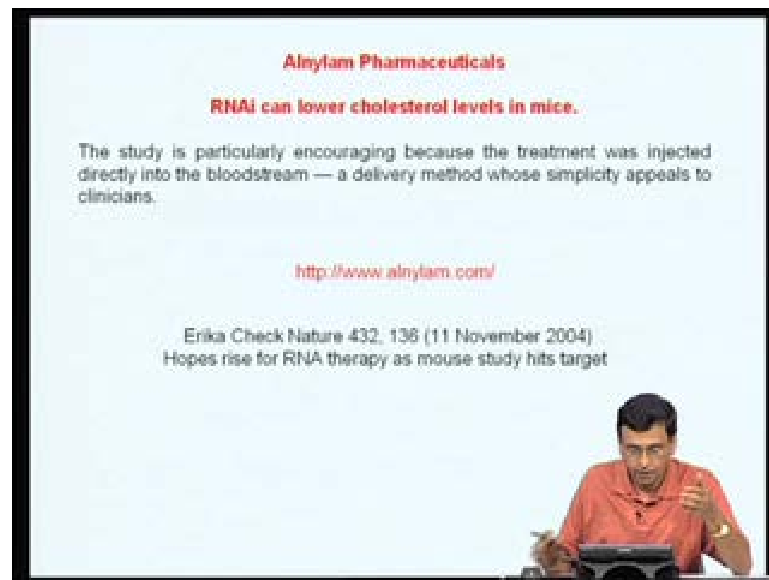


Here very interesting paper a company called Alnylam Pharaceutic ALS. Actually can show RNAI can actually lower cholesterol levels in mice. You can go to the website and then read more about this, there is also nice article on nature about how you can actually bring down cholesterol levels by RNAI.

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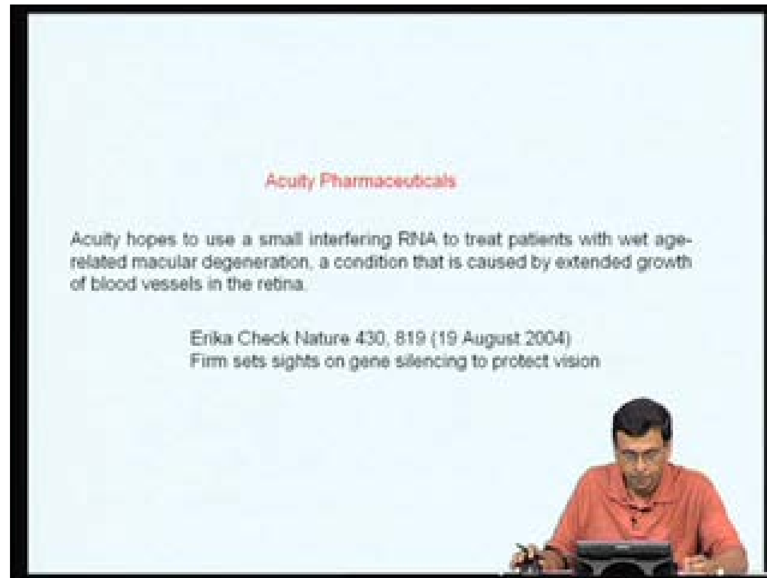


Another company called acuity pharmaceuticals, is trying to develop RNAI base therapeutics where this company hopes to use small interfering RNA to treat patients with wet age related macular degeneration, a condition that is caused by extended growth of blood vessels inside the retina. By actually inhabiting a protein called as VEGF F vascular endothelial growth factor.

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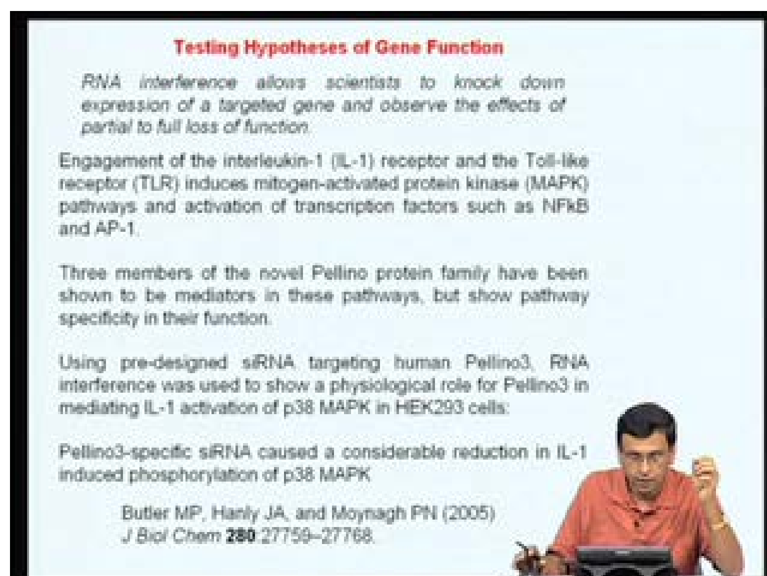


So, RNAI interference had tremendous applications in both basic and applied research. For example, you can use RNAI for testing the hypothesis of a Gene function RNA

interference allows scientist to KNOCKDOWN Expression of targeted Gene. And observe the effects of partial what happens when partial or full lock out the function of a particular Gene. I just give an example; here it is appeared in j b C in 2005. For example, interleukin one receptor and the toll like receptor induces mitogen-activated protein can his pathways and the activated transcription factor such as NFKB and AP-1.

Now, three members of a protein family called Pellino protein family were shown to be mediators of the pathways. But show specific effects, in these pathways now you want to understand how what happens if I now knock down one of the members of this Pellino protein, how does it affects the signal transaction pathway. So, when you use isRNA targeting human Pellino 3 there are 3Pellios target to only Pellino 3 it was shown to have a specific physiological role in mediating I L-1 activation of p 38 MPK in Gene in these cells.

So, by KNOCKINGDOWN specific components of a signal transduction path way you can exactly understand. What is the function of that particular of that component in the entire signal transduction pathway?

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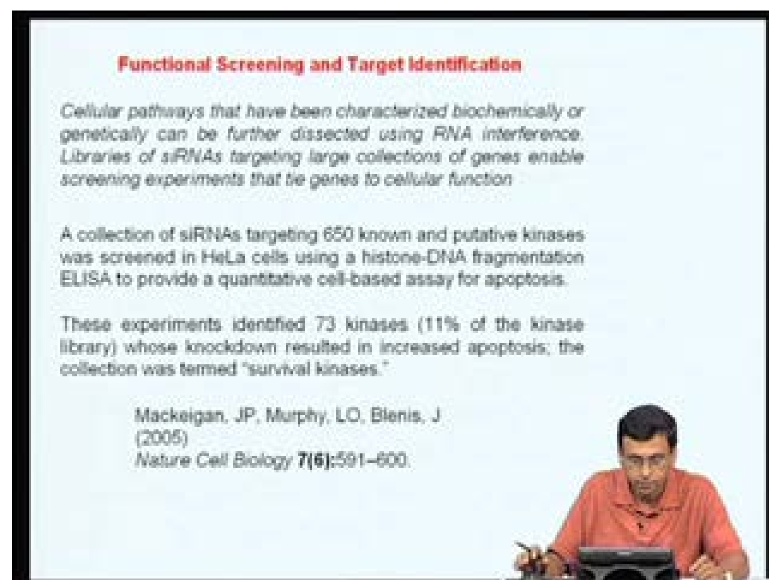


You can use a RNAI for functional scaling and target identification. For example, cellar pathways have been characterized by chemically or genetically can be further dissected using RNA interference Libraries of siRNAs, targeting large collections of genes enables screening experiments that tie genes to cellar function. I will give you one example for

example, a collection of siRNAs, targeting about 650 known putative kinases was screened in hela cells using a his tone. DNA fragmentation Elisa to provide a quantitative cell based assay to apoptosis. That is what are the genes that are involved in what are the kinases that, trigger apoptosis.

So, you make siRNAs for each one of this kinases transfer them into cells and see which of them block apoptosis. And they identified 73 that is 11% of this kinase library and when you knock down this kinases using siRNA. There was n increase apoptosis and therefore, these kinases were termed has survival kinases. So, you can see by using siRNA you can actually identify those kinases which even actually trigger apoptosis.

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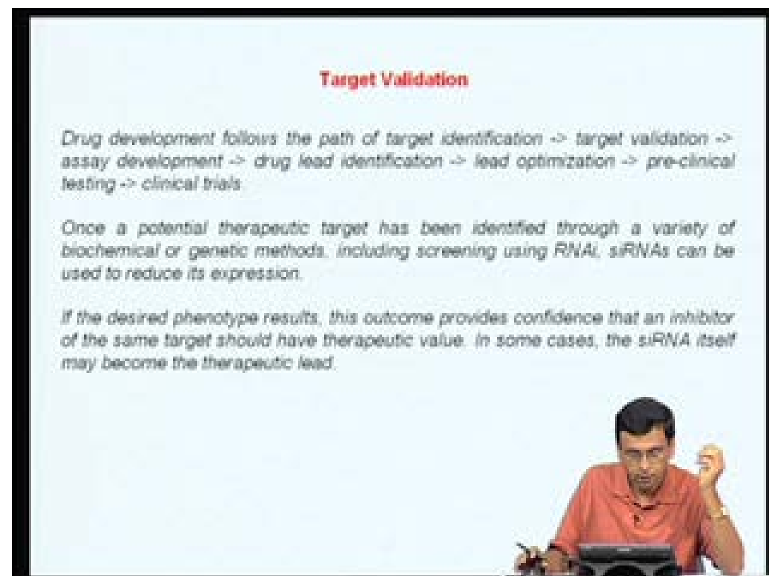


No which can increase apoptosis. Target Validation, the normal drug development involves target identification and validation assay development drug lead identification lead optimization and trusting and so, on and so, forth. Now, once your potential therapeutic target have been identified through a verity of biochemical genetically methods, screening can be done by using RNAI. And siRNA can be reduce the Expression and see whether this can be a valid target.

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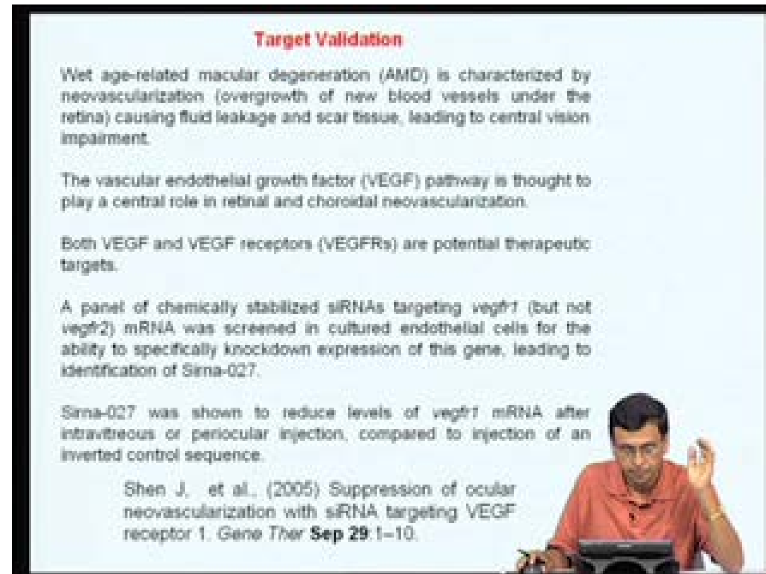


Just now, I showed you can actually use by injecting s I RNAs against a protein called as vascular endothelial, growth factor into the .I you can actually prevent a disease called macular degeneration, which is a major cause of blindness in especially in aged people. So, inhabiting the Expression of the VEGF of in the I you can actually improve vision. And prevent this decrease, the effect of this macular degeneration I am not going to the details of this paper very nicely discussed. And the paper appeared in Gene therapy suppression of ocular neovascularization with a siRNA targeting VEGF F receptor.

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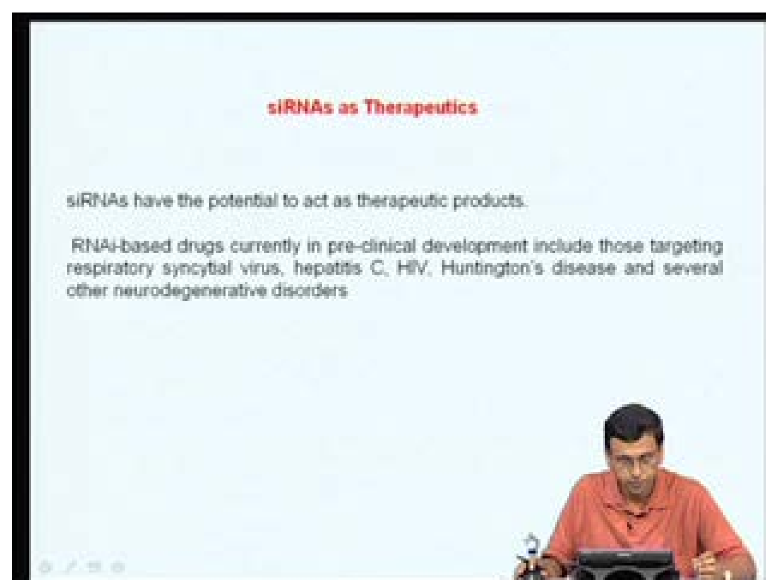

siRNA has a therapeutics a number of RNA based I based structure currently in preclinical development for targeting respiratory syncytial virusm, hepatiti C, HIV, Huntington disease and many Neuro degenerated disorders. Some of which your just discussed.

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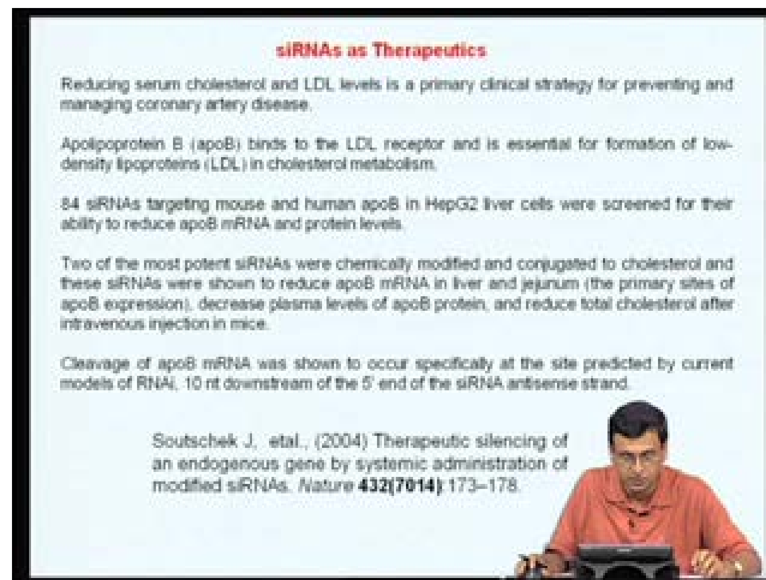


This is this is what I discussed here for example, in this case 84 siRNAs, iRNAs targeting mouse and human apoB in the protein ,where screen and two of them. Actually, were found to decrease the levels of apolpoprotein b messenger RNA. And this apoB hypo protein b actually, binds L D L receptor and therefore, essential for the formation of low density ipoB proteins. And if you decrease the apolpoprotein RNA you can prevent L D L formation.

And therefore, you can have a therapeutic effect and can prevent arthrose floroses, a very nice paper therapeutics silencing of endogenous Gene by systematic systemic administration of modified siRNAs. By simply injecting this si RNAs, against apolpoprotein you can reduce your cholesterol levels, L D L levels. The S O D 1 I again discussed about I will not go to the details one can actually read again a very nice paper lenitviral mediators silencing of SOD1. Through RNA interference retards disease onset and progression the mouse model of ALS.

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Again how viral infections can be of inhabited by using sIRNAs therapeutics. A number of new small RNAs are being discussed discovered and many novel functions are being discovered.

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For RNAs or example, again due to lack of time I will not go to the details siRNAs, again has been shown to be involved in the formation of heterochromatin a new RNA, siRNA called repeat associate RNA or RSI RNA has been shown to actually activate heterochromatic formation in eukaryotic cells. That in turn can induced in silencing

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many small RNA called tiny non-coding RNAs or T N C RNAs whose function is still not clear, have been identified small modulate RNS stem RNA s which are actually terns a transcription repressor into activators are being described.

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So, numbers of applications have come out. So, I think I will stop here and just listed in the next few slides, somehow the key papers the Nobel lecture by Andro fire is an excellent source for understanding mode. This is the first paper that I have described the double standard induce RNA Gene silencing.

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**RNAi - Key developments**

- **1990**
  - **co-suppression of purple color in plants.**
- **1995** Guo S. and Kemphues KJ.
  - First noticed that sense RNA was as effective as antisense RNA for suppressing gene expression in worm C. elegans
- **1998** Fire et al.
  - First described RNAi phenomenon in C. elegans by injecting dsRNA into C. elegans which led to an efficient sequence-specific silencing and coined the term "RNA interference".
- **2000** Zamore et al.
  - Reported processing of long dsRNA by Rnase III (Dicer) into shorter fragments of 21-23-nt intervals in Drosophila extracts
- **2001** Bernstein et al.
  - Cloned Dicer, the RNase III enzyme that is evolutionarily conserved and contains helicase and PAZ domains, as well as two dsRNA-binding domains.
- **2002** Tuschl T and colleagues
  - First described RNAi in mammalian cells
- **2003** Paddison et al. Sui et al. Paul et al.
  - Short hairpin RNAs (shRNAs) induce sequence-specific silencing in mammalian cells.
- **2003** Song et al.
  - First reported that siRNAs can be used therapeutically in whole animals
- **2004** Kawasaki and Taira Morris et al.
  - First observed that siRNA silences gene at transcriptional level possibly through directing de novo DNA methylation.

And there are number of key development that took place, in the area of RNA interference which I have listed here. Some of the very nice review articles in nature collections on microRNAs one can just click on to the website. And read some of these articles. And I have listed some more very interesting articles in the area of RNA interference.

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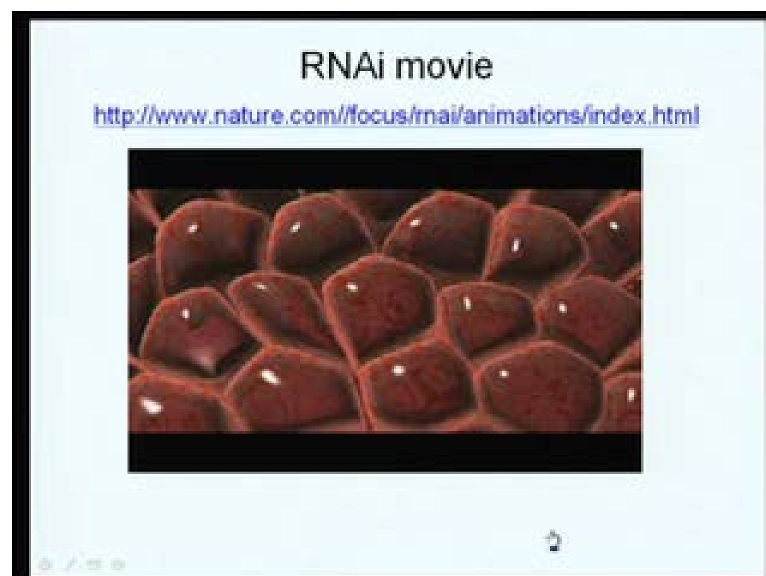

And there is also very nice movie on RNA interference one can just click on the nature animations. And you can actually, visualize how exactly RNA interference takes place by using by watching this nice movie. I think I will stop here thank you.