**Eukaryotic Gene Expression: Basic and Benefits**

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**Lecture No. # 28**

**Embryonic Stem Cells and**

**Transcription Factor-Mediated Epigenetic Reprogramming**

Welcome to this lecture series on eukaryotic gene expression basics and benefits. The last four, five hours, we have been discussing about the role of transcription factors, the way regulation of gene expression plays an important role in embryonic development. Today, we are going to discuss about what are called as embryonic stem cells and what is known as transcription factor-mediated epigenetic reprogramming.

In fact, this is going to be a very, very important lecture, because all the information we have learnt in the last four lectures about the role of transcription factors in development, ultimately, has now led to some very, very important novel and very, very fast moving field known as regenerative medicine.

And so, this class, we are going to discuss some of some, some very exciting developments that are taking place in the area of developmental biology, regulation of gene expression, as well as regenerative medicine, and let us try to understand how you can actually use transcription factors or change the genetic programming of a cell by differentially expressing specific transcription factors.

That is going to be the focus of today's lecture, and what are the advantages or what are the benefits, and how the future is going to change with these kinds of research. So, I have, basically, summarized what all we have been discussing in the last four classes, especially in the role of gene expression during embryonic development.

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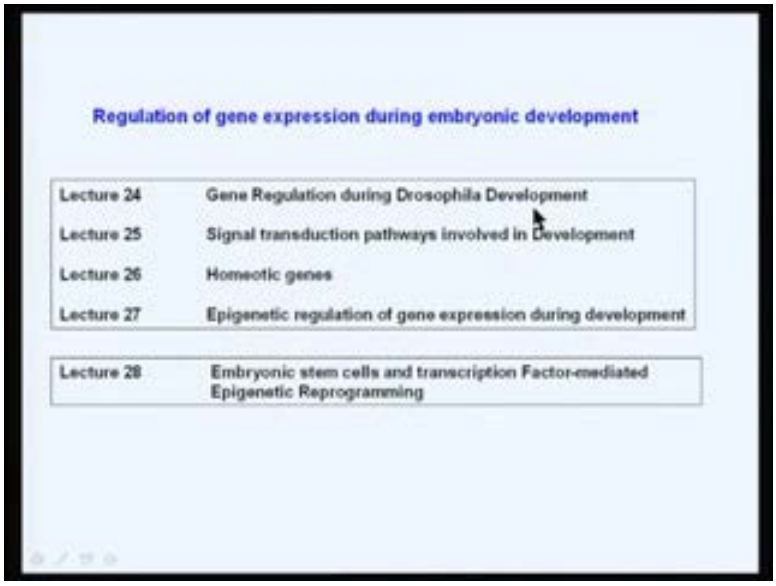


We used Drosophila as a model system, and then looked at how gene regulation plays a very important role in the regulation of gene expression in Drosophila, and then we discussed about the role of maternal genes– the zygotic genes– and how their activation– sequential activation– plays an important role in development. Then, we discussed, very briefly, about the role of signal transduction pathways in development regulation and how transcription factors turn on specific signal transduction pathways, and ultimately leading to activation or repression of transcription of various genes, and this, in turn, leads to the early embryonic development. Then, we discussed about master regulators called homeotic genes, how they play very important role in identifying organ identity, or how they act as master regulators in gene differentiation.

And in the last class, we discussed about the role of epigenetics, about DNA methylation, histone methylation, or histone modifications– how they play very important roles during embryonic development.

So, today, as I said, we are going to discuss about another very important aspect of… fascinating aspect of embryonic development, and in fact, the next decade is going to see very tremendous advantages in this area, namely, the way embryonic stem cells differentiate and how transcription factors play an important role in the differentiation of embryonic stem cells, and how, by expressing specific transcription factors, you can

actually convert an adult cell– a differentiated adult cell– into embryonic stem cell, and then re-differentiate into all cells of the body.

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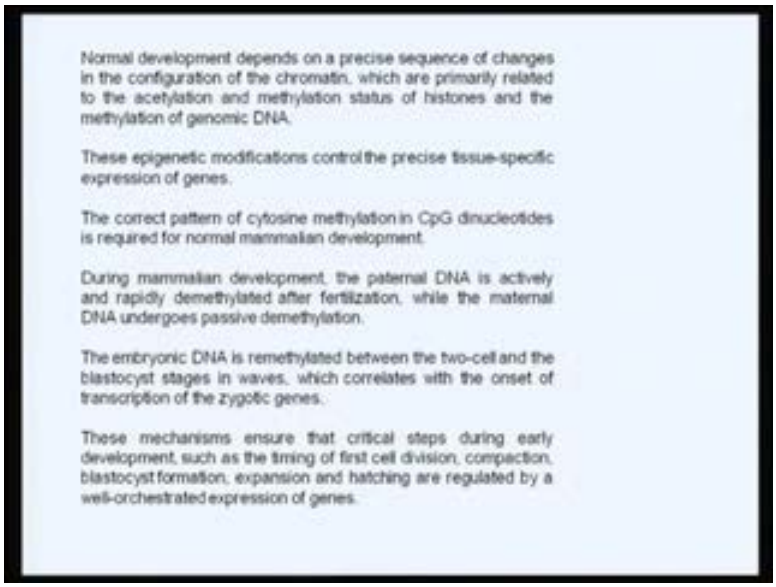


So, it is a very fascinating field of research which is now going to lead to what is called as regenerative medicine. So, let us now try to understand what are these. Now, normal development depends on the precise sequence of changes in the configuration of chromatin, which are primarily related to the acetylation methylation status of histones, and the methylation of genomic DNA. This we have discussed, both during the early stages of this course as well as in the last classes, saying that how DNA methylation and histone modification play very, very important roles in the embryonic development.

Now, these epigenetic modifications, where DNA methylation has the system modifications control the precise tissue-specific expression of genes, and a correct pattern of cytosine methylation in the CPG dinucleotides is required for the normal embryonic development. We have discussed many examples, including X-chromosome inactivation, and so on and so forth.

And also, during early embryonic mammalian development, the paternal DNA is actively and rapidly demethylated after fertilization, while the maternal DNA undergoes passive demethylation. Again, we discussed this extensively in the last class, and the embryonic DNA is remethylated between the two-cell and the blastocyst stages in waves, which correlates with the onset of transcription of the zygotic genes. So, it is a complete

epigenetic reprogramming during early embryonic development, especially in the two-cell to four-cell stage up to blastocyst. So, all this epigenetic regulation of gene expression mechanisms ensure that critical steps during early development, such as the timing of the first cell division, compaction, blastocyst formation, expansion, and hatching are regulated by well-orchestrated expression of a set of genes.

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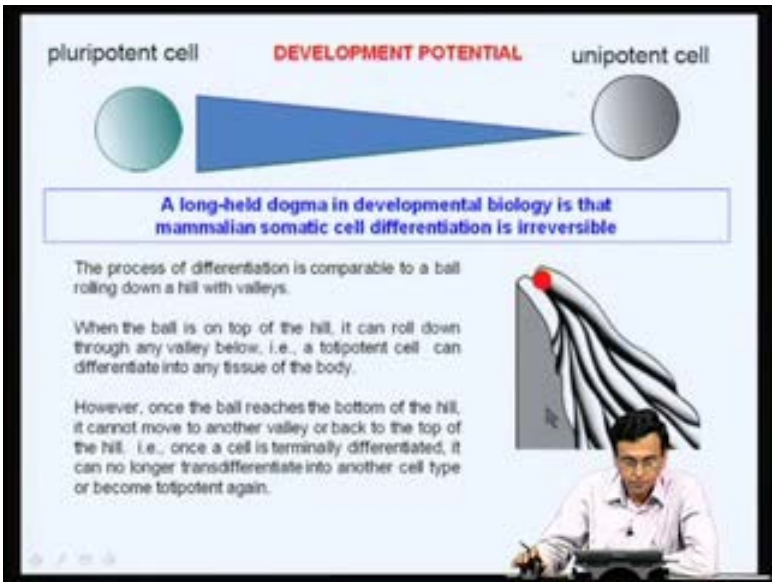


This is what is a take-home message that we learnt in the last four classes. Now, a long-held dogma in developmental biology is that mammalian somatic cell differentiation is irreversible. That means, once you have a pluripotent or totipotent fertilized egg, the deployed cell in the undifferentiated form, and once the differentiated programs sets in during embryonic development, and once you have an adult differentiated cell, it cannot go back into a totipotent stage. You cannot get a pluripotent stage, or which is seen in the early embryonic development from a unipotent or a differentiated adult cell.

This has been a dogma for a long time in the developmental biology. So, as the pluripotent cell, during early embryonic development, starts differentiating into various adult cells that during development, it loses just totipotency, and then becomes polarized to specific cell types. So, the process of differentiation is comparable to a ball rolling down a hill with valleys. So, here is a hill with valleys and here is the totipotency. So, when the ball is on the top of the hill, it can roll down to any of these valleys below.

That means a totipotent cell can differentiate into any of these different pathways. It can become a liver cell; it can become a brain cell; it can become a pancreatic cell and so on and so forth. But once the ball reaches the bottom of the hill, it cannot move back into any of these valleys on the top of the hill and become totipotent again; that means, once a cell is terminally differentiated, it can no longer transdifferentiate into another cell type or become totipotent again. So, once a totipotent cell chooses a specific differentiation pathway and becomes either liver cell or a muscle cell or a brain cell, it cannot again go back up the hill and become totipotent, again.

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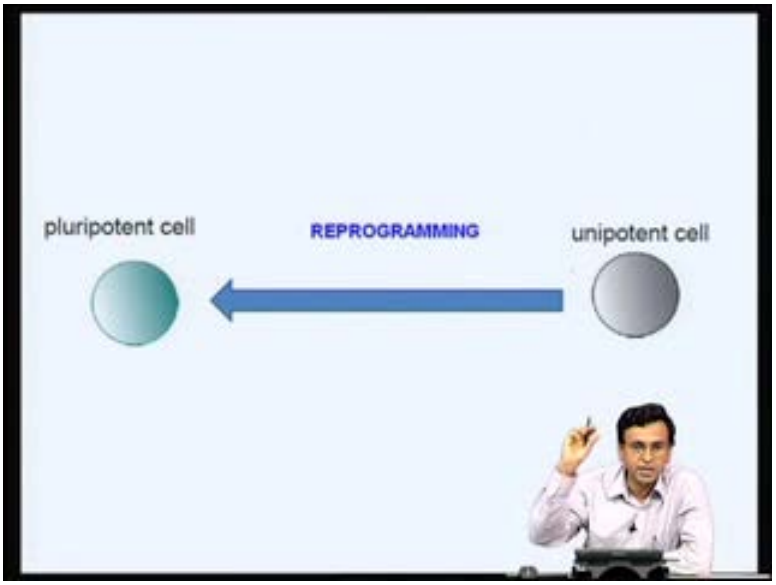


This has been the dogma in developmental biology for a long time. A lot of people, even during the in the early 90s, have, actually, tried to see whether you can take this kind of a differentiated cell or a unipotent cell and reprogram it, so that it can become a pluripotent cell. This has been one of the major challenges in the area of developmental biology. That means, can you take a differentiated cell and make it into a pluripotent cell, which we see during the early embryonic development.

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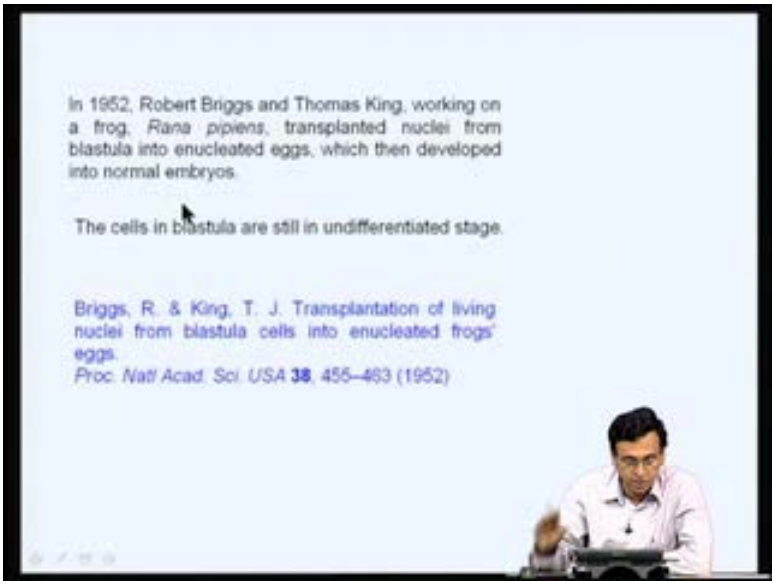


This has been a major challenge; I will give you some examples, historically, how people tried to do this; that is, can you take a differentiated cell and reprogram it, so that it now becomes a undifferentiated totipotent cell. For example, in 1952, Robert Briggs and Thomas King, working on a frog– Rana pipiens– transplanted the nuclei from the blastula into a enucleated eggs, which then developed into a normal embryo.

So, they did not go into an adult cell, but they took a nuclei from a blastula, which is a well-advanced stage of development, and the nuclei from a blastula cell was implanted or transplanted into a enucleated fertilized egg, and then showed you can get a normal frog, suggesting that this nucleus in the blastula can be programmed to a totipotent cell, and you can get a normal animal; it can differentiate into organs or tissues. But, this is not really a total reprogramming, because the cells in blastula are still not differentiated; they are still in the undifferentiated stage. So, this is not considered as a major achievement.

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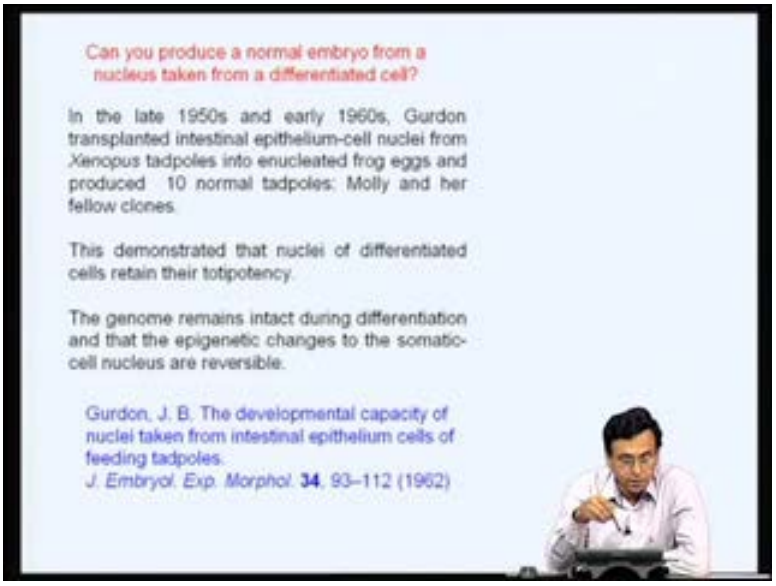


But then, the challenge was– can we actually produce a normal embryo from a nucleus taken from a differentiated cell? This was done in the late early, in the early 90s, where Gurdon transplanted intestinal epithelium cell nuclei from Xenopus tadpoles into an enucleated frog egg and produced ten normal tadpoles. So, you took a highly differentiated tissue type like an intestine; from that, you take a nucleus, and then transplant enucleated frog legs, and you got developed adult, developed tadpoles.

This demonstrated that nuclei of differentiated cells can still retain their totipotency. So, the genome remains intact during differentiation, and that the epigenetic changes to a somatic cells are reversible. So, during in the last class, I actually told how many epigenetic programs in the… are, are reversed during early embryonic development, like the DNA methylation is completely erased in the male genome, and then a totally a different set of epigenetic programming takes place in waves during early embryonic development, but these kinds of experiments, actually, showed this adult cell differentiated cell, which is epigenetically quite different from a nucleus in the fertilized egg, can now be programmed to the same status of that of the undifferentiated nucleus.

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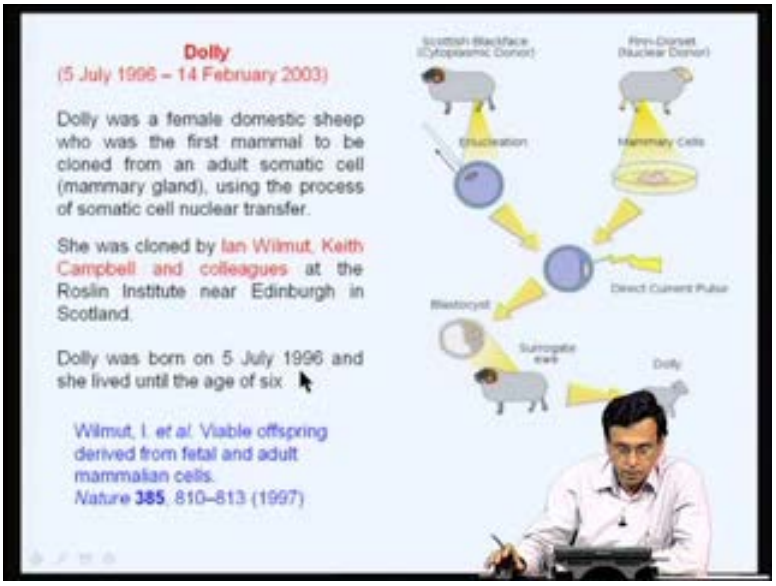


The climax in this kind of a experiment– this series of experiments– actually reached when Ian Wilmut, Keith Campbell, and colleagues in the Rosilin Institute of Edinburgh in Scotland generated, developed what is called as, say, the famous Dolly. I am sure every one of you have heard of this term– Dolly– which is a sheep, which was generated by somatic cell nuclear cloning. What they did, they actually took the nucleus from the udder or the mammary gland of the sheep, and put this adult differentiated nucleus into a enucleated fertilized egg, and actually demonstrated you can generate or develop an entire sheep from an embryo, which contains…, which contains the nucleus from an adult differentiated cell, rather than the egg of a fertilized egg generated by…, than the nucleus generated by a fertilization.

So, Dolly was a female domestic sheep who was the first mammal to the cloned from an adult somatic cell using the process of, what is called as, a somatic cell nuclear transfer. The dolly lived up to an age of six, but it had a lot of other problems, clearly telling that the epigenetic reprogramming is not really equivalent to what is seen in the normal development.

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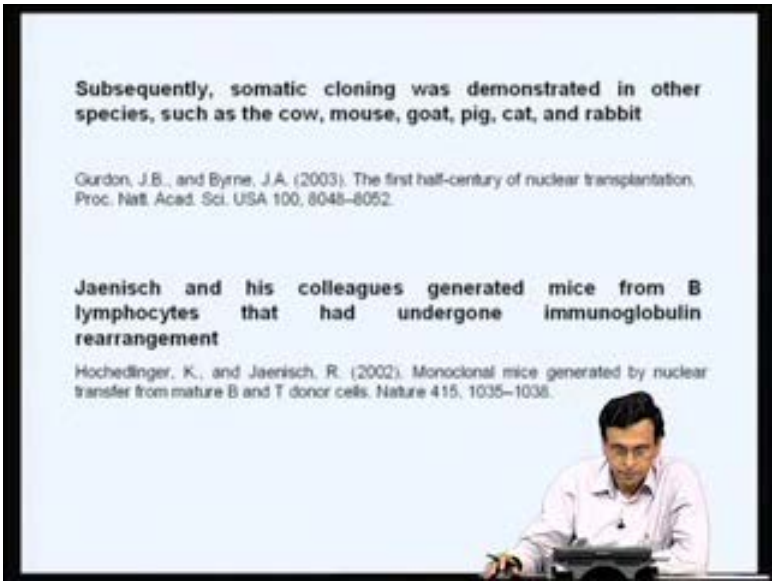


Subsequently, this kind of a somatic cloning that is where you take nucleus from an adult tissue, put it in an enucleated fertilized egg, and then develop a complete animal, was initially shown with Dolly, but then, later, was shown in a number of other species like cow, mouse, goat, pig, cat, rabbit, etcetera; a number of papers were actually published. In fact, this a nice review in PNAS by Gurdon and Byrne in 2003.

What does some of this very interesting paper for example, Jaenisch and his colleagues actually generated mice from B-lymphocytes that had undergone immunoglobulin rearrangement. You know, in all the sides of the body, the immunes as the B cells; that is, the immunoglobulin rearrangement. Even such genome nucleus in which the immunoglobulins are rearranged can be now reprogrammed to develop a…, and you can get a complete animal from using nuclei from such reprogrammed adult cells.

So, all these experiments clearly told that this dogma, that once the embryonic differentiation development had seen and self starting differentiating, and you get a differentiate as a cell type. They cannot be reprogrammed into a totipotent cell is reversible, and it is not unidirectional. You can reprogram these adult cells back into early embryonic cells, which are pluripotent.

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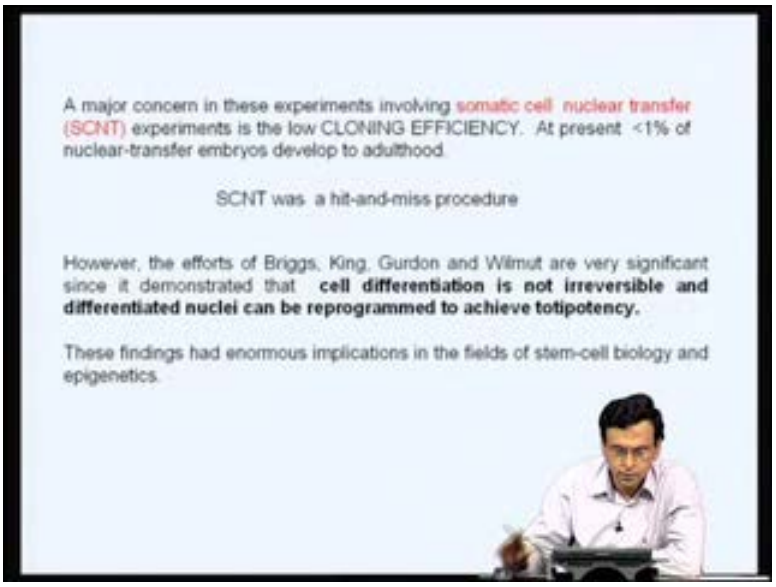


But one of the major problems with this kind of a somatic cell nuclear transplantation experiment was that the efficiency of this cloning was very, very low. For example, only one percent of such nuclear transfer embryos actually developed into adults. So, the failure rate was very, very high.

So, basically, somatic cell nuclear transfer was a hit-and-miss procedure; you try some 100 or 200 different nuclear transfers, and one or two may work, but nobody knows why it is failing. So, although the the efforts of Briggs, King, Gurdon, Wilmut– they are all very, very significant, because although the failure rate was very high, it was significant because it demonstrated for the first time that cell differentiation is not irreversible, and differentiated nuclei can be reprogrammed to achieve totipotency. This was one of the most important observations that came out of all these experiments, although the failure rate was very, very high. These findings had enormous implications in the fields of stem-cell biology, as well as epigenetics and developmental biology.

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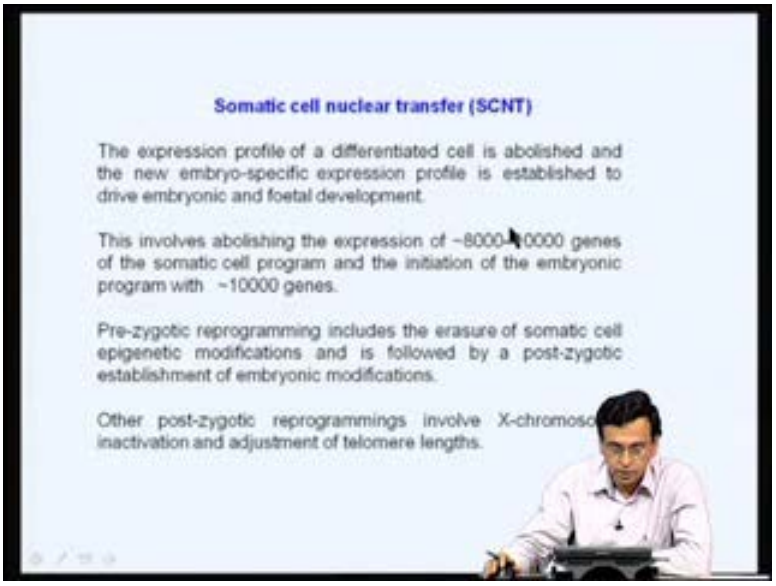


Now, let us see what exactly is happening in this somatic cell nuclear transplantation experiments. Now, the expression profile of a differentiated cell is completely abolished and a new embryo-specific expression profile is established to drive embryonic and foetal development.

In adult cell, is it they…, the set of genes which are expressed in a liver cell is different from that of a muscle cell, which is different from a brain cell, but all these program transcription programming has to be erased and a totally new program has to be developed, so that this genome can now go and then give rise to small cell types of a body.

It is a major challenge. Basically, this involves abolishing expression of about 8000 to 10000 genes of the somatic cell program and initiation of embryonic program with approximately 10000 genes, and you can see the challenge here; the expression about 8000 or 10000 genes, which are specific for that type of tissue– tissue type– has to be abolished, and a new set of genes have to be expressed, which lead to normal embryonic development. Pre-zygotic program includes, reprogramming includes erasure of somatic cell of epigenetic modifications and followed by a post-zygotic establishment of embryonic modifications.

So, all these things– unless all these things happen correctly, you will not get a proper embryonic development. That is why the failure rate is very, very high. In addition, other

post-zygotic reprogrammings, which involve X chromosome inactivation, adjustment of telomere length, all this has to happen if an adult nucleus has to differentiate into, develop into a normal embryo, has to be generated from a adult nucleus, differentiated cell nucleus. So, while these experiments where going on, so on one hand scientists were trying, trying to see if you take a nucleus from a adult differentiated cell and put in back in an a fertilized egg environment, can it give rise to all cell types? Can it give rise to a adult normal embryo leading to adult?

While this is going on, somatic cell nuclear transplant is, at present, going on. On the other hand, people who are also trying to understand how pluripotent stem cells or what are called embryonic stem cells develop into various, various cell types. Now, so the story of stem cells was being very actively pursued by another group of scientists. Now, let us see what are these stem cells.

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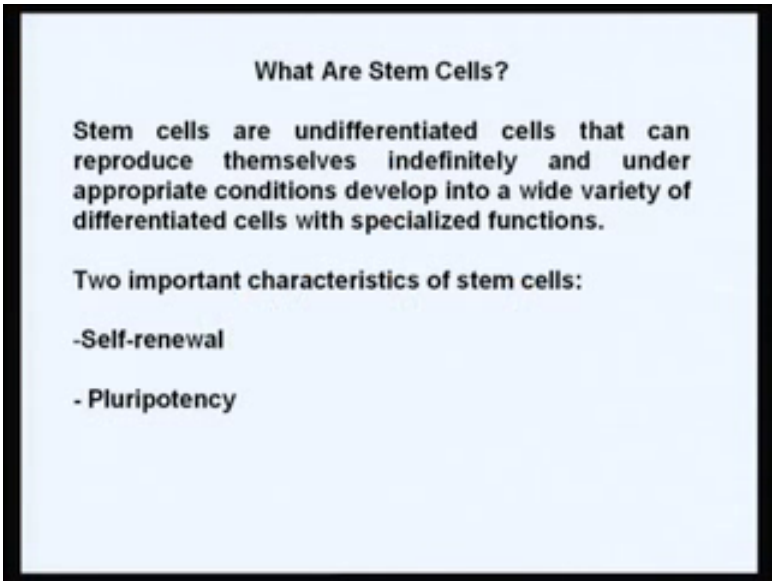


Stem cells are nothing but undifferentiated cells that can reproduce themselves indefinitely, and under appropriate conditions, develop into a variety of differentiated cells with specified functions. This is what happens during the normal embryonic development as the fertilized egg develops, starts dividing, and keeps on developing into a ball of cells like morula, blastula, and so on and so forth.

Slowly, the cell starts losing totipotency and then become to go, go towards unipotency. So, at T or certain stage of embryonic development, the cells– all the cells of embryo–

will remain totipotent; that means they can differentiate into all three germ layers– ectoderm, endoderm, and mesoderm. So, the such cells, which have totipotency, are known as the embryo, an embryonic stem cells, or the ES cells.

The two important characteristics of such stem cells are self-renewal; that is, they can keep on dividing in a totipotent stage and pluripotent stage. They, they have to keep on dividing, but they should also retain their pluripotency. These are the two important characteristics of a stem cell.

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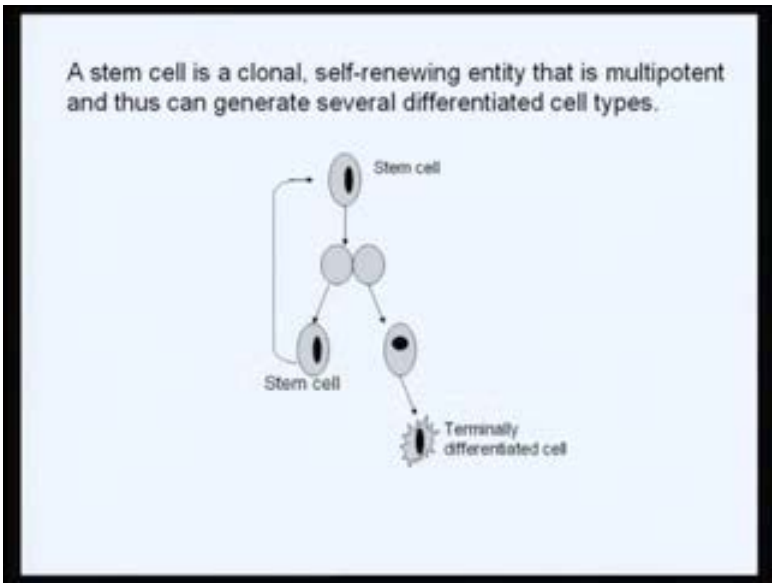


So, a stem cell is a clonal, self-renewing entity that is multipotent, and thus can generate several differentiated phenotypes. So, as the stem cell keeps on dividing, it should be able to generate the same– its own kind– of stem cells again and again. This is what is the self-renewal. At the same time, was some population of the stem cell should be reprogrammed into terminal differentiated cells. These are the two important properties of these stem cells. It can, on one hand, it should keep on repopulating its own stem cell phenotype. On the other hand, some of these stem cells should differentiate into specific phenotypes.

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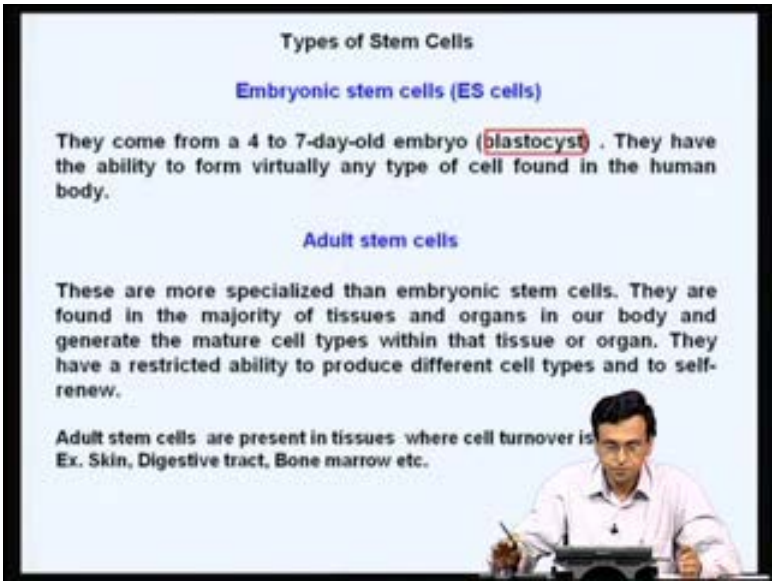


There are two major types of stem cells; one are called the embryonic stem cells, and the other called as the adult stem cells. The embryonic stem cells, usually, are derived from four to seven day old embryo, usually, when the development has proceed up to a stage of blastocyst, and they have the ability to form virtually any type of cell found in the human body. So, these are called embryonic stem cells.

Adult stem cells– the adult stem cells are much more specialized than embryonic stem cells. They are found in the majority of the tissues and organs in our body, and are generated from the mature cell types within that particular tissue or organ. They have a restricted ability to produce different cell types and to self-renew. For example, adult stem cells are present in tissues where cell turnover is very high– skin, for example, there is a continuous loss of cells in the skin. Therefore, new skin cells have to keep on getting regenerated.

So, you need to have stem cells in the skin. Otherwise, if there is some damage happens on skin, you can have get a new skin. Similarly, bone marrow; the bone marrow has to continually produce various blood cells, various cell types in the blood, and therefore, there have to be stem cells in the bone marrow.

Similarly, digestive tract– there is a continuous turnover of cells. In the digestive tract, old cells are getting destroyed and new cells are being formed. So, in many of these adult

tissues where there is a very high turnover, you need to have stem cells so that these cells have to be repopulated again and again.

But the difference between an adult stem cell and embryonic stem cell is that a stem cell which is present in the skin cannot give rise to intestinal cell. Similarly, a stem cell, which is from bone marrow, can only yield the various blood cells. It cannot go and form a skin cell. So, their ability is very, very restricted, unlike an embryonic stem cell, which can differentiate into any cell type. So, these are the two major distinctions between an embryonic stem cell and an adult stem cell.

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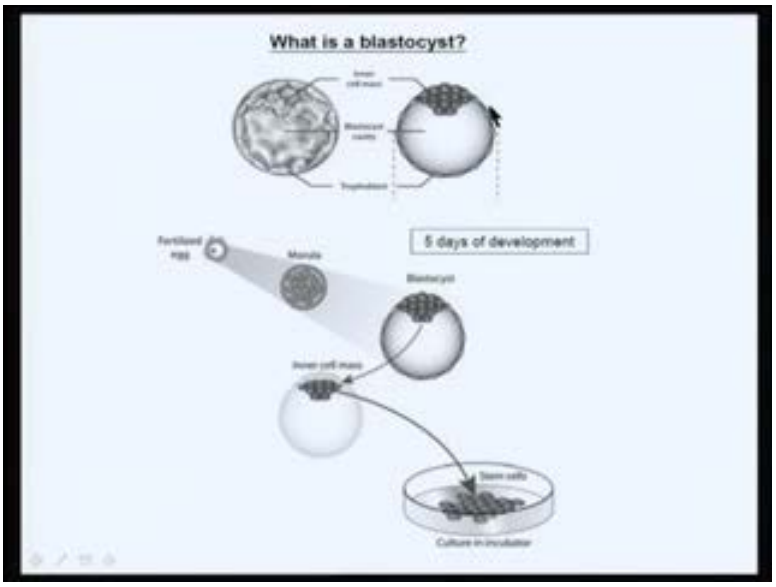


So, let us now concentrate on the embryonic stem cells. As I said, the embryonic cells are derived from when the embryo has, actually, reached the blastocyst. As the fertilized egg starts dividing, you get a ball of cells called morula; then it regits a starts a blastula, and in the blastula, you have what is called as a inner cell mass, and it is in this inner cell mass which has the pluripotency.

And it is the cells of the inner cell mass which are known as the embryonic stem cells, because if you now take these inner cell mass cells and culture them appropriately, they can differentiate into cells of all three germ layers, namely, ectoderm, endoderm, and mesoderm.

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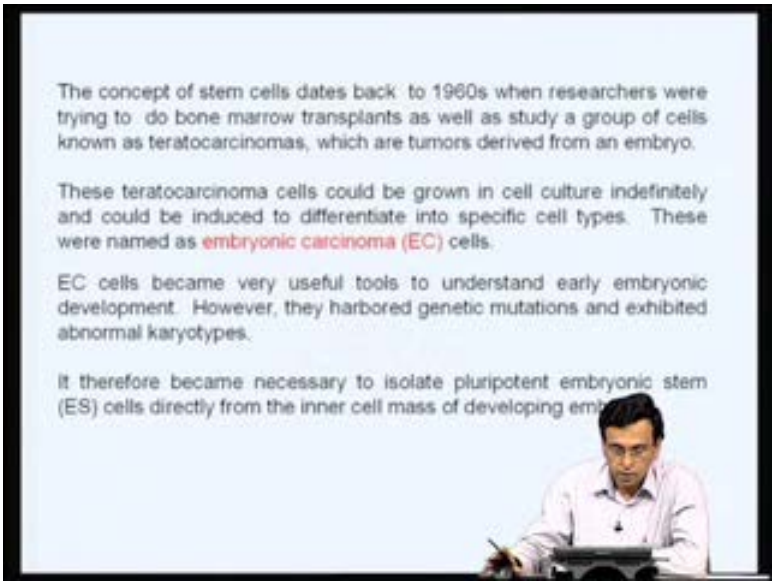


So, the concept of stem cells– that is, during embryonic development, there are what are called as stem cells dates back to 1960, when researchers were trying to do bone marrow transplants as well as study of group of cells known as teratocarcinomas, which are basically tumors derived from embryo. The tumors which are derived from developing embryo are called as teratocarcinomas, but these teratocarcinoma cells could be grown in cell culture indefinitely, and they could also be induced to differentiate into specific cell types, and therefore, these are known as the embryonic carcinoma cells or EC cells.

Remember, EC cells are different from ES cells. The EC cells became very, very useful tools to understand early embryonic development; however, they harbor genetic mutations and therefore, they exhibited abnormal karyotypes.

It, therefore, became necessary to isolate pluripotent embryonic stem cells directly from the inner cell mass of developing embryo, because the EC cells were not really giving the actual picture that is happening during embryonic differentiations, because these are actually tumour cells and they, their karyotype is quite different, and they did not exactly behave like the embryonic stem cells of a normal embryo.

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So, three people made a very important contribution for the study of embryonic stem cells and the applications. These are Mario Capecchi, Martin Evans, and Oliver Smithies. All of them were awarded, jointly, Nobel Prize in the year 2007 for their discovery of introducing specific gene modifications in mice by using embryonic stem cells. Now, let us now focus, for this lecture, only on Martin Evans, and see what, exactly, did he do.

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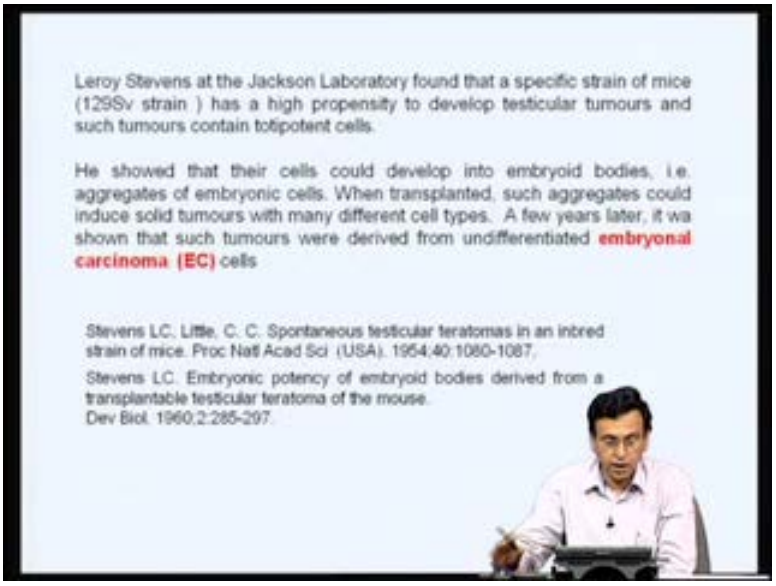


Now, the background for Martin Evans' work comes from Leroy Stevens laboratory in Jackson laboratory. What he actually found is that when one particular strain of mice–

they have a very high propensity to develop testicular tumours– and such tumours actually contain totipotent cells.

This was the observation from the laboratory of Leroy Stevens; this what is showed is that these cells from this particular mouse develop into embryoid bodies, that is, aggregates of embryonic cells, and when transplanted, such aggregates could induce solid tumours, which contain many different cell types. That means, although it is tumourigenic, these cells had the capacity to differentiate into any different cell type, namely, the ectoderm, endoderm, and mesoderm divide cells.

So, a few years later, it was shown that such tumours were actually differentiated from undifferentiated embryonal carcinoma cells or the EC cells, which we just discussed. So, now, these are important papers which, actually, were published during the early 1950s and 60s.

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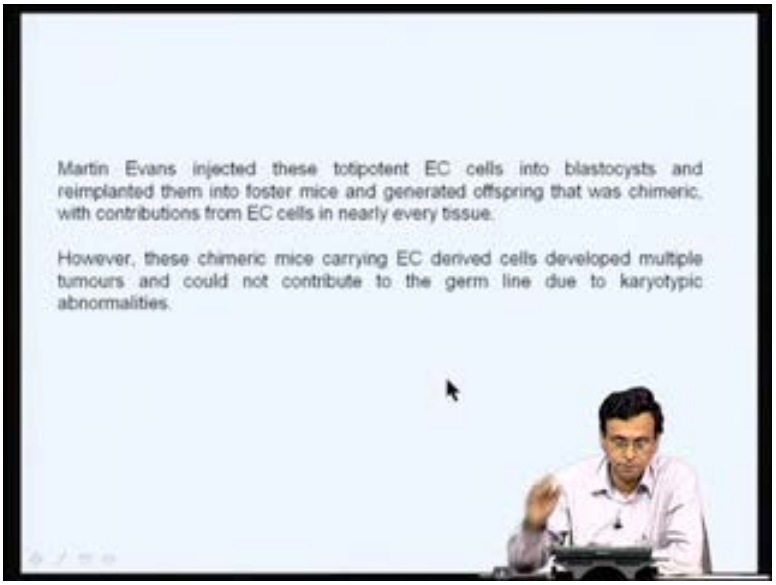


Martin Evans found this observation very, very interesting; that is, there are certain tumour cells which behaved like embryonic stem cells, because they can differentiate into any cell type. They can give the cells of ectodermal origin, endodermal origin, mesodermal origin; that means, they are totipotent. So, he took these EC cells from Leroy Stevens lab, and when injected them into blastocyst and reimplanted them into foster mice, and generated offspring that were chimeric with contribution from embryonic cells in nearly every tissue.

These are very, very important experiments what Martin Evans did– he took this embryo carcinoma cells and then injected into blastocysts, and found that these embryonal carcinoma cells can actually repopulate all the tissues of the body, although, because they are of tumour origin, they actually develop tumours.

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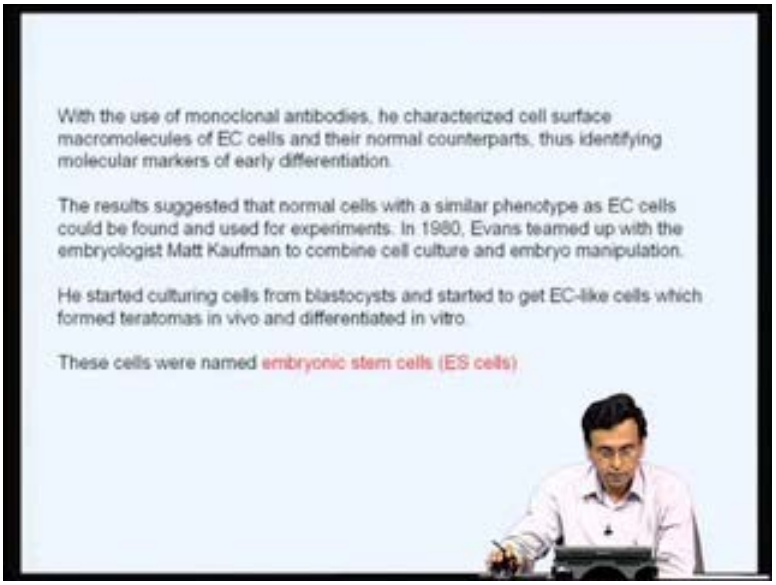


So, but this– the fact is that these embryo carcinoma cells are totipotent, and they can, actually, give rise to all the cells of a body gave a very important thing, that these EC cells, probably, have specific markers that are very, very important for the embryonic stem cells. So, using monoclonal antibodies, Martin Evans actually characterized cell surface molecules which are present in this embryonal carcinoma cells and normal counterpart, thus identifying what are the molecular markers for early differentiation.

So, these are some of the very, very important work that went on to characterize embryonic stem cells. So, Martin Evans actually identified what could be the molecular markers that are expressed on the surface of early embryonic development and embryonal cells which are pluripotent. The results suggested that normal cells with a similar phenotype as EC cells can be found and used for experiments, and in 1980, Evans teamed up with the embryologist Matt Kaufman to combine cell culture and embryo manipulations.

So, once you have you identified a specific markers on the EC cells, which are also present in certain embryonal cells of a very, very early development stage, he started to

see whether you can culture such cells which can retain their pluripotency. So, he started culturing cells from blastocysts and started to get EC-like cells, which formed teratomas in vivo, and with differentiated into various cell types in vivo, and these are the cells which named as embryonic stem cells or the ES cells.

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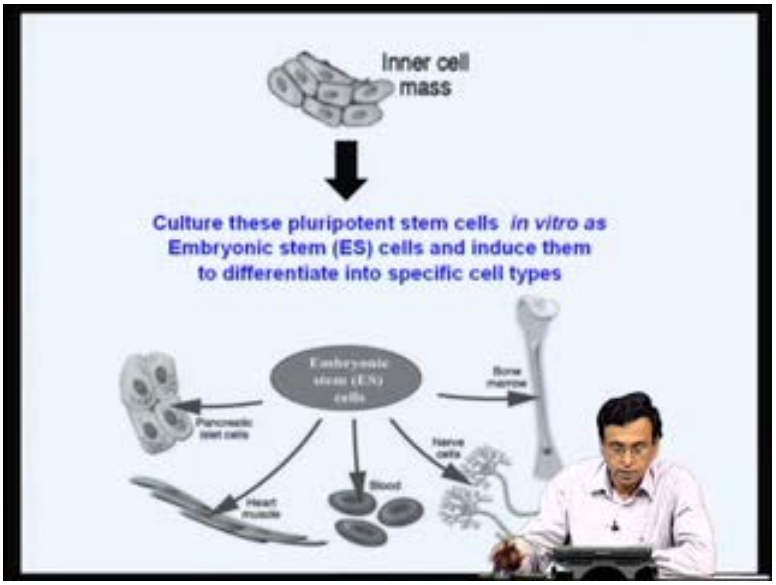


Now, what is the importance of these ES cells? Why people are after these ES cells? You have an inner cell mass in the blastocyst, and if you now take this inner cell mass, if you can now find some culture conditions by which you can able to maintain their pluripotency and make them divide indefinitely, then if you now can add specific factors and induce this pluripotent embryonic stem cells into specific cell types, you can make them induce to either into pancreatic cells producing insulin, or you can differentiate into heart muscle cells, or you can derive blood cells from them, nerve cells, bone marrow cells. If you can do this, then you have achieved wonders.

So, if you can have a condition by which these embryonic stem cells can be cultured indefinitely in a laboratory rather than inside the blastocyst, then you can actually use these ES cells to understand how these ES cells can be differentiated into various cell types; what kind of actors are actually required for the differentiation of embryonic stem cells, and once you identify these factors, you can virtually derive any specific cell type using these embryonic stem cells.

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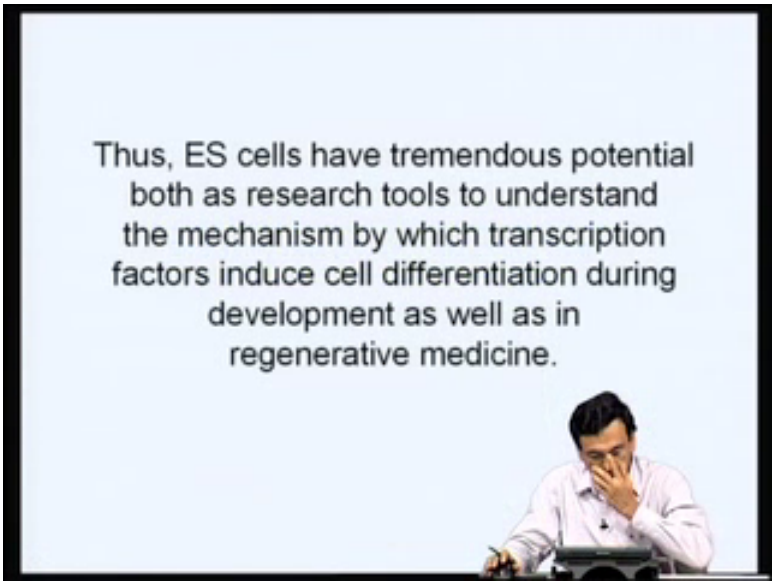


This has very, very great applications in regenerative medicine. So, the ES cells have tremendous potential both as research tools to understand the mechanism by which transcription factors induce cell differentiation during development, as well as in regenerative medicine.

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So, the ES cells became very, very important, and the problem was how to culture these ES cells without losing their totipotency. This was a major challenge. Slightly, if you

vary the culture conditions, they would lose their totipotency, and they would not express those markers which are characteristics of totipotent ES cells.

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So, one has to develop very, very careful culture conditions to retain the totipotency of these ES cells. So, in the year 1991, the ES cells were, actually, isolated from mouse embryo, and they, actually, published the exact culture conditions by which these mouse ES cells could have be cultured by threatening their pluripotent state.

These are the two classic papers that appeared in Nature in 1981– establishment in culture of pluripotent cells from mouse embryos and isolation of a pluripotent cell line from early mouse embryos cultured in a medium conditioned by teratocarcinoma stem cells. So, this paper which was published in 1981 demonstrated that it is possible to culture this embryonic stem cells from the inner cell mass of the blastocyst, in a still retaining that pluripotent… pluripotency. Remember, this was in 1981; they could demonstrate the mouse stems embryonic stem cells can be cultured in, in a totipotent state, but it took… it was only in the year of 1998, a similar, similar thing could be achieved with human embryonic stem cells.

So, it was only in the year 1998, researchers lead by James Thomson in the University of Wisconsin developed a technique to isolate and group human embryonic stem cells in culture. So, from 1981, it took these many years, to upto 1998, to culture, actually, human embryonic stem cells in the same way Martin Evans demonstrated for mouse

embryonic stem cells. So, it is not very easy to culture these embryonic stem cells of mouse or human origin exactly the way as they develop during embryonic development in the blastocyst retaining pluripotency was a major challenge.

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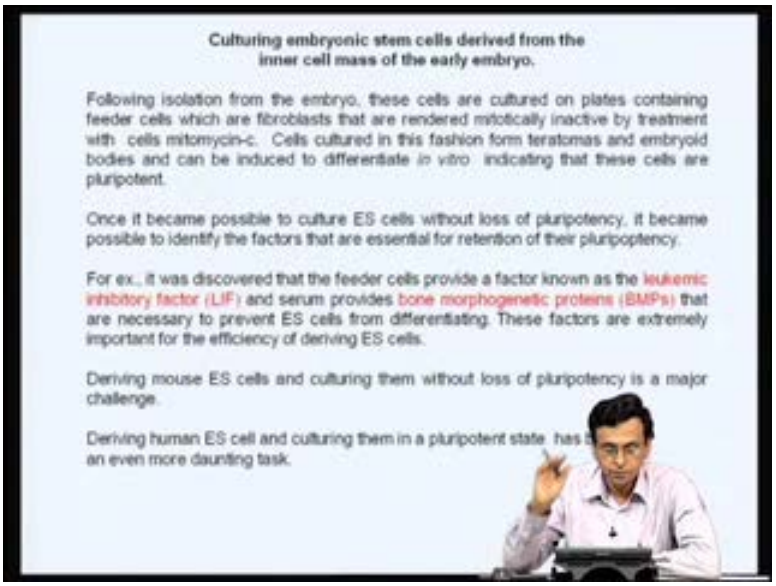


So, following the isolation of the from the embryo, these embryonic stem cells or ES cells are cultured on plates containing what are called as feeder cells. You cannot just put these embryonic stem cells on to a normal cell cultured plate. You have to you have what is called as a feeder cells, which are nothing but fibroblasts, which are rendered mitotically inactive by treatment with mitomycin-C. So, you have a feeder layer cells, which cannot divide because they have not been treated with the compound called mitomycin-C. So, they cannot divide, but if you now see, embryonic cells is over this feeder layer. Only embryonic cells will divide, but the feeder cell layer, actually, does not divide, but actually provides these nutrients that is essential for the embryonic stem cells to multiply. So, cells cultured in this fashion form teratomas and embryoid bodies can be and can be introduced in vitro, indicating that these cells are pluripotent.

So, once it became possible to culture ES cells without the loss of pluripotency, it became possible to identify what are the factors, which are essential for retention of the pluripotency. So, you can see, these are very, very important. Now, if you want to understand what are all the factors which are required for the pluripotency of embryonic stem cells inside a blastula, you cannot do it, but once you have a culture system, in

which you can culture these ES cells in a… in a laboratory– in a culture dish– in a totipotent state, now you can ask questions as to what kind of factors are, actually, essential for retaining the totipotency.

In fact, it became very clear, but these feeder layers, which are mitomycin-C treated fibroblast– mouse embryonal fibroblast– they actually synthesize and secrete a factor called as leukemia inhibitory factor or LIF, and this LIF, as well as certain factors present in the serum such as the bone morphogenic protein are very, very essential if you have to keep these ES cells in an undifferentiated state. So, certain secretory factors that are secreted by this feeder layer or the feeder cells, such as the LIF, and the morphogenic proteins keep these ES cells in a different undifferentiated state.

So, these factors are extremely important for the efficiency of deriving ES cells. So, if we have to maintain these ES cells in a pluripotent state, you have to grow these cells in the presence of this LIF and bor morphogenic factors. Only then, the moment you remove them, they differentiate into various different phenotypes, clearly indicating that these factors play very important role in the retaining the pluripotency or the undifferentiated state of the embryonic stem cells.

Deriving mouse ES cells and culturing them without loss of pluripotency is a major challenge. As I said, in 1981, Martin Evans published his papers, and it took more than 17 years– only until 1998– that a similar thing could be achieved with human ES cells. So, the conditions by which either the mouse or the human ES cells could be cultured in a pluripotent state was a major challenge, and it took a lot of time to achieve actual culture conditions by which these embryonal stem cells can be cultured, indefinitely, in a laboratory in a pluripotent stage

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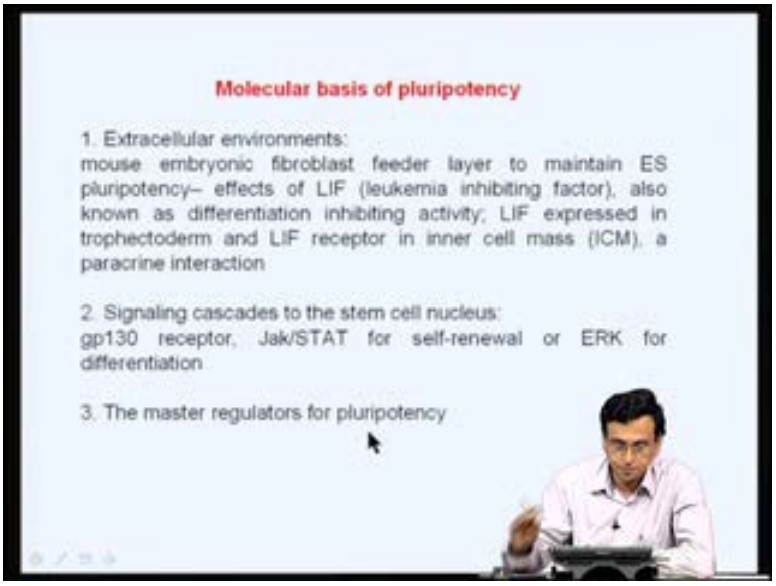


So, once they had such culture conditions, people started asking the question– what are the molecular determinants of pluripotency? For example, just like… just as I mentioned in the last slide, you have the leukemia, leukemia inhibiting factor or the LIF, is a very, very important factor for keeping the totipotency or pluripotency of the embryonic stem cells.

Similarly, in specific signaling cascades from the stem cell nucleus involving gp130 receptor, certain JAK-STAT signaling pathways or the ERK pathways were found to be very, very important for this retaining this totipotency, and many master regulators of pluripotency could be identified once you had a system for culturing these ES cells in a pluripotent stage.

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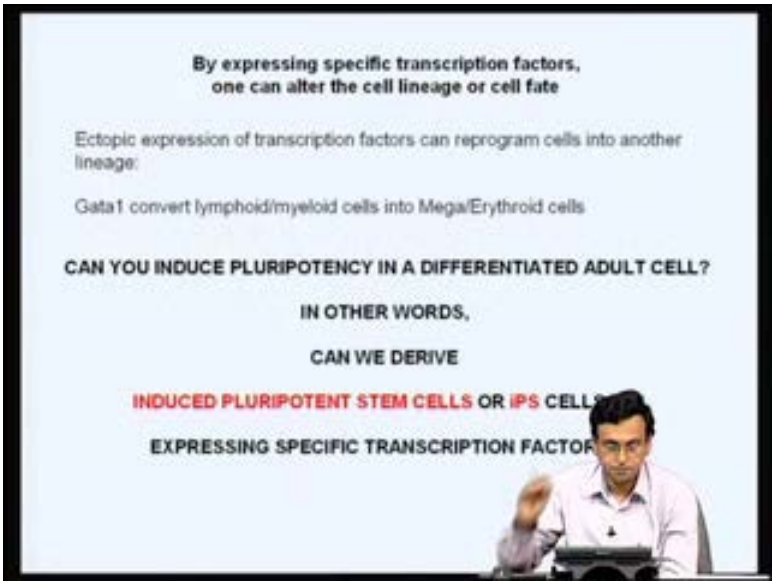


So, people started asking the question– by expressing specific transcription factor, is possible to alternate this cell lineage of these ES cells? For example, while people are working with the ES cells– embryonic stem cells– culturing them, there are also people who were actually taking these adult stem cells, which I told you in the, in the previous slides, there are other stem cells which are present in skin, in the intestine, in bone marrow and so on and so forth. So, people took this adult stem cell from these tissues and asked the question– can you now reprogram, reprogram into different type of cells by expressing the specific transcription factors?

And such studies, actually, demonstrated that if you express a transcription factor called gata1, you can actually convert certain bone marrow cells, which are of the lymphoid or myeloid lineage, you can now convert them into a megakaryocytes or the erythroid lineage. That means in the bone marrow cells, as the bone marrow cells started differentiating into various blood cell site into different lineages, you can alter this lineage of this bone marrow derived stem cell into one lineage, into another lineage by expressing specific transcription factors.

So, people started asking the question– can you induce pluripotency in a differentiated adult cell? See, a myeloid lineage cannot be changed to erythroid lineage, but people are asking the question– can you now induce a pluripotent, derive a pluripotent stem cell from a differentiated stem cell by actually expressing specific transcription factor? So,

this concept of converting an adult differentiated cell into a pluripotent stem cell is known as the induced pluripotent stem cells or iPS cells.

So, a major challenge was– can you take an adult differentiated cell and induce pluripotency into the adult differentiated cells, or this of such, and if you can achieve that, such stem cells are known as the iPS cells. So, you have the embryonic stem cells where adult stem cells. Now, you are have a new kind of cells called as iPS cells or induced pluripotent stem cells, which are nothing but adult differentiated cells which have been reprogrammed by expressing specific transcription factors, so that they can now become pluripotent stem cells. These are called as iPS cells. Is it possible– that was the question that was asked.

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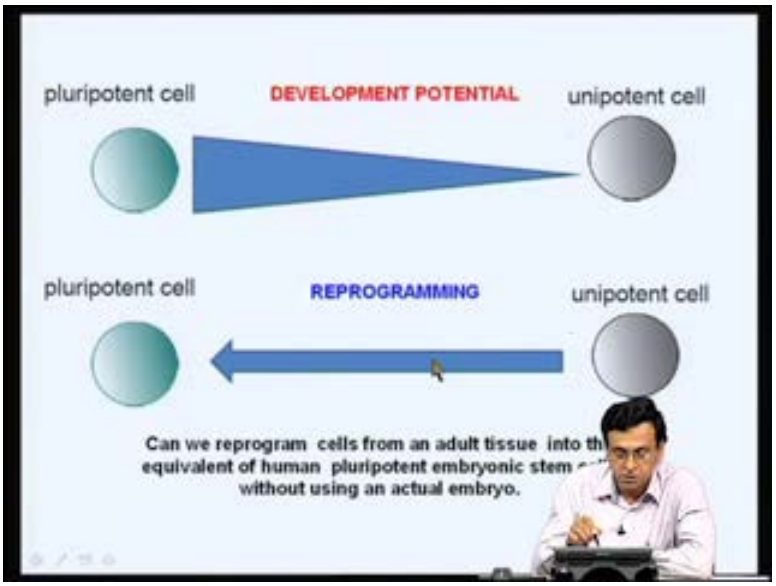


So, you have a pluripotent stem cell. During the course of development, they lose their pluripotency and they became a unipotent cell. This is what is happening in the normal course of development. Now, what we are asking– can you now take such a differentiated unipotent cell, and by expressing specific transcription factors, can you now reprogram it into a pluripotent cell? This is the question we are asking now.

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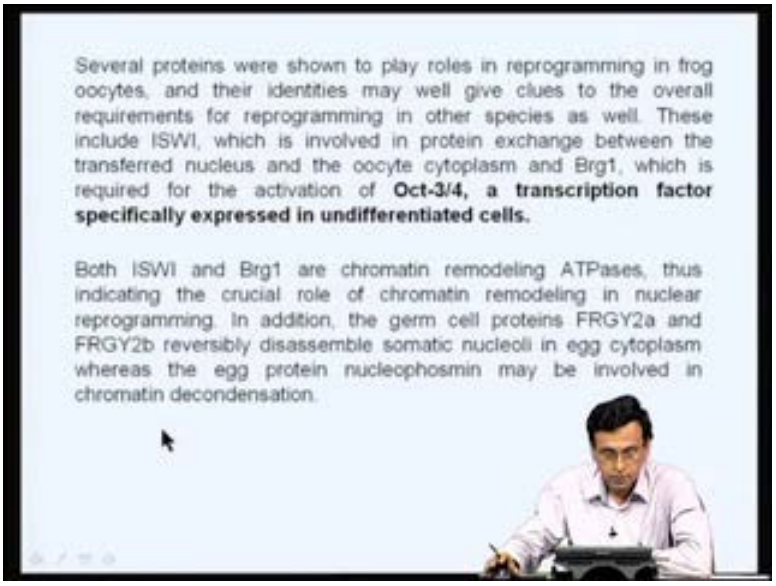


So, once you have people using have a all these cell culture techniques available, people started looking at all these things and see what kind of conditions can be… can induce any adult differentiated cell into a totipotent or a pluripotent stem cell, and such studies revealed that several proteins play very important role in reprogramming frog oocytes and their identities may well give clues to overall requirements for reprogramming in other species as well.

So, people were using a wide variety of model systems– frog oocytes– or people were using adult stem cells or embryonic stem cells– EC cells– all these cells are being used to ask the question, what are the factors that are involved in retaining the pluripotency and what are the factors that are involved in the differentiation of these stem cells into various differentiated phenotypes?

For example, if ISWI, a protein has been shown to be involved for the activation of the Oct3/4 transcription factor, which was found to be expressed only in the undifferentiated cells. So, the expression of Oct3/4 became a marker for an undifferentiated stem cell, because this Oct3/4 was never expressed once this stem cell differentiates into the differentiated cell type.

So, like this, by looking at various proteins that are getting expressed in undifferentiated cells, people started identifying markers which are…, by which by simply looking at the expression of this markers, you can say whether this cell is totipotent or pluripotent or

not, and proteins like ISWI and Brg1 were actually chromatin remodeling ATPases, indicating that crucial role… the chromatin remodeling has a very important role in this reprogramming these adult stem cells– adult differentiated cells– into specific stem cells.

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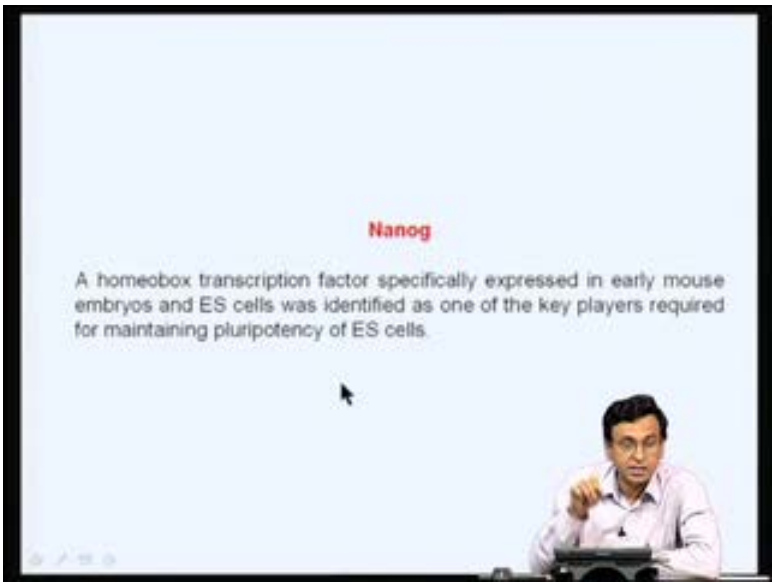


Another very important thing that came out of some of these studies is that a transcription factor called nanog, which is nothing but a homeobox transcription factor, was found to be specifically expressed in the early mouse embryos as well as ES cells, and is one of the key players that is required for maintaining the pluripotency of ES cells.

So, by looking or what kind of factors are getting expressed in the undifferentiated cell versus differentiated cells, people started slowly identifying what are the crucial transcription factors, which are required for maintaining the pluripotency, as well as those which are required for differentiation of this pluripotent stem cell to various cell types.

So, Oct-4 is one transcription factor that was shown to be expressed only in the undifferentiated cells; nanog is another transcription factor, which is shown to be expressed only in the early embryonic cells which are pluripotent. Many studies, now, so more studies were actually conducted to see what is the effect of this nanog expression on these ES cells.

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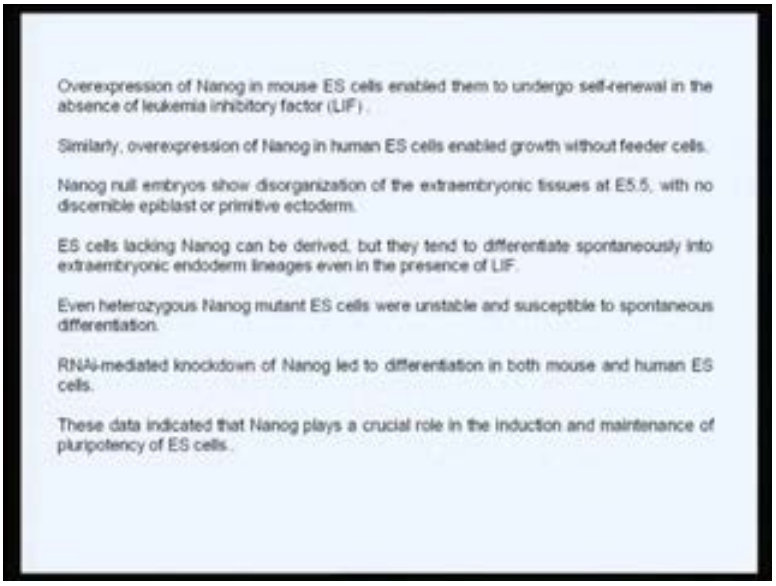


They found over expression of this nanog in mouse ES cells enabled them to undergo self-renewal even in the absence of leukemia inhibitory factor. I told you, the feeder layer actually produce a leukemia egg inhibitory factor or LIF, and this LIF is actually essential for keeping these ES cells in a undifferentiated state.

But now, people found out, would not require LIF if you express the nanog in the ES cells; that means, the function of LIF can now be taken over by nanog. Similarly, overexpression of nanog in human ES cells enabled growth without feeder cells; you do not even need a feeder cell if you can have ES cells, which are expressing nanog. Nanog can keep the ES cells in undifferentiated cell, which is actually done by LIF secreted by the feeder layer.

In fact, nanog nul embryos show disorganization of extraembryonic tissues at 5.5 day old embryos, with no discernible epiblast or primitive ectoderm, regarding that, this nanog plays a very important role in the differentiation. Similarly, ES cells lacking nanog can be derived, but they tend to differentiate spontaneously into extraembryonic endoderm lineages, even in the presence of LIF, clearly indicating that if ES cells do not express nanog, it is spontaneously differentiated into other cell types. They lose their totipotency. So, even heterozygous nanog mutant ES cells were unstable and were susceptible to spontaneous differentiation.

Similarly, RNAi-mediated knockdown– we will discuss some of these things in the next few classes. Basically, if you block the expression of nanog, it can lead to differentiation of both mouse as well as human ES cells. So, all this data clearly indicated that one of the factors which is very, very essential for retaining the pluripotency of both mouse as well as human ES cells is nanog. So, if you do not have nanog, the ES cells spontaneously differentiate and lose their totipotency.

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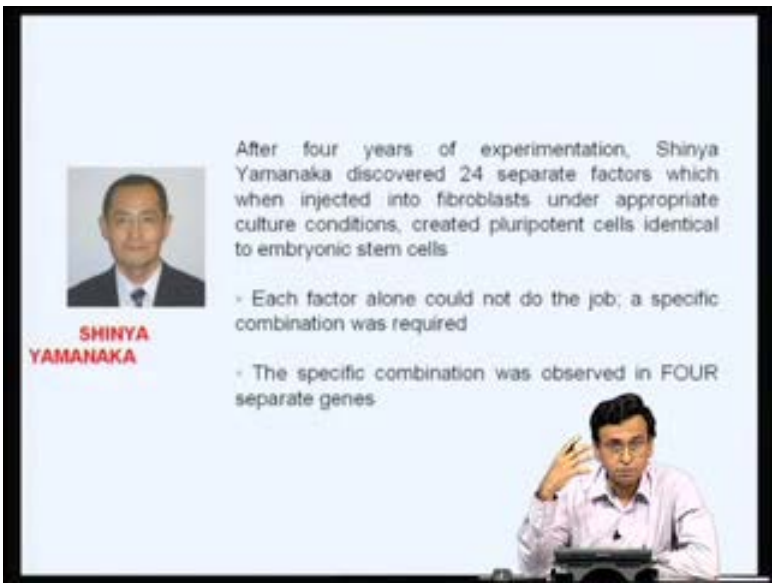


So, but see, the pluripotency is unlikely to be mediated by just one factor. To retain pluripotency, it probably requires many transcription factors. So, people started asking what are these combination of factors, which are expressed in these ES cells, that is essential for retaining in the pluripotency of these embryonic stem cells.

So, after experimenting with a number of transcription factors, which are expressed during the early embryonic development in the…, in the ES cells, Shinya Yamanaka, for example, discovered about 24 separate factors, which when injected in a fibroblasts in appropriate culture conditions, created a pluripotent cell identical to embryonic stem cells. So, people arrived at about 24 different transcription factors which, probably, are very, very important for the pluripotency. Each factor– each one of them alone– could not do the job, but a specific combination from these 24 factors is what is required to keep these ES cells in a pluripotent stage. So, a specific combinations soon by trusting a various combination of this factors among these 24, Yamanaka soon came out with a

combination of four transcription factors, which if you now express in ES cells, which can be sufficient for pluripotency.

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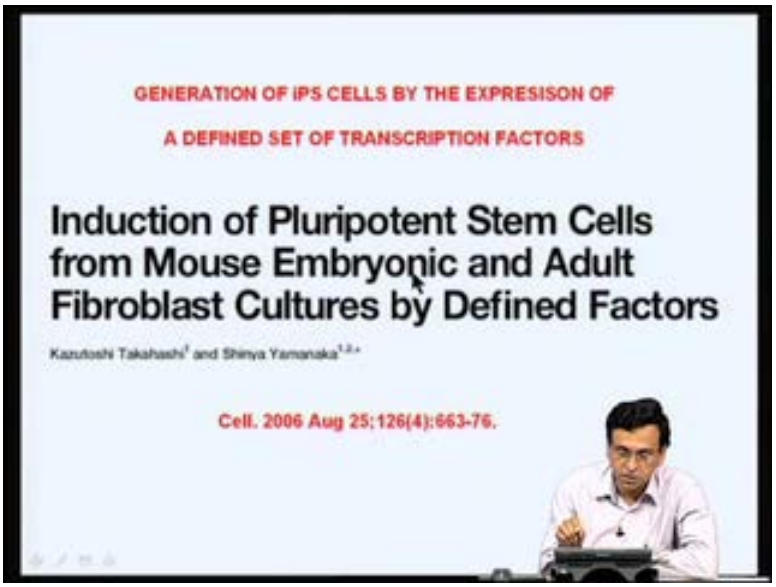


So, this was the semano paper which was published in the 2006– induction of pluripotent stem cells from mouse embryonic and adult fibroblast cultures by defined factors. What Yamanaka actually showed is that you can generate induced pluripotent cells– induced pluripotent stem cells– by expressing four different transcription factors. What is shown in this paper is that you can take adult fibroblasts, which are highly differentiated adult cell types, and if you now express four transcription factors in this adult fibroblast, you can now convert them into stem cells, which are identical to the embryonic stem cells. So, it is possible to derive inducible pleuripotent iPS cells or inducible pluripotent stem cells by expressing four transcription factor in an adult differentiated cell.

So, these four transcription factors can bring about the recursive genetic reprogramming that is necessary to induce pluripotency, which is required for the normal embryonic development. So, this was a major achievement in the area of developmental biology, regulation of gene expression, as well as regenerative medicine– the demonstration of the fact that four transcription factors can reprogram an adult cell type into a pluripotent stem cell.

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So, these are the 24 different transcription factors which he studied, and from these 24 transcription factors, he demonstrated that four factors– Oct4, Sox2, KLF, c-Myc, and KLF4– if you have these four transcription factors are expressed, you can now convert a fibroblast cell into a stem cell– pluripotent stem cell. So, it was a major achievement. So, overexpression of these four transcription factors– Oct3/4, Sox2, c-Myc, and KLF4– transforms mouse fibroblasts into embryonic stem like cells.

And these four factors soon came to be known as Yamanaka factors, named after the Yamanaka. So, now, let us try to understand what these Yamanaka factors are. This was shown in the mouse; that is, mouse fibroblasts can be transformed into stem cells by expressing these four factors, but will the same thing be true for human stem cells? Can you also derive, similarly, the human iPS cells?

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In fact, the derivation of the human inducible pluripotent cells were demonstrated in November 2007, both by Yamanaka as well as by another person called James Thomson in University of Wisconsin. Both published papers in 2007 November and December, where they actually shown that by expressing four different transcription factors, you can also convert a human adult cell type into a stem cell.

So, using retroviral vectors, Yamanaka expressed Oct3/4, Sox2, KLF4, c-Myc– that is the same four transcription factors which he showed, if expressed in mouse fibroblasts, can be converted into a pluripotent stem cell or the iPS cell, and the same four transcription factors can also convert a human fibroblast into a human stem cell or human iPS cell, whereas Thomson used a slightly different combination of transcription factors. He used Oct4, Sox2, but instead of using KLF4 and Myc, he used nanog and another gene called as LIN28. So, using these four transcription factors, he could generate human iPS cells from a differentiated cell.

These are known as the landmark papers in the area of developmental biology and regenerative medicine, where they could demonstrate, by simply expressing four transcription factors, you can convert an adult differentiated cell type into a stem cell-like phenotype, capable of generating all three different germ layers and all tissues of a adult animal , and you can generate an entire embryo, entire adult, from these kinds of reprogrammed cells.

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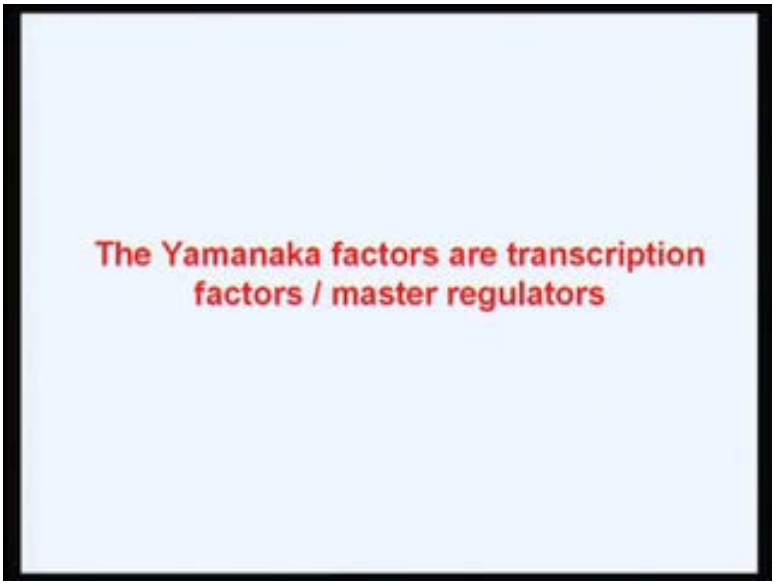


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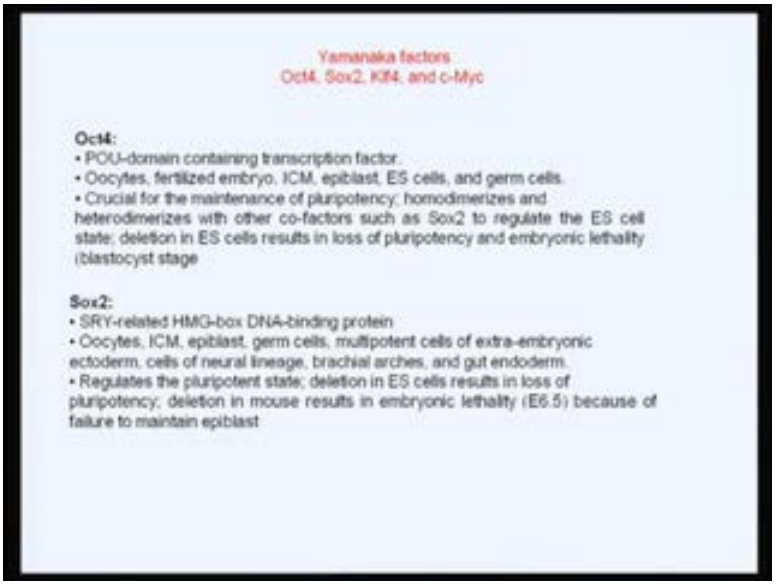


So, what are these Yamanaka factors? They are nothing but the transcription factors, or they are master regulators. Let us briefly understand what these Yamanaka factors are. As I said, Oct4, Sox2, KLF4, and c-Myc are the four factors which when expressed in adult differentiated cell, you can convert them into a… now, you can reprogram them into a pluripotent embryonic stem cell-like stem cell, which are known as the inducible pluripotent stem cells.

Now, Oct4 is nothing but it is a POU- domain containing transcription factor. Oocytes, fertilized embryos, ICM, epiblasts, ES cells, and germ cells, all of them express this Oct4. It is very, very crucial for maintenance of pluripotency. It homodimerizes and heterodimerizes with other cofactors such as Sox2 to regulate the embryonic stem cell state, and deletion in the ES cells results in the loss of pluripotency and embryonic lethality. So, these are the important functions of Oct4.

Now, Sox2 is nothing but an SRY-related HMG-box containing DNA binding protein. It is also expressed in oocytes, inner cell mass, epiblast, germ cells, multipotent cells of extra embryonic endoderm, as well as cells of neural lineage, brachial arches and gut endoderm, and it regulate the pluripotent state, and deletion of Sox2 in ES cells results the loss of pluripotency, and also results in the mouse embryonic lethality.

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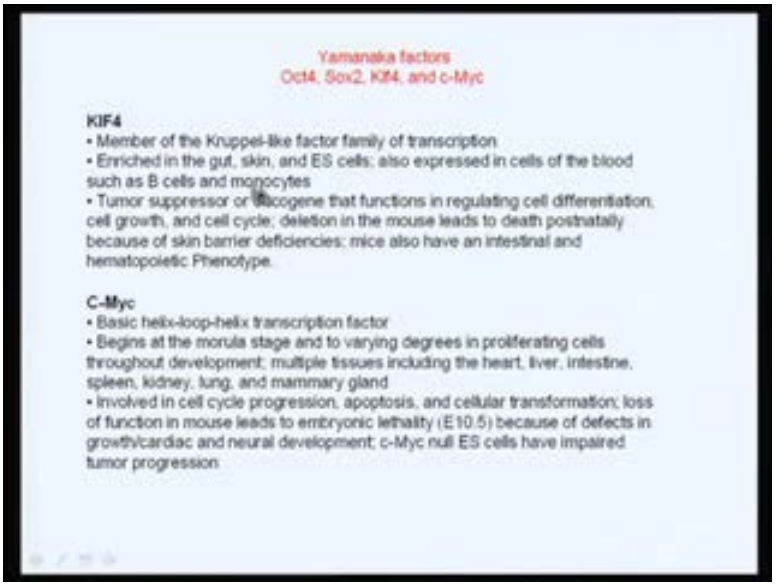


KLF4, again, is a member of Kruppel-like factor of family of transcription factors. It is enriched in the gut, skin, and ES cells. It is also expressed in the cells of blood, such as B-cells and monocytes. It is a tumour suppressor or oncogene that functions in regulation of cell differentiation, cell growth, and cell cycle. Again, if you delete KLF4 in the mouse, it leads to postinatally, primarily because of skin barrier deficiencies, and mice also have an intestinal and hematopoietic phenotype.

Think that they are all important master regulators. Similarly, c-Myc is a basic helix-loop-helix transcription factor. Its expression begins at the morula stage, and it is

expressed varying degrees in proliferating cells throughout the development in various tissues of the developing embryo. It is involved in cell cycle progression, apoptosis, and cellular transformation. If again, if you do not have c-Myc, it leads to embryonic lethality. The embryonic development do not, does not proceed beyond 10.5 in the mouse embryonic development, because of defects in growth, cardiac, and neural development.

And c-Myc-null ES cells have impaired tumour progression. c-Myc is an oncogene, basically. So, it basically tells you that all these are very, very important transcription factors, and if you delete them, you do not get normal embryos, and Oct4, nanog, are essential for maintenance of pluripotent state of ES cells, and are expressed only in pluripotent cells.

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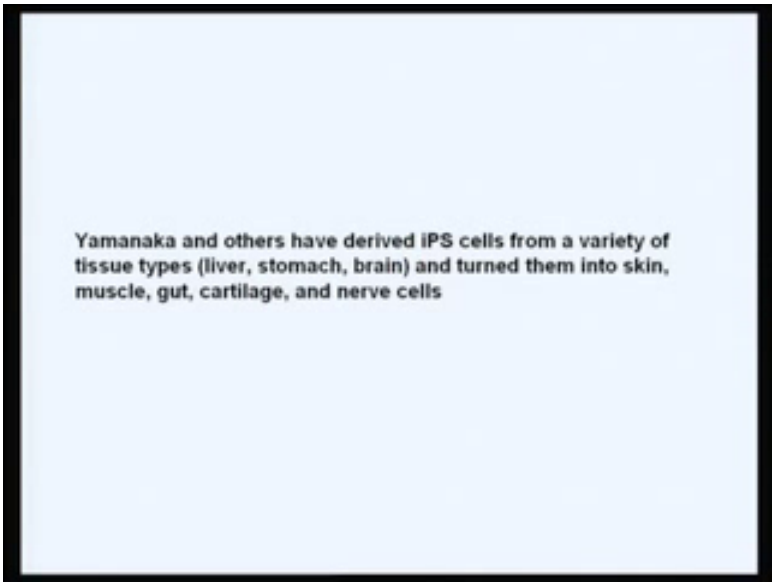


So, these are very, very important markers for pluripotent cells. So, Yamanaka and others, once these two landmark papers were published, following this, Yamanaka as well as many other others have derived, actually, iPS cells from a variety of tissue types, either liver cells stomach or a brain, and turned them into various other cell lineages.

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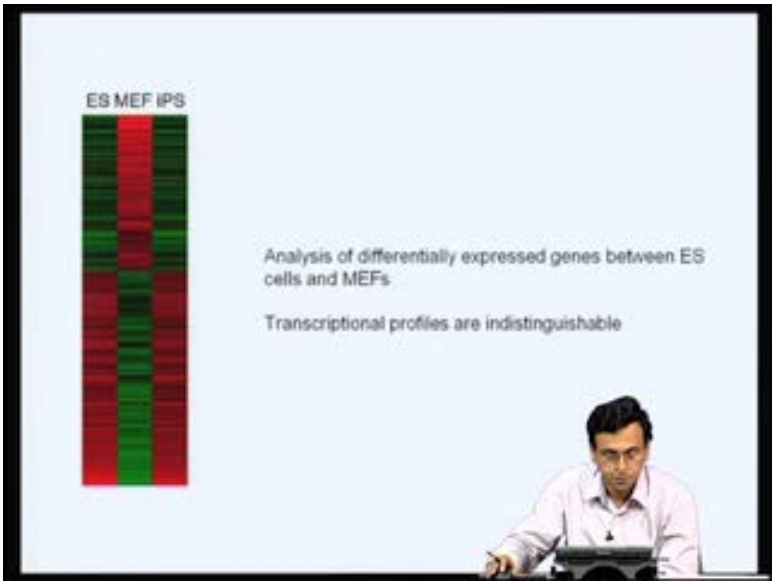


They could derive these iPS cells from a number of other adult tissue cell types, and in fact, one that major challenges was to see that the stem cells that have derived by programming into adult cell types into stem cell like cells, are they identical to embryonic stem cell which are normally present in the blastocyst? So, the best way to do this is to compare the gene expression profile of normal embryonic stem cells, which are present in the blastocyst, and the gene expression profile of an iPS cell, which is, actually, a reprogrammed stem cell derived from the adult cell.

So, what you do? You do what is called as a microarray experiment. Again, we will discuss this in the later stages of our class. So, basically, you get what kind of genes are expressed in the embryonic stem cells, and what kind of genes are expressed in these iPS cells. When they did that, you can see, the embryonic expression profile of ES cells is very, very similar to that of iPS cells.

That means the gene expression profile of these iPS cells is very, very identical to that of embryonic cells, whereas the mouse embryo fibroblast, from which the iPS cells were derived, have a completely different set of gene expression profile. So, by expressing four transcription factors, you can change this particular gene expression program into this program, which is very, very identical to that of embryonic stem cells. So, these are total reprogramming, the entire gene expression profile has been changed to that of ES cells and that of a adult cell size.

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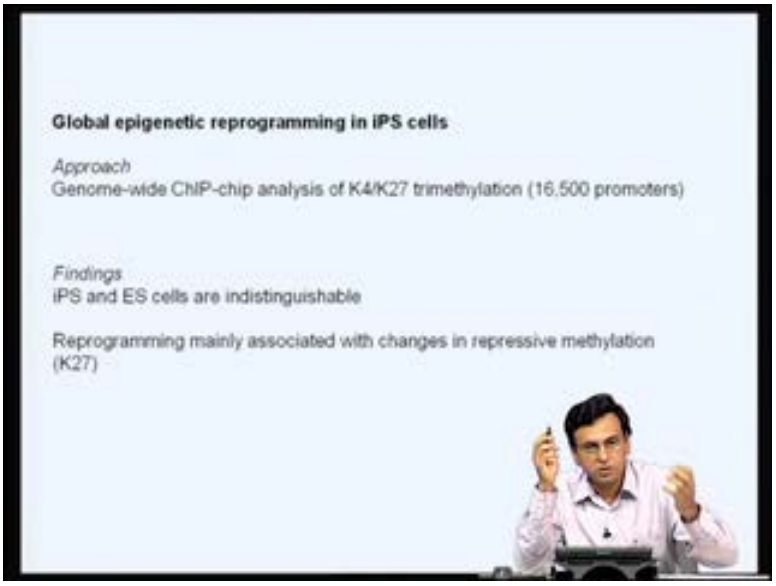


So, the transcription profile of a iPS cell is very, very similar to that of AES cell. They also asked about what about epigenetic reprogramming of this iPS cells? So, you do what is called as a chromatin technique. Again, we discussed in the previous classes, and actually seen, examined, for example, the trimethylation of the lysine 27 of, or lysine 4, and when did that on about 16500 promoters, again, they found that this lysine 4 and lysine 27 trimethylation pattern is exactly the same in both iPS cells as well as the ES cells, clearly indicating that you have these iPS cells are very, very identical to that of the embryonic stem cells.

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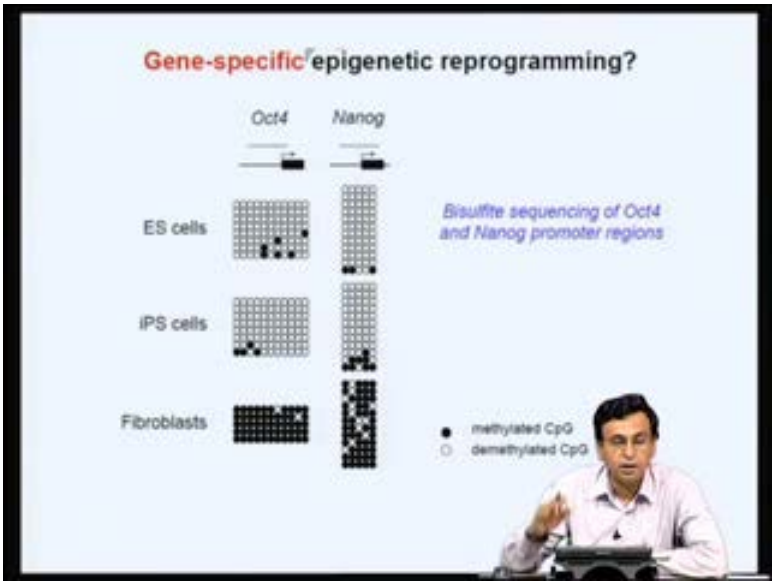

They also looked at the… the methyl DNA methylation in the promoters of Oct4 and nanog, and you found that if you now look at the fibroblast from which the iPS cells are derived, you can see, it is the cytosines here– the CPS are high methylated in the fibroblast in the Oct4 nanog promoters, but the moment you convert these fibroblasts into iPS cells by expression of these four transcription factors, these two promoters are highly hypermethylated, and it is very similar to that you normally see in the embryonic stem cells. So, the DNA methylation pattern, the histone methylation pattern, the gene expression profile are all identical to that of embryonic stem cells in the iPS cells.

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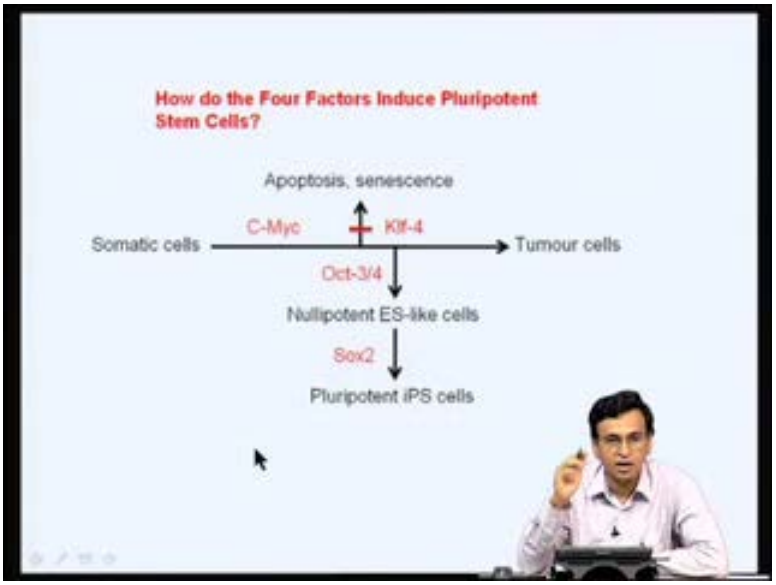


So, how do these four factors convert a fully differentiated cell into a embryonic stem cells? So, this hypothesized that when you have this normal somatic adult differentiated cell, if you now express just c-Myc– c-Myc is an oncogene– and if you over express an oncoprotein like c-Myc, it should now become a cancerous cell or tumour cell.

That's what happens if you express only c-Myc, but now, along with c-Myc, not only it converts somatic and tumour cells, it also induces apoptosis or senescence of the… when you over express c-Myc. But, along with c-Myc, if you now express KLF4, it actually prevents the apoptosis or senescence of the somatic cells. Now, in addition, now if you express Oct3 or 4, instead of somatic cells now becoming tumour cells, in the presence of these three, now the somatic cells now become multipotent ES-like cells.

So, myc alone will convert somatic cells into tumour cells. It also induces apoptosis and senescence, but if you have KLF4, you can block this, but if you have…, if you have Oct3/4 also expressed along with these two, instead of somatic cells becoming tumour cells, they now become ES-like cells.

And if you now express one more factor– Sox2– these ES cells, now, really become the fully pluripotent iPS cells. So, this is the mechanism by which these four transcription factors are converting a differentiated somatic cell into pluripotent iPS cells.

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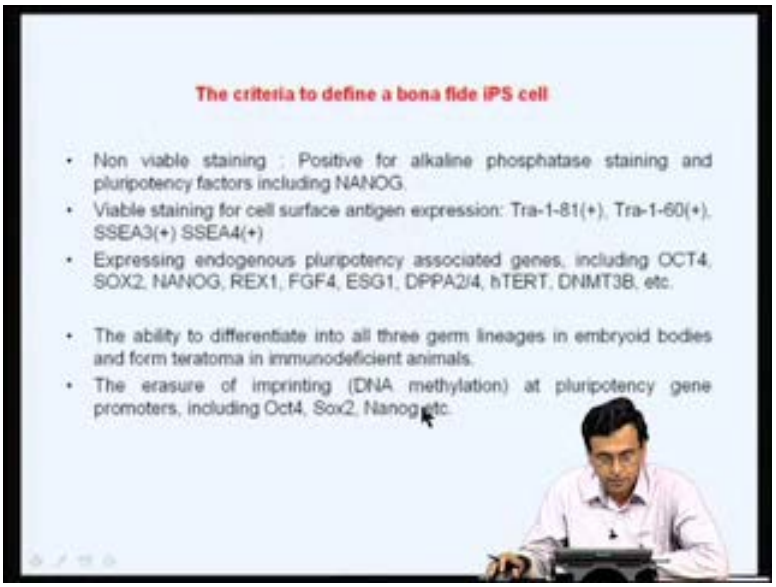


There are number of criteria to, actually, demonstrate that you have actually derived an iPS cell. I will not go into the details. For example, a truly inducible pluripotent cell should be positive for alkaline phosphatase staining, and it also expressed pluripotency transcription factors like nanog. It also should have expressed some very specific cell surface antigens like Tra-1-81, 1- 60, SSEA3, SSEA4. Only then, you can say, you have actually derived true iPS cell.

A many endogenous pluripotency associated genes I have listed here, like Sox2, nanog, they all have to express. Only then, you can say you have actually derived a iPS cell. It should also have the ability to differentiate into all three germ lineages in the embryoid bodies to form a teratoma in… That is if you take these iPS cells in... infect into a…, inject into a nude mouse, it should form a tumour, and this tumour should have all three germ layers– ectoderm, endoderm, and mesoderm, and the DNA methylation pattern

should be completely erased, and the… as I as I shown in the previous slide, the promoters such as Oct4 and Sox2 should be completely hypermethylated– it should not be methylated– these promoters are methylated only in the differentiated stage, but not the undifferentiated pluripotent cells.

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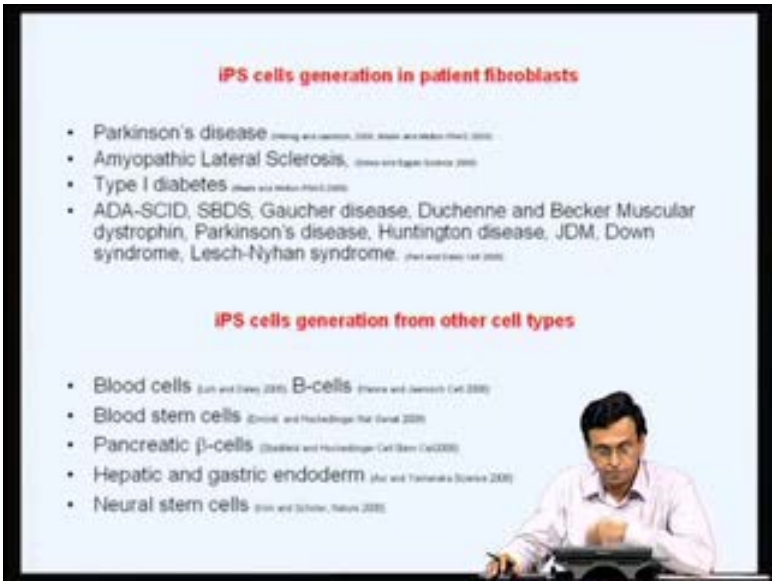


So, having generated this kind of iPS cells by simply expressing four transcripton factors, people now started deriving this kind of iPS cells from a number of diseased patients– people suffering from Parkinson's disease, people suffering from amyo lateral sclerosis, type I diabetes, and so on and so forth. From all these things, we simply take either skin cells or various adult stem cells and express these four transcription factors and see, can you now convert, derive iPS cells?

That means can you derive iPS cells which are patient-specific? And for example, if you are suffering from type I diabetes, if you now take these skin type cells from you, express these four transcription factors, and convert them into stem cells, and then can you now convert them back into insulin-producing pancreatic beta cells, and can now…, then you can put them back, so that you will now continue producing insulin. So, this is a field called as regenerative medicine, which has tremendous implications in this century.

Similarly, iPS cells have been generated from blood cells, pancreatic beta cells, hepatic cells, gastric endoderm cells, neural stem cells, so on and so forth. The field has exploded in the last two years.

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So, to summarize, reprogramming patient cells into pluripotent stem cells provides the best matching and most abundant source of a tissue regeneration; you do not have to worry about tissue matching. Among all different methods, the most achievable is direct reprogramming by introducing pluripotency associated transcription factors into a primary tissue culture.

Direct reprogramming generates induced pluripotent stem cells that are functionally and phenotypically identical to embryonic stem cells. So, you do not have to go to the embryo to derive pluripotent stem cells. You can simply take any adult cell type, simply express four transcription factors; you can now convert them into a stem cell, which is identical to the ES cell.

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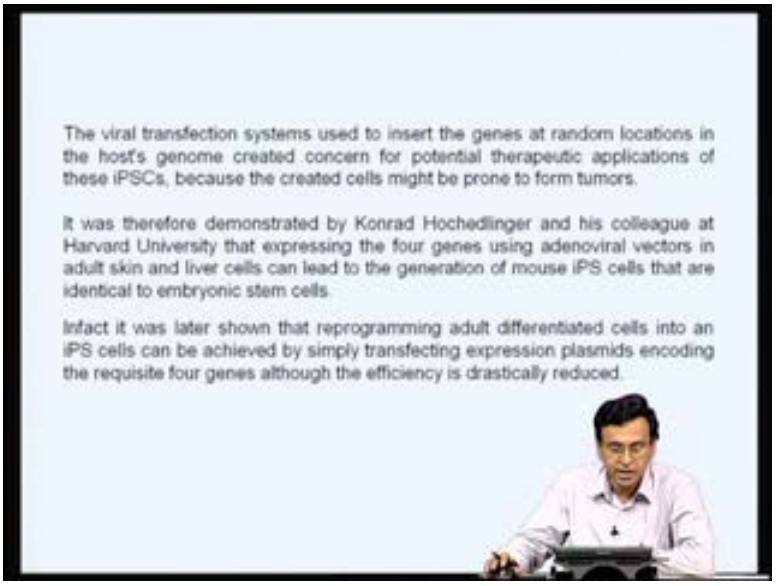


A lot more work is required; there are still problems associated to the iPS cells. We are not going into the details; the one of the major problems is that the way you are expressing these four transcription factors in these adult cells is by using what are called as viral vectors.

We will discuss some of these things in the next few classes, when we talk about the gene therapy and so on and so forth; that means, to introduce these genes into these adult cells– the four transcription factors– you have to use viral vectors to express them, and many times, what happens is these viral vectors, especially when you have what are called as retroviral vectors, they go and integrate in the genome, and there is a possibility that when they go and randomly integrate in the genome, they may activate certain oncogenes, or they may inactivate some tumour suppressor genes and you may end up with cancer. So, you have that problem. So, people are trying to see where you have the systems can be used to avoid these particular problems.

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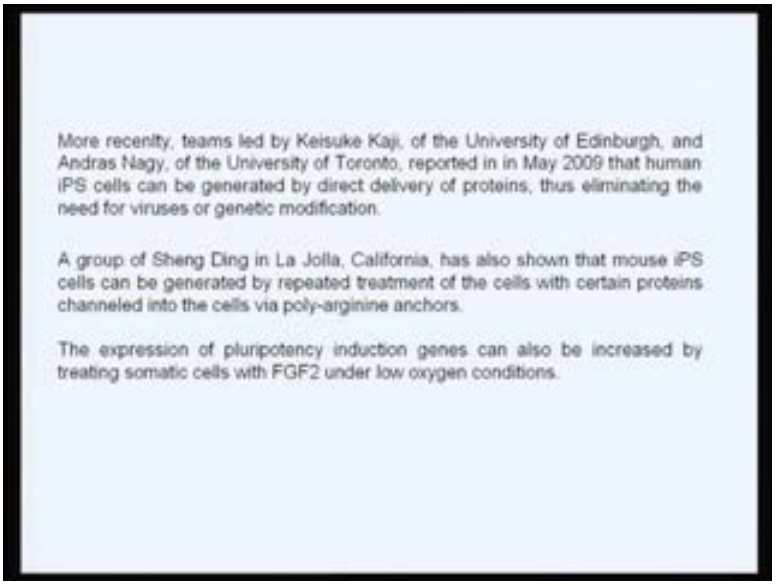


For example, people have been using, instead of retroviruses, can you actually use adeno viruses for these purposes, or there are certain other people are actually using proteins themselves– can it be directly transferred to express genes? Can you express, for example, nanog directly? Can you express Oct3/4? Can you express? Oct protein itself can be directly transfused into the adult cells and they can be induced into iPS cells.

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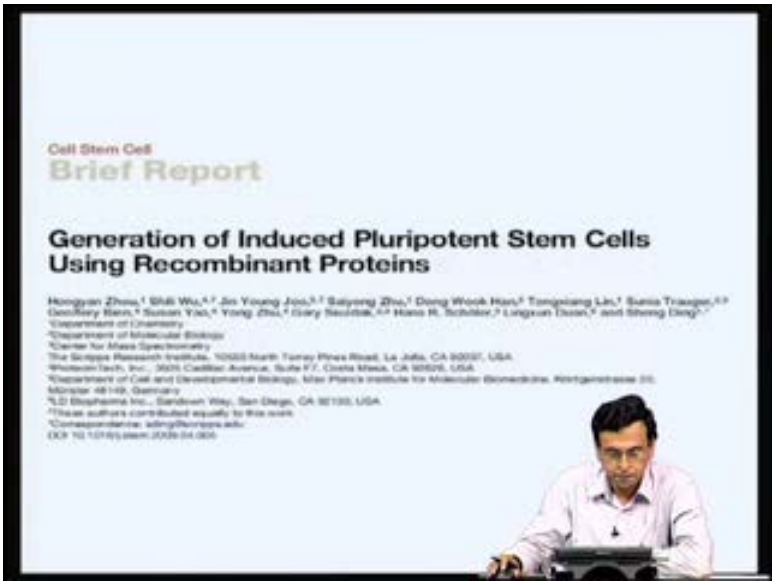


This is a paper exactly shows generation of induced pluripotent stem cells by using recombinant proteins. You do not have to transfer genes anymore; you can directly

express recombinant proteins encoding these transcription factors, and convert an adult cell into a iPS cell.

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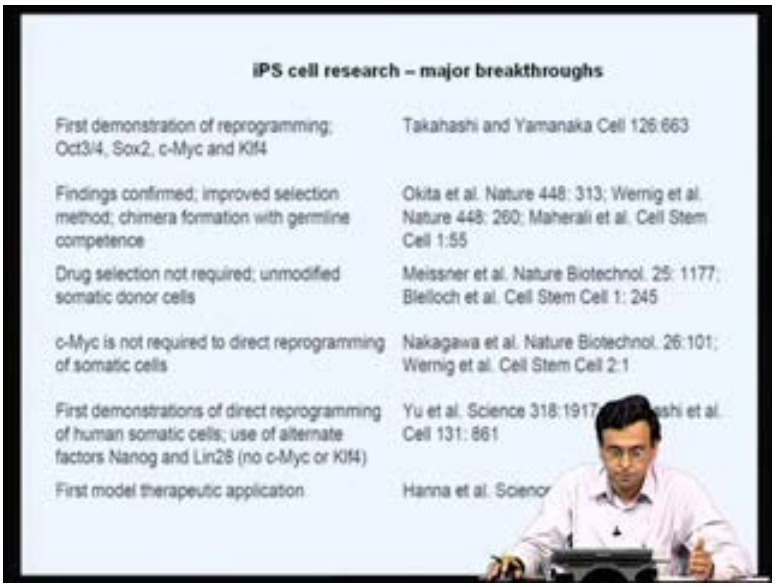


Several major breakthroughs have been achieved in the iPS cell research. I have just listed some of these things here; I will not go through its details, but I also given the various references, and how, from this stage of demonstration of by Yamanaka, how four transcription factors can be introduced in the adult cell and convert them into iPS cell, a number of improvements have been made to see how you can make this iPS cell generation more safe.

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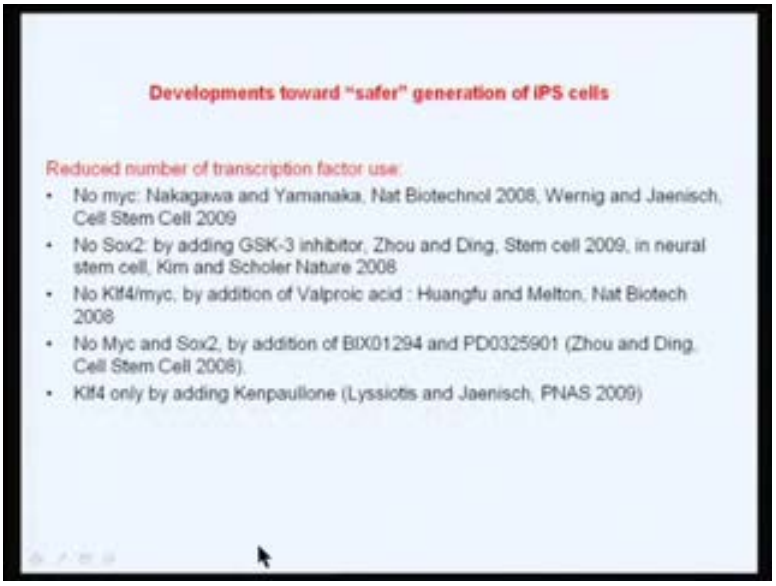


As I said, can you reduce a number of transcription factors? Instead of four, can you make it two? Can two factors make the job? A number of papers have appeared between 2008 to 2010– how you can induce the number of transcription factors. For example, myc is an oncoprotein. Now, myc no longer is required for inducing iPS cells. You can do with myc, myc can only Sox2, and by addition of two other factors, you can generate in iPS cells.

Similarly, no KLF4 or myc– if you can just add a compound called valproic acid, you do not have to transfer KLF4 or myc. So, several variations of this is been tried out to generate safe iPS cells.

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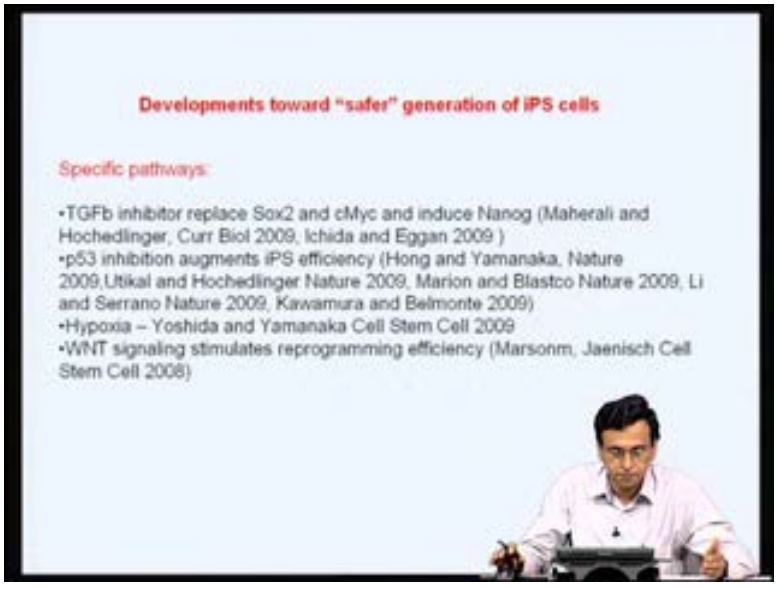

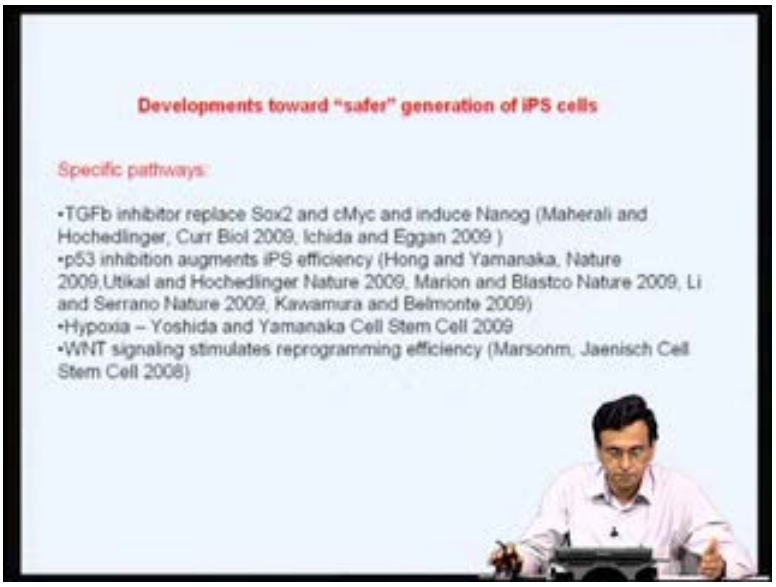


Specific pathways– what kind of specific signal transduction pathways are being activated when iPS cells are being generated is being studied. Can we use better vectors? Again, a number of papers have appeared to see how you can generate safe or better iPS cell generation.

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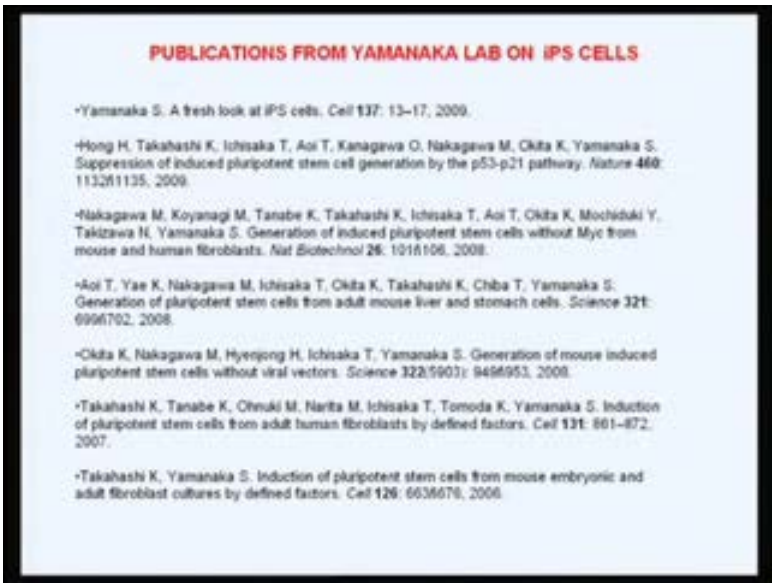


A number of papers have been published. Even this, I just show how many papers have been published by Yamanaka lab alone, for deriving this various iPS cells in various journals.

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There is a very nice review in Scientific American, which tells turning back the cellular clock– a farewell to embryonic stem cells. This a beautiful review; one can try to understand the implications of these iPS cells.

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These are the two landmark papers one must read by Yamanaka, and also by JA Thomson, on the derivation of the human ES cells.

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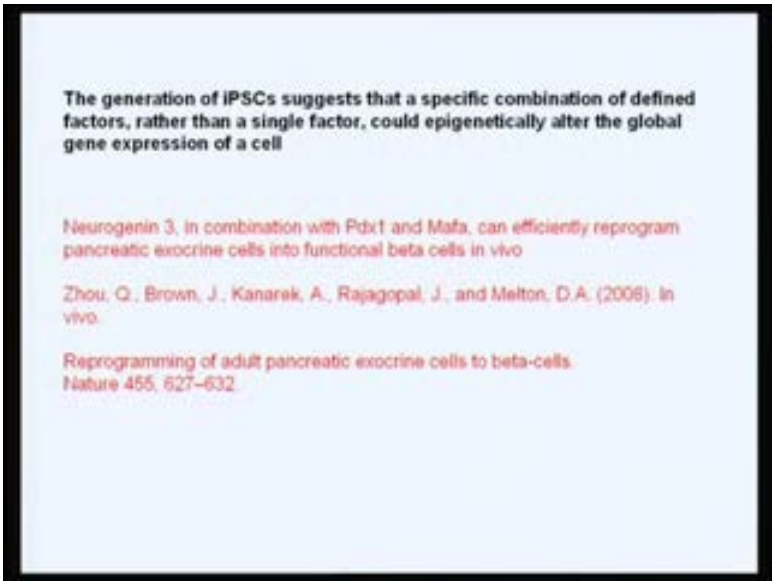


People are now converting various cell types. For example, if you express a protein called neurogenin 3 in combination with Pdx1 and mafa, you can reprogram pancreatic exocrime cells into functional beta cells. That means, exocrine pancreatic cells, which do not produce insulin, now they start producing insulin.

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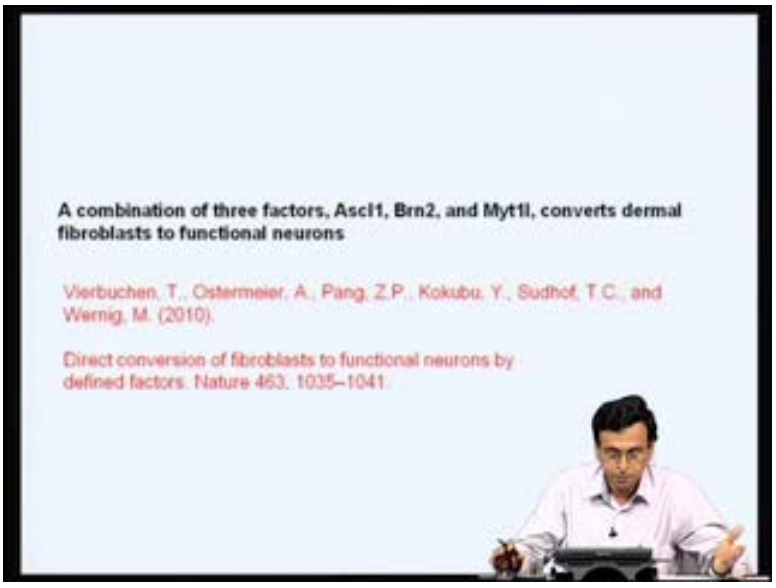


It has tremendous implications for people suffering from type I diabetes. Similarly, by expressing a combination of three factors, you can convert dermal fibroblasts into functional neurons.

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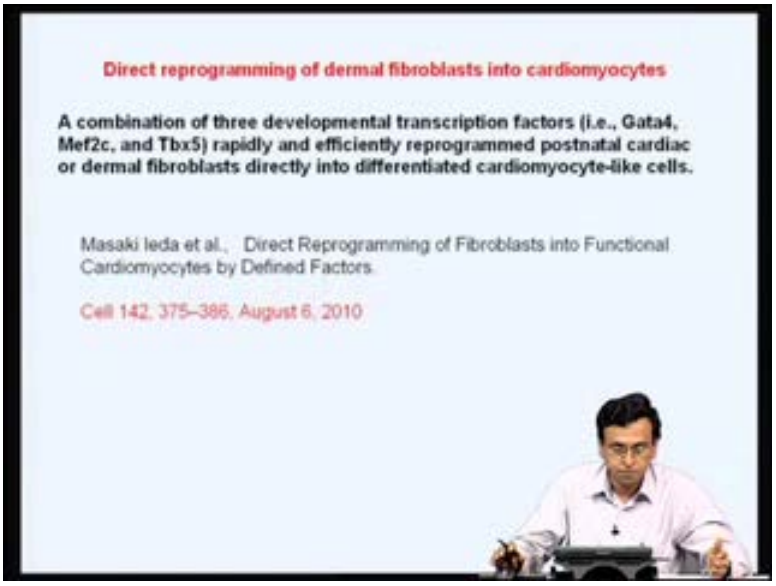


You see, there are tremendous implications as far as biomedical sciences are concerned or as diseases are concerned, and this a landmark paper which just appeared in August 2010– just last month, where direct reprogramming of dermal fibroblast into cardiomyocytes. That means you do not have to convert fibroblast into ES cells or iPS cells, and then convert them back to cardiomyocytes. You can directly reprogram dermal fibroblast into cardiomyocytes.

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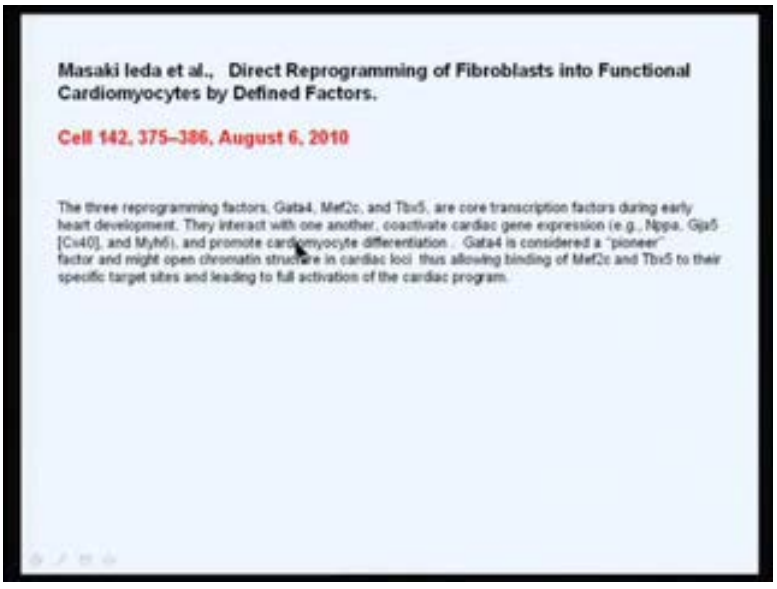

One can go and read some of these factors. This is a landmark paper that appears in Cell. By simply expressing three transcription factors, you can directly reprogram fibroblast into cardiomyocytes. So, I think I will stop here, and what I told you, so far, is a fascinating area of developmental biology, and by how expressing specific transcription factors, one can reprogram an adult cell type into the iPS cells and induced pluripotent stem cells, and how these have tremendous implications in the area of regenerative medicine.