**Biomaterials for Bone Tissue Engineering Applications**
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**Module 4**
**Lecture No 18**

So we will continue the discussion on the stem cell differentiation in this module also. So in the, in the, while discussing the stem cell differentiation I have mentioned that there are two approaches to induce or to guide the stem cell differentiation.

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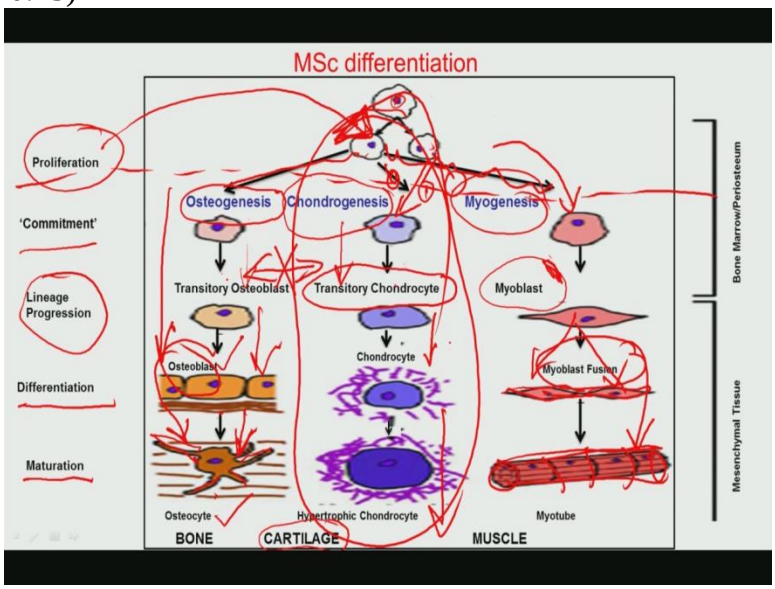


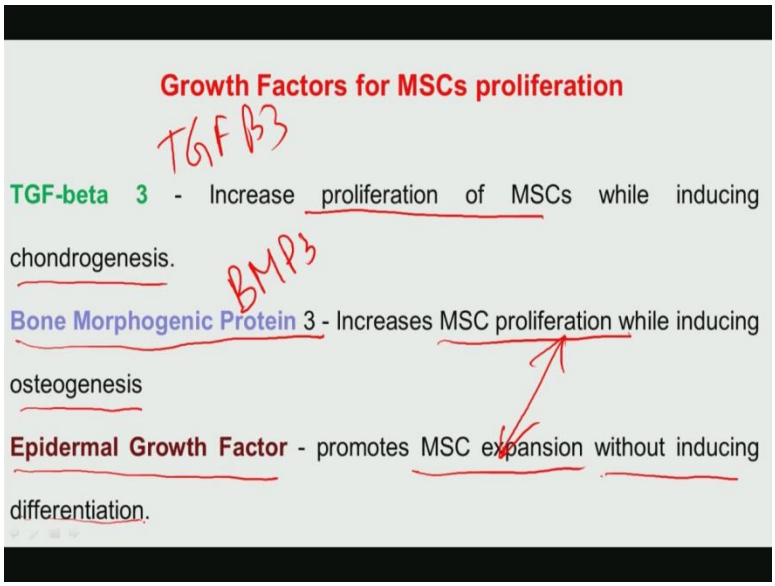

One of the approaches is to use some soluble growth factors or biochemical growth factors and three examples are mentioned; these growth factors are plenty, are available in large numbers. And it is not possible to cover so many growth factors in this course. Therefore I try to restrict the discussion on growth factors in reference to three examples. One is the transforming growth factor, beta 3 that is TGF beta 3. It is many books, it is also mentioned like TGF beta 3.

It increases the proliferation of mesenchymal stem cells, while inducing chondrogenesis. Chondrogenesis is the second lineage or that I have mentioned; this is your chondrogenesis lineage, okay? This chondrogenesis essentially leads to the chondrocyte cell formation as well as the cartilage, as well as the cartilage tissue. Second one is the BMP 3, that is the bone morphogenic protein 3 and BMP as you see that it is 3, so BMP has also several varieties.

So it increases the MSC proliferation while inducing the ostrogenesis. So this is kind of can be expected for the name itself, like if it is BMP 3 protein, that means it can be used as a growth factor to induce ostrogenesis. So why it shows that MSC proliferation in the first case it shows that, MSC proliferation while inducing chondrogenesis, that means to make a tissue you just you just one or two cells of the chondrocytes is not sufficient.

Now if you increase the proliferation of MSC and make them committed towards this chondrogenesis, then this TGF beta 3 will essay will ultimately lead to the formation of cartilage tissue. So if more number of MSC is are committed towards that ostrogenesis or chondrogenesis then it is helpful to form finally cartilage tissue. Same is true for BMP 3 because it essentially instructs that MSC proliferation while inducing ostrogenesis like when when forming this bone tissue.

Third one is epidermal growth factor. So it is, it promotes the MSC expansion, so expansion and proliferation, these two things are synonymous. In cell biology in biomaterials literature, this proliferation and expansion particularly in the context of MSC people tend to use the word expansion more often than proliferation. So MSC expansion and proliferation they are like synonymous words, or they are used interchangeably.

Without so epidermal growth factors essentially promotes MSC expansion without inducing any differentiation. So you can use it just to maintain the stemness without differentiation.

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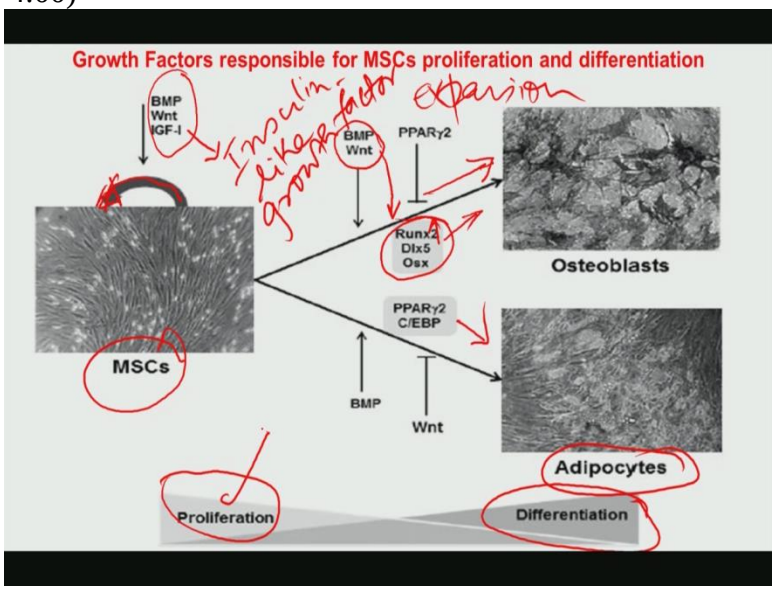


So these are the, so different growth factors are essentially responsible for either MSC is expansion, that either use the word expansion or differentiation. Just to show the two cases; one case you know it goes to the osteoblast like ostrogenesis, another case it goes to adipocytes; like it can be used to store fat cells and fat compounds.

Now what are the growth factors that is used here? Bone morphogenic protein WNT, insulin like growth factor 1, IGF stands for insulin like growth factor. I think I have mentioned this insulin like growth factor and this was mentioned while I have discussed the cell signalling processes in one of the earlier modules. Now this arrow essentially means that it helps in that expansion of the stem cells, so it is still remain, it still remains as mesenchymal stem cells but it is not yet differentiated to specific cell type.

Now when you use this when you add this WBMP and WNT and then you see that what is the expression of ostocalcy in runx2 this kind of genes which I have mentioned in the last to last module while discussing the cell culture assays or differentiation assays. And then you see that if runx2 and all this is upregulated or their regulation is up, then osteoblasts can be confirmed. Similarly in the other case you can confirm this adipocytes.

Other things that I have mentioned about the proliferation and differentiation, proliferation means it goes to the cell cycles and then it goes through like 1 to 2, 2 to 4 this kind of cell

division process. But if you want differentiation to take place, that somehow you cannot allow the cells to undergo expansion and proliferation in an unlimited manner. So somewhere some of the cells need to be stuck at the G1 or airspace itself at the checkpoint so that it can undergo differentiation to give different cell types.

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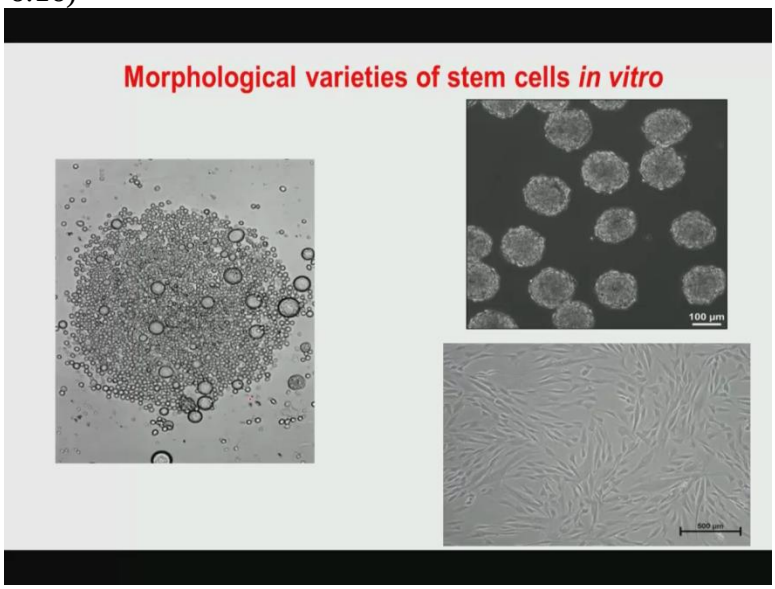


So these are like different morphological varieties of stem cells in invitro conditions. So this is the stem cell invitro, how they appear in the invitro conditions.

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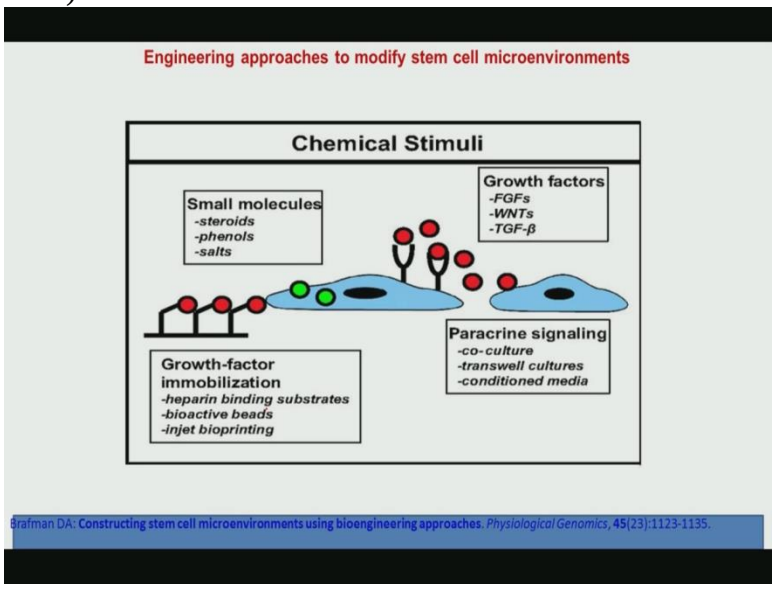

So now coming to the engineering approaches to modify stem cell micro environments now this slide is important in the sense that it shows the different signalling approaches or different signal molecules and growth factors and growth factor immobilisation. And subsequently I will show you that how elastic stiffness and other, then other physical cues also can be effectively used to guide stem cell differentiation.

Now the small molecules can be either steroids or phenols. Now growth factors can be fibroblast growth factors or transforming growth factor beta like which essentially allows stem cell proliferation while inducing chondrogenesis. You have paracrine signalling, now if you remember the different type of signalling mechanism I have mentioned before, you have autocrine, you have paracrine, you have endocrine signalling.

Paracrine essentially means from stem cell type, the signalling between the two different type of cell types. And the fourth one is the growth factor immobilisation.

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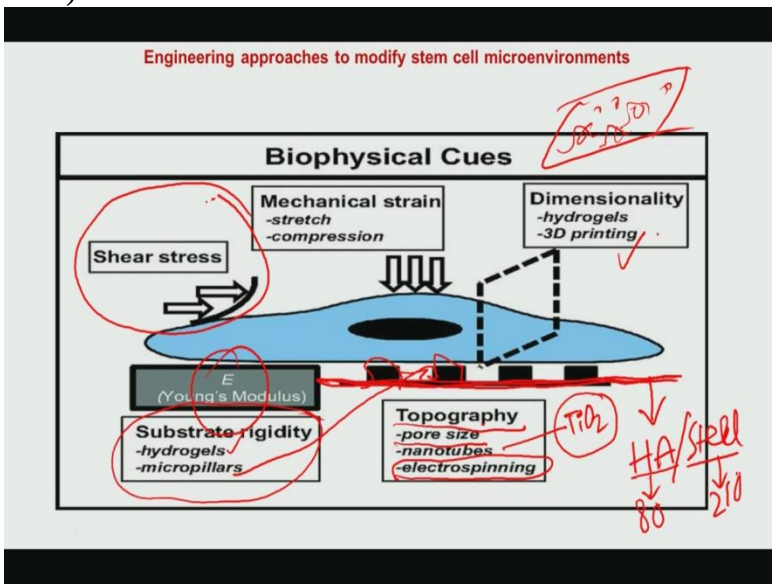


Now biophysical cues essentially means that you have certain cues, it is a combination of biological origin as well as the physical cues. Now I have shown some examples, specific examples here. Now one is that you can have, you can allow the stem cells to grow on a specific substrate, now biomaterial substrate.

Now this can be hydroxyapetite substrate which is ceramic and therefore it is elastically stiff or it can be steel substrate which is even stiffer than hydroxyapetite. Because hydroxyapetite has a around 70 to 80 gigapascal elastic stiffness, steel has a elastic stiffness of 210 gigapascal. So this is like examples of the elastic stiffness. Another example can be the substrate can be elastically compliant like polymeric substrate but you can do certain patterning on the substrate.

Like you can introduce certain surface topographical features either in the form of pore size. So when you have pores in the substrate now if you have, this is the substrate now if you have different pores, so essentially these pores will introduce local roughness in the substrate, right? Because this pores essentially means certain discontinuity in the structural features. So therefore pores will essentially introduce certain surface rough.

Then you have nano tubes like titanium oxide nano tubes people use, like they can grow titanium oxide nano tubes by certain chemical processing on the titanium substrate itself. And this nano tubes have certain physical features, like it can be tubular in shape, it has certain diameter, it has certain aspect ratio and so on. You can make materials with different topography by electro spinning, which has been very widely used in the biomaterials.

Okay, the, second one I have mentioned is that you can, you can have a control, over the dimensionality of these features, like topographical features using three different things. Like which is one of the additive manufacturing technique. And the substrate rigidity can be in the form of either micro pillars, like this kind of micro pillars which is example of micro pillars on the substrate itself. Or hydrogels that is another kind of substrates.

So what are the things that I have mentioned here ? That one is that elastic modulus or they call it elastic stiffness, they call it young is modulus, typically material science or metallurgy people they use that young is modulus quite often. And this elastic stuff, elastic modulus is essentially slope of the stress to strain in the elastic region of any stress-strain plot.

And elastic stiffness however is little different because it involves some tensorial notations and it involves certain, when the elastic modulus is very anisotropic then typically people use that elastic stiffness to describe this elastic property. Then apart from this you can have some external

stimulation that is kind of sheer stress, like typically if you consider that blood cells, when this blood cells are present within a blood vessel so you have a certain blood flow.

And so blood cells and endothelial cells like endothelial tissues are this smooth muscle tissues they always experience certain kind of sheer in their in this flow conditions or physiological flow conditions. So under that sheer stress conditions the cells will experience some kind of mechanical strain conditions. It can be either stretching like tensile strength if it can also experience compression strain.

So under this kind of mechanical strain conditions also the mesenchymal cells can undergo differentiation. So I am trying to kind of explain here that what are the different possible scenarios , what are the different possibilities that biophysical cues can exert on the stem cells to guide them to differentiate in different lineages.

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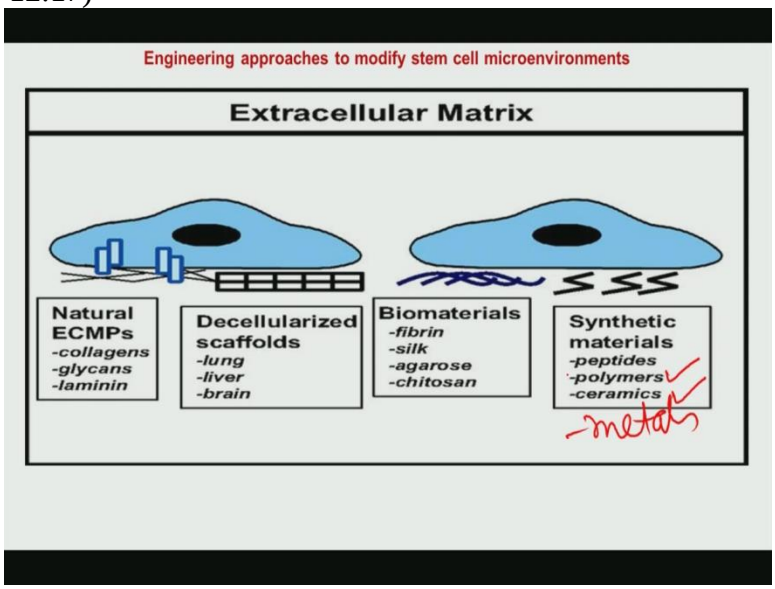


So taking the first one, is the extra cellular matrix. Sso this is one of the examples. Now you can use different scaffolds and you can use different synthetic materials as well. So some of the decullularized scaffolds is being mentioned. Like decellularized means the scaffolds where cells are kind of extracted and separated away and these scaffolds can be used for this guiding stem cells.

Like lung, liver or brain and you can have the natural ones like collagens, glacuns and laminins as a coating on the substrate itself. Now you have the different natural biomaterials like the biomaterials of natural origin like fibrin, silk, agarose or kytosen and you have synthetic materials also like polymers, ceramics or metals.

Now depending on what is the kind of material or what is the kind of substrate you are using, each substrate will have certainly different elastic modulars. So therefore biophysical constraints or biophysical cues also would be different.

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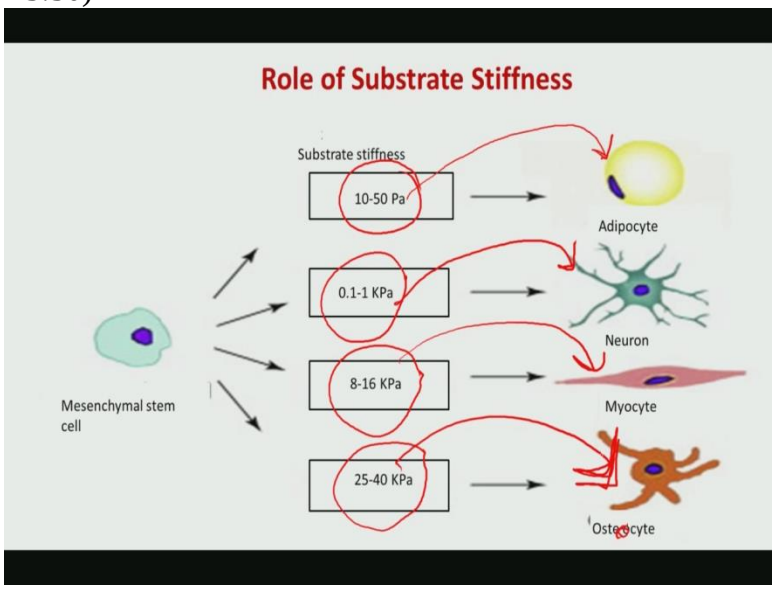


So just to give an example that how different substrate stiffness can guide stem cells to different lineages. Now in one of the classical study by Inglor Ettel, I think it was in a pecibin or penstead, so that was published quite a few years ago. So they have varied the stiffness of the substrate in different window. For example some of the substrates they have used and they have grown stem cells on those substrates having elastic stiffness of 10 to 50 pascal.

Some of the substrates that they have used in their experiments have a different substrate stiffness, that is 0 dollar 1 to 1 kilopascal. They have also used substrates of 8 to 16 kilopascal and another set of substrates they have used 25 to 40 kilopascal. So now by varying the substrate stiffness they were trying to understand that how bone marrow derived, even mesenchymal stem cells can differentiate in a stiffness dependant manner two different cell types.

And what they found is quite intrigue in nature that when they are growing that when they grow the stem cells on 10 to 50 kilo 10 to 50 pascals it is less than 100 kilo less than 100 pascal, the stem cells were differentiated to adipocyte. In 0 dollar 1 to 1 kilopascal there were very characteristic features like neuron life cells, neuronal neurogenic differentiation 8 to 16 pascal kilopascal monocytes, 25 to 40 osteocytes.

So that is like bone cells, so matured bone cells. So essentially to grow or to guide differentiation to osteogenic lineage, or to induce osteogenesis, you need substrate stiffness much higher than what you need for neurogenic differentiation or neuro or what you need for adipogenesis. So this is very clear in this classical study.

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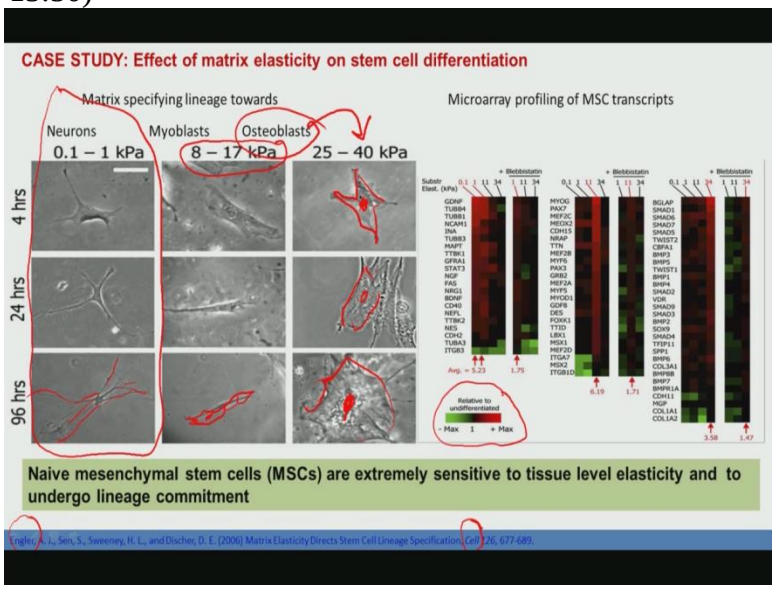


So as I said that this particular study was made by Inglor, and that was that appeared in 2006 almost like 10 years ago, in of the most reputed journal called Cell. So and they have confirmed that all this different all this differentiation by extensive cell biological as well as micro array profiling for all the different genes which are specific to the different type of cell types that they have finally confirmed.

So this is some examples like you know they have grown the stem cells upto 96 hours that is 4 days on substrates which have a stiffness of 0 dollar 1 to 1 kilopascal. And you can see it little here although it is not that clear, that on these fluorescent images that it goes very characteristic

neuron type of cell. When they have grown the stem cells, MSC is on 8 to 17 kilopascal it shows more a spindle like shape.

Like you know that spindle shape and this spindle shapes are like more like muscle cells like myoblasts. In the case of 25 to 40 kilopascal elastic stiffness they show signature of more flattened cell type, and this flattened cell type also shows some extra cellular matrix synthesis and so on and this is like classical case for osteocytes or matured osteoblasts.

Now these things are also mentioned here that in different genes that they have used and they have all also used some housekeeping genes and these different colour essentially shows that whether what is that quantitative they extend relative to the undifferentiated cell type, whether it is upregulated or down regulated. So essentially naive mesenchymal cells are extremely sensitive to tissue level elasticity to undergo lineage commitment.

What is the meaning of that? That essentially when stem cells are grown in this kind of substrates they essentially feel that as if they are getting the substrate the tissue like substrate. Like in case of neurons they are getting the feel that this stiffness is good enough so that I can grow or I can differentiate to neurons, because the neural tissue also have similar stiffness value.

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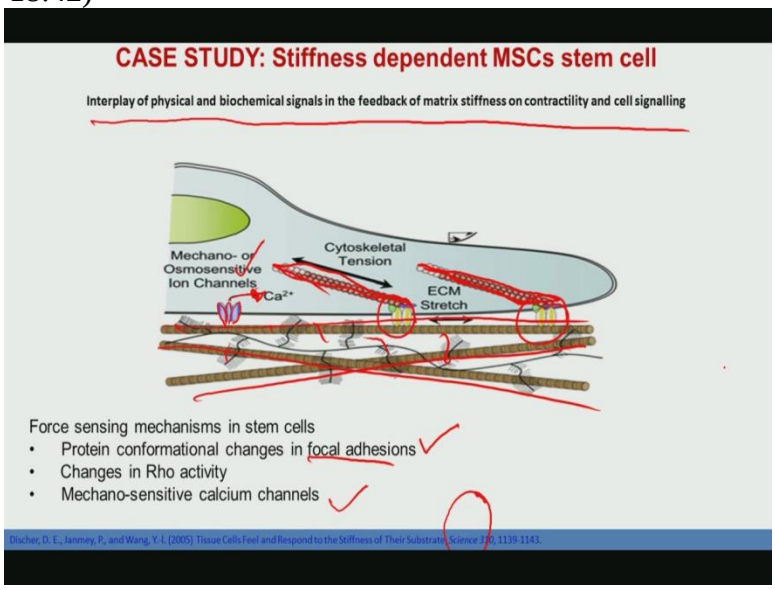


So this is the little bit more discussion on the stiffness dependant mesenchymal stem cells differentiation. So people have studied other things also, that is one another famous study which

was published more than 10 years ago, which was published in the journal Science which is again the most reputed journal in the field. And that is the work by Descheir, Jamie and Wang and the name of the paper is “Tissue cells feel and respond to the stiffness of their substrate”.

Now here they have investigated the interplay of physical and biochemical signals in the feedback of matrix stiffness on the contractinity and cell signalling. So what are the things that they have done? They have used some ACM like substrate and then they have grown, they have used certain coatings then they have grown stem cells on the substrate. Now these are some of this voltage gated calcium channels, if you remember I have mentioned that in a cell membrane has specific channels or specific porous channels which allows the transport of specific ions.

Like certain voltage gated channels they allow the transport of calcium ions, similarly other voltage other channels they allow the transport of potassium or sodium ions. So the channels which allow potassium and sodium ions will not allow the entry or exit of the calcium channels, so they are essentially ion specific channels on the cell membrane. So what has been shown here?

Because of the presence of this mechano or osmo sensitive ion channels this when they are grown on a specific material scaffolds then there is cytoskeletal they would be reorganised and this cytoskeletal reorganisation is necessary for changing the cell shape and at the same time the extra cellular matrix like substrate also can stretch. Now this fore sensing mechanism in stem cells there essentially take place either by protein combo conformational changes in the focal adhesions.

Now these are the focal adhesions that is the cluster of some biological molecules at the interface of the cell and substrate. Now this focal adhesions are important for the cell adhesions and cell migrations. So this forcee sensing mechanism is actually the origin for this stiffness dependant MSC is differentiation.

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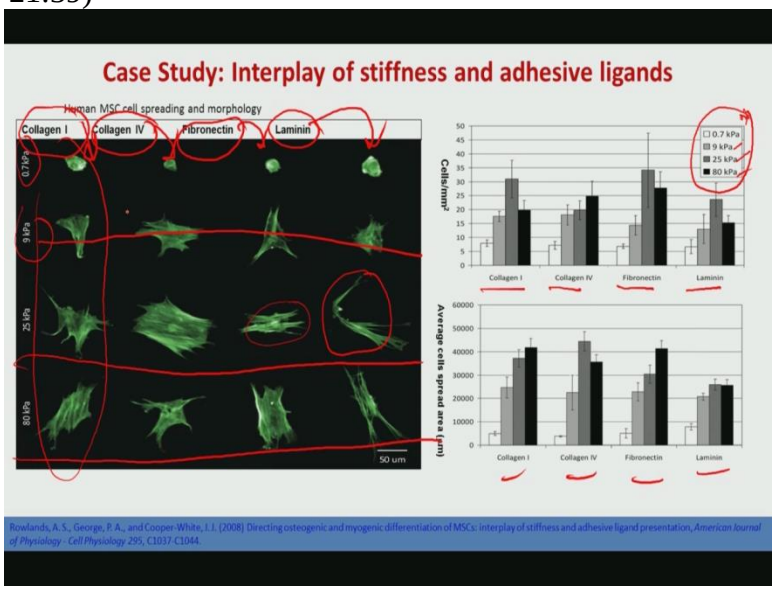


This shows little bit more details like people have used the different substrate stiffness which are mentioned here. Like some substrates have stiffness of less than 1 kilopascal some has 9 kilopascal, some has 25 and some has 80 kilopascal. They have grown the stem cells, human mesenchymal cells they have grown it and they have used the different extra cellular matrix like collagen 1, 4, fibronectin and laminin coated surfaces.

And then this is that cellular images like they are when they are growing in the smaller one like less than 0 dollar 7 kilopascal, this is the second row is for 9 kilopascal, third row is for 25 kilopascal, and fourth row is for 80 kilopascal. Certainly their morphology the way it appear is quite different and it is shown to be dependent on the substrate stiffness, on which they are growing, that is extremely clear.

So this is again essentially showing so the fourth column is for laminin, third column is for fibronectin, second column is collagen type 4, and first column is collagen type 1. So essentially it again reconfirms that such reported public research results again reconfirm that yes indeed the substrate stiffness has an important role on the way the stem cells differentiate.

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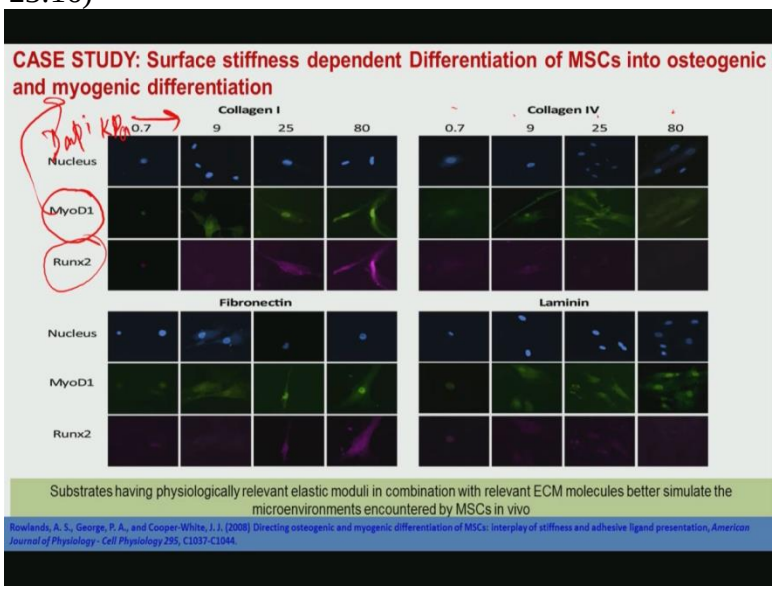


Okay. This is the another paper, some of the selected results are taken. This is from Rowlands Ettel, so this is the three authors, Rowlands, George and Cuperboy; this was published in the American journal of Physiology and Cell Physiology. So this was published in 2008 quite a few years back. Now what is shows here is that a, after this human mesenchymal stem cells were grown on collagen type 1 coated substrate, collagen type 4, fibronectin, or fibronectin and laminin coated substrates, these were like stained, cells were stained differently.

Cells were stained either with Dhappy that is for the nucleus, or they have used mioD1 that is just to show there is a miogenic differentiation markers, runx2 more for the ostrogenic marker. So if you remember in some of the earlier modules I have mentioned for that differentiation of the bones, or the bone cell specific differentiation you need to use different markers or different gene expression.

I have mentioned in particular that ALP assay and runx2 assay, so ALP and runx2 expression takes place in early stage f differentiation. Whereas ostocalcin that is that expression takes place at the late later stage of differentiation. So at early stage of differentiation certainly the runx2 is expressed and you can see that how they are expressed and how they are the signature of this this after they are treated with runx2 that is certainly different in terms of the what is the substrate stiffness that you are growing on.

So along these top one, your elastic stiffness is changing in kilopascal unit; 0 dollar 7, 9, 25 and 80 and similar things 0 dollar 7, 9, 25 and 80. So this column wise these are like different elastic stiffness and row wise it is like what you were seeing, mostly this nucleus mioD1 and runx2.

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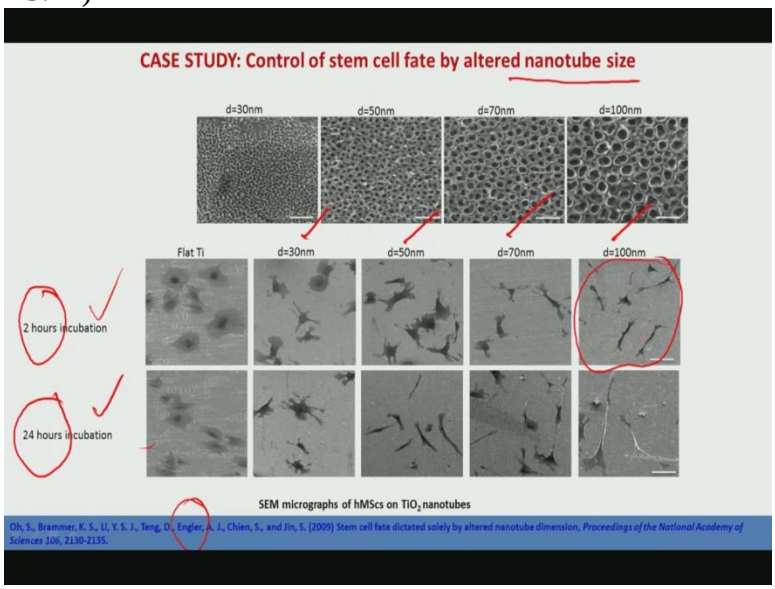


In some slides before, I have mentioned about that biophysical cues, so one of the physical cue is that nano tubes. This nano tubes and specifically I have mentioned the titanium oxide nano tube. Now titanium nano tubes can be grown on titanium substrate by certain chemical synthesis route and now for the time being you just consider that you have the titanium oxide nano tubes and by chemical synthesis route you can vary certain synthesis parameters to get nano tube diameters of different size.

Let us say 30 nano meter, 50 nano meter, 70 nano meter or 100 meter. Essentially you are growing this nano tube of 100 nano metre or below. And you are comparing with pure titanium as a substrate without any nano tube. Now when you grow the stem cells this for 2 hours and 24 hour like during initial stage and then after one day of this one, if you look at the morphology of the stem cells, you suddenly see that nano tunes indeed have some influence or some observable influence on the stem cell morphology after it is grown from 24 hours.

This work is from the group of Jean, Inglor is also one of the author. It was published with Proceedings of National Academy of Sciences in 2009 also quite a few years ago.

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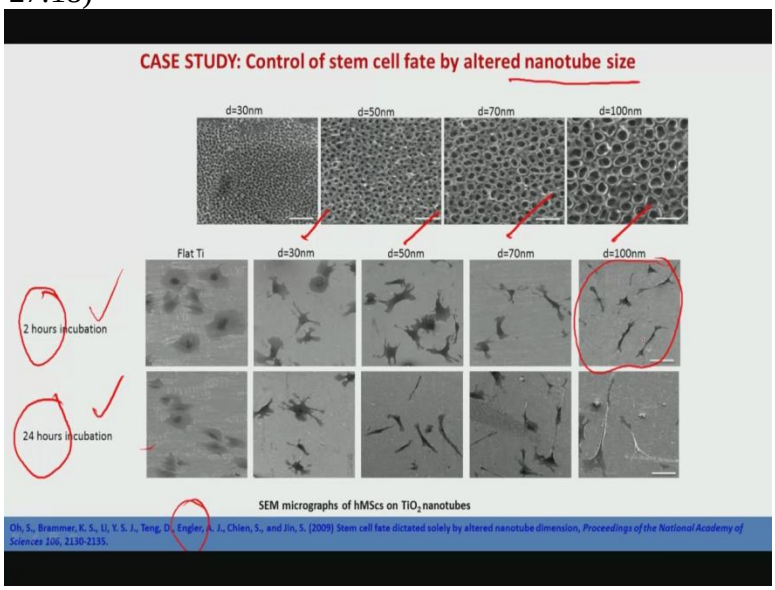


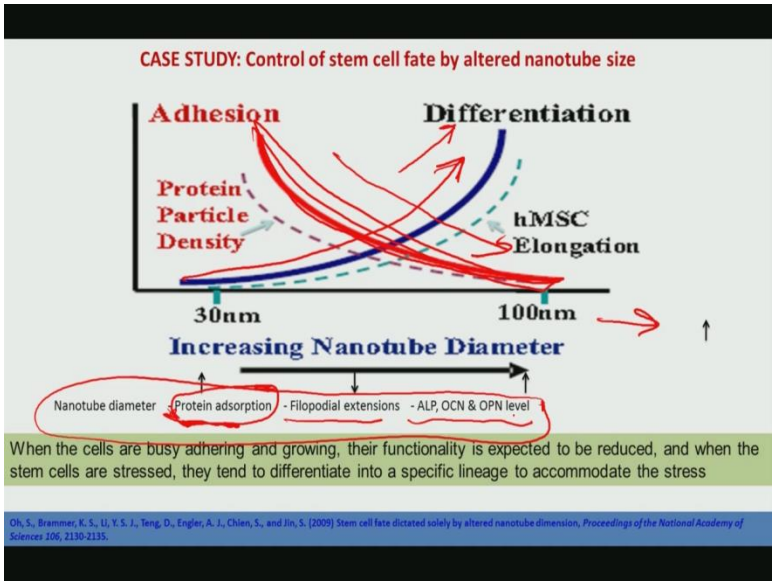


So what was the final conclusion or what was the kind of results or their understanding which is kind of explained in the form of a schematic plot. In the schematic, this is the schematic plot essentially is qualitative description as how stem cell adhesion and differentiation varies as you change the nano tube diameter, titanium oxide nano tube diameter along the x axis.

So if you go back to the earlier slide you have seen that this four sizes atleast were investigated 30, 50 ,70 and 100 And this two time scale, they have investigated 2 hours for adhesion and 24 hours just to see some signature of the differentiation. And so essentially stem cell adhesion

decreases qualitatively decreases along this line as you increase the nano tube diameter, nano tube size. Your differentiation is increased when your adhesion decreases, your differentiation increases.

So what it means is that, that if there are less number of cells on this let us take an example of 100 nano meter. So since larger the size less is the adhesion, but less is the adhesion means the more less is the number of cells which are able to attach to the substrate. Now less is the number of cells that will be attached so then each of the cells will have much more space in this particular substrate to alter their morphology so that they those kind of situational scenario will allow the stem cells to maximise their morphological changes, altering their morphological changes.

That is what I am trying to explain on the basis of this qualitative description, that that as the adhesion cell adhesion decreases kind of in a non linear manner as you increase the diameter, as you increase the nano tube diameter (())(29:37) nano tube diameter your differentiation process or your differentiation is enhanced in a in just an opposite manner. So less is the adhesion more is the differentiation, simply because of the reason that I have shown I have mentioned that physical space that each stem cell has in the surroundings is much more so that this the way the stem cell would like to differentiate that is possible.

The other things that I have mentioned in this slide towards the lower side; that nano tube diameter, now if you increase the nano tube diameter what it means is that larger protein absorption also takes place because nano tubes typically facilitate or typically allow more and more protein like different like cell specific proteins like bovine serum albumin or fibronectin or vitronectin or those kind of proteins. Now more the proteins on this nano tube substrate then that also gives more that also facilitates more fallopian extension and after that during the bone cell differentiation this group by this group led by I think Jean, as well as that involving Inglor they have shown that bone cell specific genes are upregulated, like ALP alkaline phosphates, OCN stands for osteocalcine, OPN stands for osteopontin.

So these three specific genes these specific genes are over expressed and therefore are upregulated. Therefore it essentially shows you that this differentiation process has taken place. So I hope that I have shown you that all the things that I have mentioned earlier, that what is the

role of this biophysical cues towards the stem cell differentiation and then I have given some specific examples as how if you change the elastic stiffness or elastic substrate stiffness then how differentiation through different lineages are possible.

I have also shown you some specific examples from published literature that if you change the nano tube diameter if you use the smaller nano tube diameter or larger nano tube diameter then how that will help in inducing mesenchymal stem cell differentiation to the oestrogenic lineage.