Introduction to Developmental Biology
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Lecture No – 25
Early Mammalian Development (Part 2 of 2)

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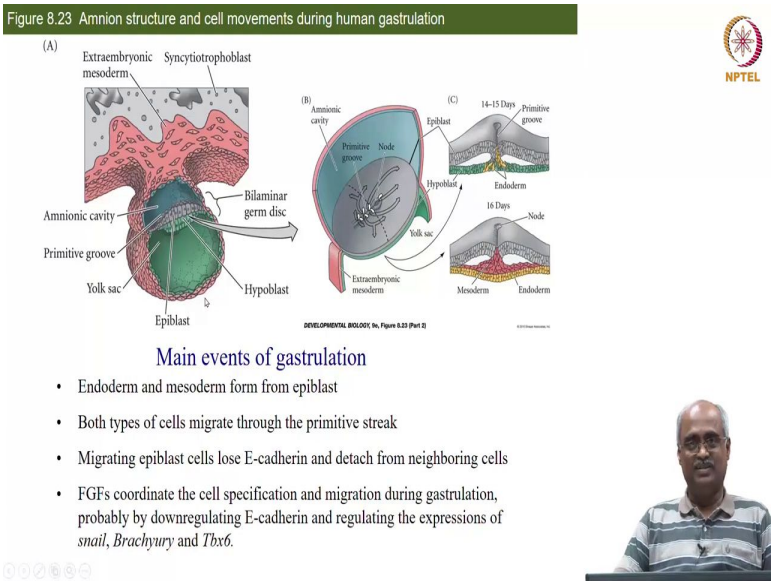

Students welcome back to the developmental biology course. In the last class, we were discussing the very early part of the mammalian embryonic development, up to the end of blastocyst. And then, we looked at the various you know names like epiblast, embryonic epiblast, amnionic ectoderm, hypoblast and so on. So today we are going to continue that, from gastrulation onwards. So one important thing that we discussed in the last class is the molecular events that determine who is going to become trophoblast and who is going to become the inner cell mass.

So this inner cell mass and you know the trophoblast are the key initial differentiation that happens in the very early stage of the embryonic development. So today we continue from there. This is another view of whatever we have seen. So yesterday, you saw a cartoon like this.

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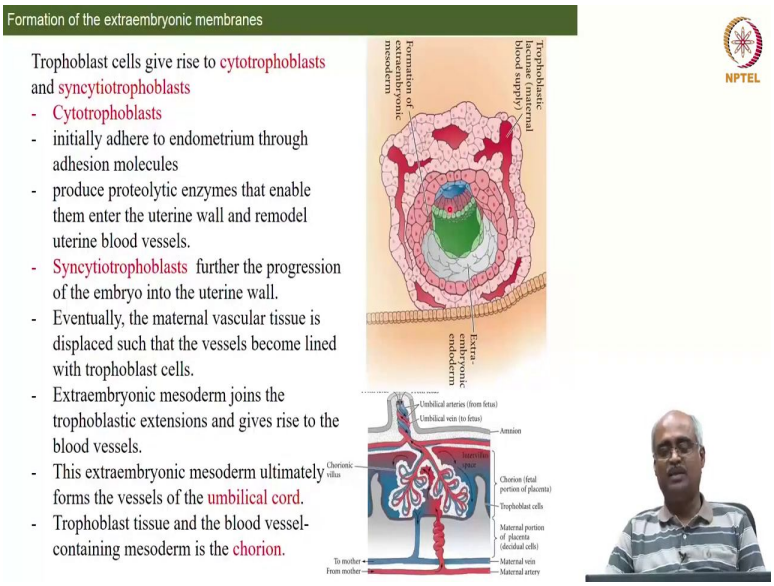

The same thing, slightly different in a sort of 3D view is what you have in this. So this is the embryonic epiblast after having given rise to this amnionic ectoderm and this is the amniotic cavity. This is the yolk sac. So this is the extra embryonic mesoderm that came from the hypoblast (green cells). So these two layers of cells here, if you can imagine a 3d structure, it is going to be like a disc-like thing. So here, this is another view where you are seeing the surface of this grayish area.

So this is the embryonic epiblast and the greenish thing below, so these two discs together are called the bilaminar germ disc from which everything comes primarily. And so this is our starting point of gastrulation. So the gastrulation gets over by the 15th day in humans, after gestation. So the main event of gastrulation is basically, these cells start migrating in between the two layers as shown here.

So the formation of this, what we call as the primitive streak, so this is the posterior and this is the anterior and this is the node. So this node and primitive streak are structures through which the cells are going to go inward between these two layers (the gray and the green layers) and that is how we are going to have the endoderm and the mesoderm. So that is the key event, you know endoderm and mesoderm formation from the epiblast.

And the migration direction is shown by these arrows, darkened just to highlight the point that they are beneath the gray cell layer and this white arrow says from the top, where it is going to go like this. And so if you take a cross-section along these dotted lines and look at them, you are going to see like this. So this is 14 to 15th day and this is the 16th day or the end of 16th day. So here what you see is, from the epiblast cells you get these endoderm cells.

These yellow colored cells coming down are going to be later followed by mesodermal cells. So these cells are actually epiblast cells and some of those cells lose E-cadherin expression and therefore, their adhesion is lost and they become migrating epiblasts and these are detached from the neighboring layers of cells and then they migrate down. An important signaling pathway that regulates is the FGF. So FGF coordinates the specification of these two types of cells i.e endoderm and mesoderm as well as, help their migration during gastrulation.

And one of the potential downstream targets of FGF is the E-cadherin. So E-cadherin level is probably down regulated by the FGF signaling and therefore, that facilitates the detachment of these cells and also it regulates the expression of these transcription factors *Snail*, *Brachyury* and *Tbx6* and these are essential for those fate specifications. So this is the main point of gastrulation. So eventually this endoderm that is coming out, is going to replace these hyperblast cells and that is going to form a layer here.

So much of our understanding of mammalian embryonic development actually comes from studying the mouse embryo development. And the mouse embryo, at this stage, is not a flat-disc like structure. Instead, this disc starting at the node is folded up. So this gray sheet is folded up along with the green below and therefore this endoderm here is going to be on the outer side of the embryo. So, that we will see in the next slide.

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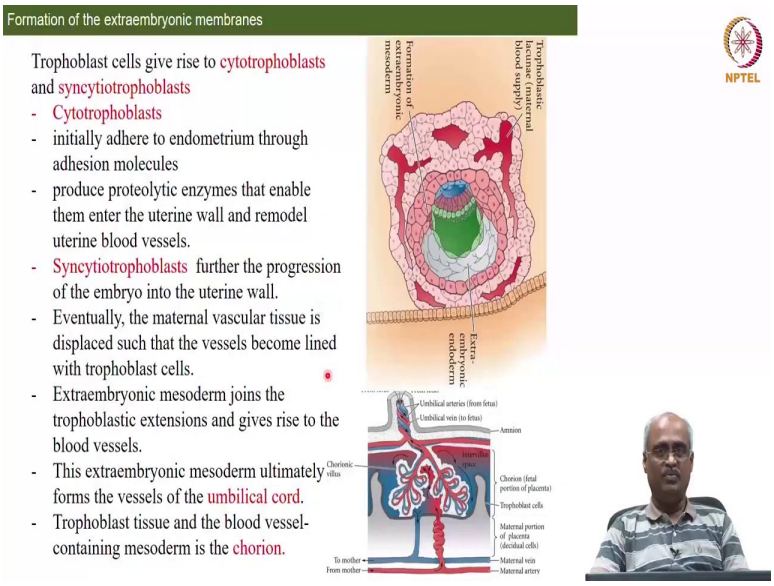

But before we go further to that step we need to look at what is happening outside the embryo. So far I was talking about these two cells and we should not forget about these trophoblast cells. What are they

doing simultaneously while the embryonic gastrulation is happening? So they are busy setting up the connection with the mother and that is explained in this. So if you recall from the last class, the outer layer i.e the trophoblasts form a layer which we call syncytiotrophoblast.

And they produce the proteases that help in dissolving ECM and getting attached to the endometrium. Then, they give raise to a set of cells where they undergo cyto-nuclear division without cytokinesis, forming multinucleated cells called the syncytiotrophoblast (shown in light pink color). These are the ones that end up setting up the actual connection. So, they form the chorion of the placenta. So this detail we need to understand clearly and that is the focus of this slide.

So, the trophoblast cells give raise to cytotrophoblasts and these are the ones that are important for attaching to the endometrium initially and they accomplish this by producing proteolytic enzymes and that enable them to enter into the uterine wall and remodel the uterine blood vessels and this job is taken up at further extent by the syncytiotrophoblast. So these are called syncytio, primarily because these are multinucleated like syncytium. So syncytiotrophoblast further the progression into the uterine wall.

So eventually what they are going to do is, they are going to line the maternal vascular tissue. So we have a close-up view in the next slide.

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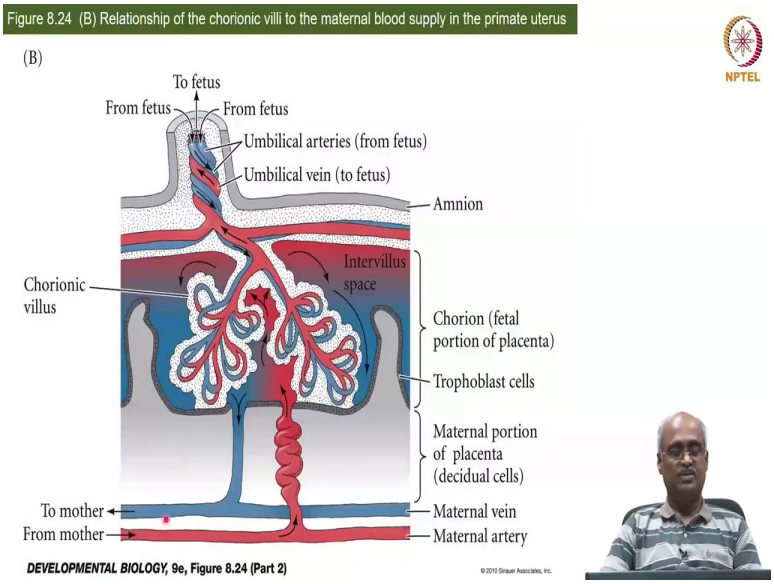

So this is the blood vessel from the mother's tissue and these trophoblast cells are going to line those blood vessels. The blood vessels basically now end here and it opens up into a wider area rather than being a tube-like structure. It opens in a wider area lined by these trophoblast cells.

So, the trophoblast cells and the extra-embryonic mesoderm that we saw earlier are the ones that are eventually going to form the blood vessels from the fetus side and that is what is going to give rise to the blood vessels within the umbilical cord. So umbilical, as you see here, is the connection between the fetus and the mother. So the umbilical cord itself and these blood vessels inside this are formed by these extra embryonic mesoderm. So they form these blood vessels and these actually differentiate into loop-like structures which we call as the chorionic villus and that increases the surface area a lot.

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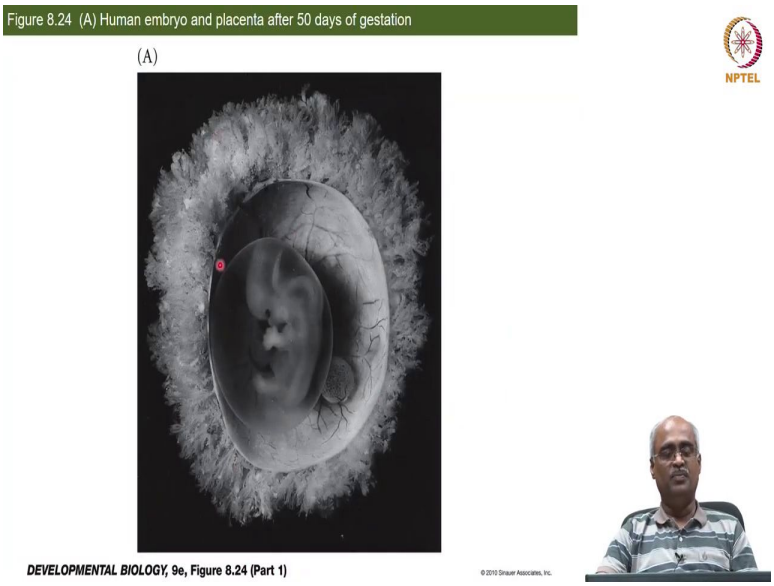

And this is quite clear when you see in this actual photograph of an embryo. So this is the amniotic sac inside with the embryo and this is the trophoblast cover and this is the chorionic villi. So that is formed by the extra embryonic mesoderm and so that is what forms the chorion. So along with this trophoblastic layer as well and the mother's blood baths this area.

So these blood vessels of the mother and the fetus are not connected. They are not continuous tube-like structures, instead they exchange blood in this area in which both of them diffuse. So the oxygen and other food supply from the mother's circulation comes into this area, from which this chorionic villus takes up. And similarly urea and carbon dioxide from this diffuses into this area, from which it drains into the mother's vein. So this is how the placental structure actually forms.

So this portion which is modified by the endometrium is the decidua and (mother's part) and this portion coming from the trophoblast-derived cells and extra-embryonic mesoderm forms the chorion and that is the fetal component of the placenta and this connecting thing is what we call as the umbilical cord. So I

hope you have a clearer idea of what is chorion and what is decidua and what actually placenta is. One point I want to highlight is, there is no direct blood vessel connection here.

So, they both have a common area which allows the diffusion between the two circulatory systems. So, this gives you a closer view of the structure that we just discussed. So you have the maternal artery blood flowing into this area and then the maternal vein that drains from this area. So you actually have an umbilical cord, two arteries but only one vein.

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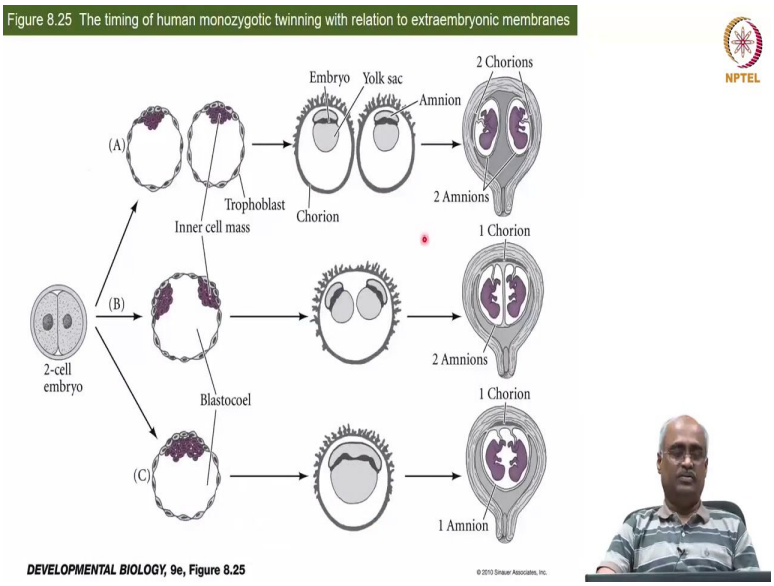

So let's start from inner cell mass once again to understand one specific aspect of inner cell mass. So as we discussed several lectures ago while talking about different kinds of specification, I mentioned that all vertebrates follow what is called conditional specification leading to regulative development.

So the interactions among the neighboring cells are critical. No cell comes specified by the maternal inheritance. Therefore if you remove any one cell in the inner cell mass, the remaining cells will replace its part. So each inner cell mass is actually capable of forming an entire embryo. They are completely undifferentiated except that they do not make trophoblast. Each one of the inner cell mass cells are capable of making the entire embryo.

So there are certain implications of this property and that is highlighted in this slide and in the next slide as well. For example, if you start with a two-cell embryo and let us say when they reach the blastocyst stage. And suppose let us say it split into two groups before this step itself. So between the two-cell stage

and the morula stage, if it is separated into two, they are capable of forming two embryos; those cells are capable of making both the trophoblast and inner cell mass.

If the embryo is split into two parts that early like for example in an eight-cell stage or earlier you know then they end up making two trophoblasts and two inner cell mass leading to making two chorions because chorions come from trophoblast. And then the inner cell mass give rise to epiblast from which you get the amnion and as a result you get two amniotic cavity as well.

So two chorion, two amnion and you get this set of zygotic twins. So these are not fraternal twins. Fraternal twins arise from two oocytes getting fertilized by two sperm at the same time and developing in one pregnancy; when you have two fertilization events that is fraternal twins. So we are not talking about that here, we are talking about twins that arise from single fertilization and this is possible simply because of the totipotency of early embryonic cells.

Now suppose let us say the split happens later after the trophoblast formed and the inner cell must split into two groups and now they are going to make two epiblast and hypoblast; and as a result that epiblast is going to give rise to to two amniotic ectoderm and therefore you will end up making two amniotic cavity but this happened after the trophoblasts separated and due to that you are going to have a single chorion.

So a single trophoblast population and as a result a single chorion but two inner cell mass and as a result you get two amniotic cavities. So here you can see this shaded area, that is a trophoblast and that is the chorion formed from the trophoblast and this white area is the amniotic cavity so your two amnion with one chorion. Suppose if it happens even later or like for example the embryonic epiblast separate into two groups after the amniotic ectoderm is formed and therefore a single amniotic cavity is present but the embryonic epiblast split into two.

Now you get two embryos in a single amnion and a single chorion. At all these stages, the cells are capable of forming an entire embryo. So if splitting is possible then mixing also should be possible right and that is what is illustrated in the next slide.

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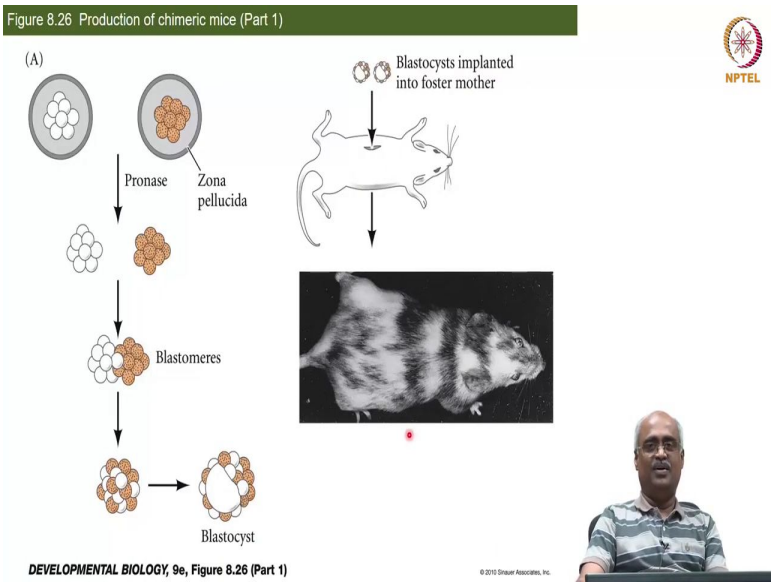

So here you have two different pigmentation, two different fur colors. So if you take embryos from the two different fur colored mice and open up the zona pellucida and allow the cells to mix, they form via regulative development. So therefore they form a single inner cell mass and form an embryo that develops into an adult mouse with mosaic color pattern.

The cells of both genotypes are interspersed and resulting in this mosaic color pattern and you can take this further. Suppose let us say this mouse mates with a homozygous recessive organism, that is the gene for which this fur color is encoded is recessive and does not produce any pigments and that is the white color. Why is it having homozygous recessive phenotype? Let us see what happens.

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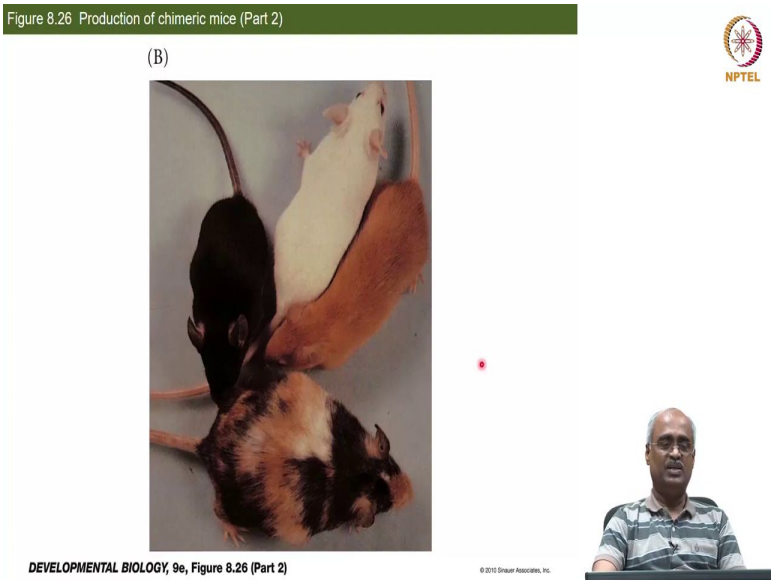

So in this experiment actually three different pigmented morulas were taken and fused into one and they form three different colors. You see white, brown and then the black. These cells contributed to the germ line as well. You know the germ cells have been derived from these cells and as a result you have three different genotypes of germ cells. And when oocytes formed from those three different types, developing in a single mother, are fertilized by sperm from a homozygous recessive, you end up getting all three colors separated in the progeny.

So when the black gene fuses with a sperm having a white allele, it gives black progeny. And if the germ cell had the brown allele, then with the recessive allele from the sperm, you have a brown fur. So both of these very clearly show the pluripotency of the inner cell mass to embryonic epiblast stage.

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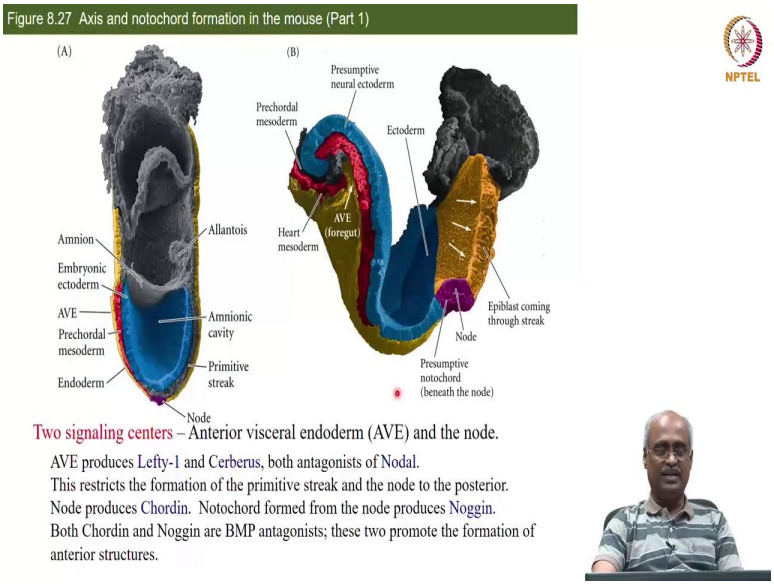

So now let us get back to the development of the embryo. So this is the cup-shaped embryo, this is the node and the posterior side, the primitive streak is here and this is the anterior part.

So the endoderm is outside and this AVE (anterior visceral endoderm) that actually formed from the hypoblast initially, is at this anterior most end. Posterior is often called caudal. So this is the caudal side and this is the anterior side where the head is going to form and the node is right in the middle at the bottom.

So this is the primitive streak, between this blue and greenish gray area. Between these two you have the migrations of the cells happening as shown here. So this is a later stage and for now our focus is the

gastrulation. We sort of understood the endoderm and mesoderm started to migrate through the primitive streak and the node; and now how the head to tail axis is formed is the focus of the next few slides.

There are two signaling centers, basically one is formed by this anterior visceral endoderm near the head area where the head is going to form and then at the bottom of this cup shape you have the node. So that is the one from where the cells are going to fold and form a tube-like structure that will finally connect from here to the head and that is going to be the notochord. And this node and AVE form the two signaling centers that determine the head to tail axis.

So we have not understood this process in much detail as we saw in drosophila anterior-posterior axis formation but some details are emerging already. For example, this AVE produces lefty and cerberus proteins and they antagonize nodal expressed in the epiblast cells and therefore nodal is not going to be expressed in this area. And that seems to be key for positioning the node and primitive streak in this part and not here.

And so that is one key aspect i.e. production of lefty and cerebros by AVE which suppresses the nodal to the posterior part. Later, when the node starts producing chordin and when notochord is produced from the node area, this node formation itself is induced by the underlying trophoblast cells and they seem to be key in specifying a node. The node is derived from the mesodermal layer and the cells coming from that are this precaudal mesoderm.

Once node produces the notochord, notochord produces noggin. This chordin and noggin are key along with AVE for the head structures to form. And what they do is they are antagonists to the BMP signaling. So BMP gets restricted to the posterior and so chordin and noggin are important for the head structures to form including the neural tube. So this is illustrated by the phenotypes of the chordin and noggin knockout mice shown here.

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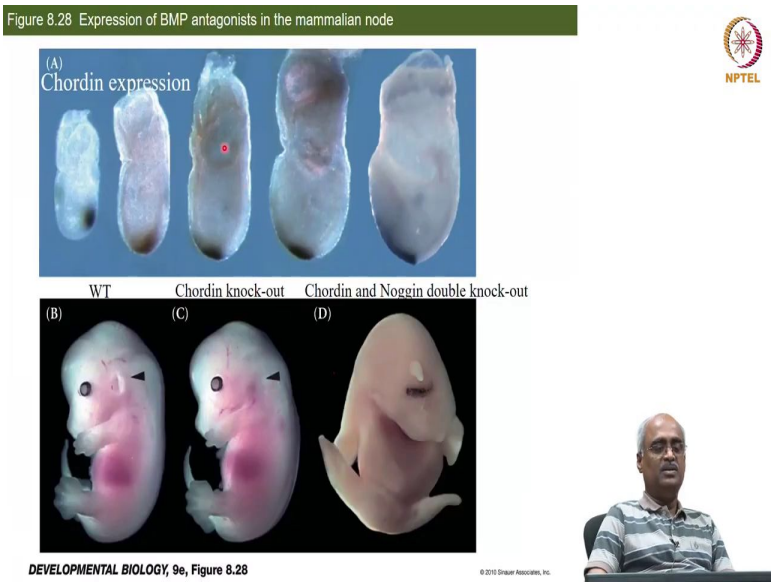

So this shows a whole embryo stained for chordin expression. So chordin is there in the anterior part of the primitive streak and then it is there in the node and in the anterior structures here. So that is what you are seeing as the embryonic development continues. So that is the chordin expression pattern and if chordin is knocked out, this ear development defect occurs and in the wild type, the head structure formation appears to be all right.

But if both chordin and noggin are knocked out, the head structure does not develop right. There is no jaw formed and only one eye is present which is below the nose-like protrusion. So, no clear head structure forms, indicating that these probably function in a redundant fashion for the head structure to form. And another important molecule that plays a role in this head to tail axis formation is the vitamin, retinoic acid.

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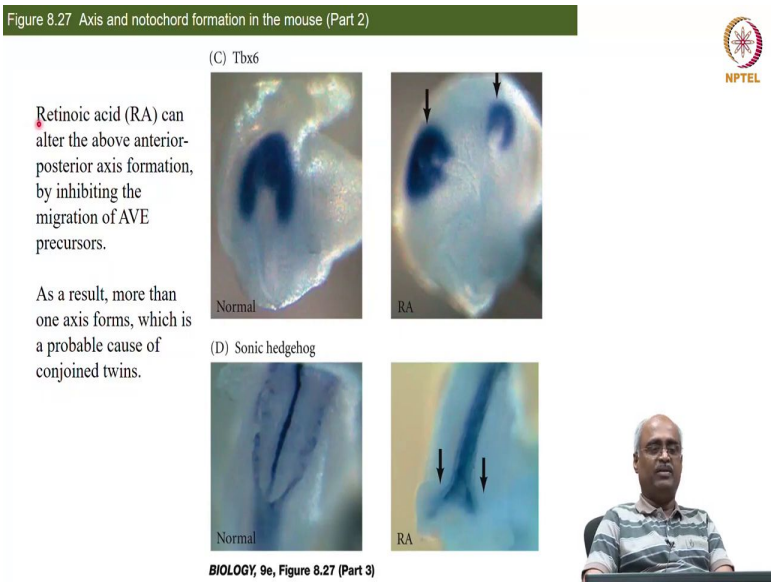

Vitamin-derived signaling molecule, retinoic acid can alter this anterior-posterior axis if it is introduced at the stage of gastrulation. So when the embryo is incubated with retinoic acid, you have these alterations which is illustrated here. So Tbx is a transcription factor normally expressed in the anterior primitive streak.

So this is where Tbx6 is normally expressed and when embryo is bathed in retinoic acid, it alters the anterior-posterior axis formation such that it is duplicated into two axis. Another critical signaling molecule, the sonic hedgehog is expressed strongly in the notochord here and now with retinoic acid application, it gets split into two notochords.

And this is probably one of the causes for the formation of conjoined twins. In TV, you would have seen the images of some children, even grown up adults where they are joined at the head. So that is due to partial duplication of the head to tail axis and that is what leads to the formation of conjoined twins.

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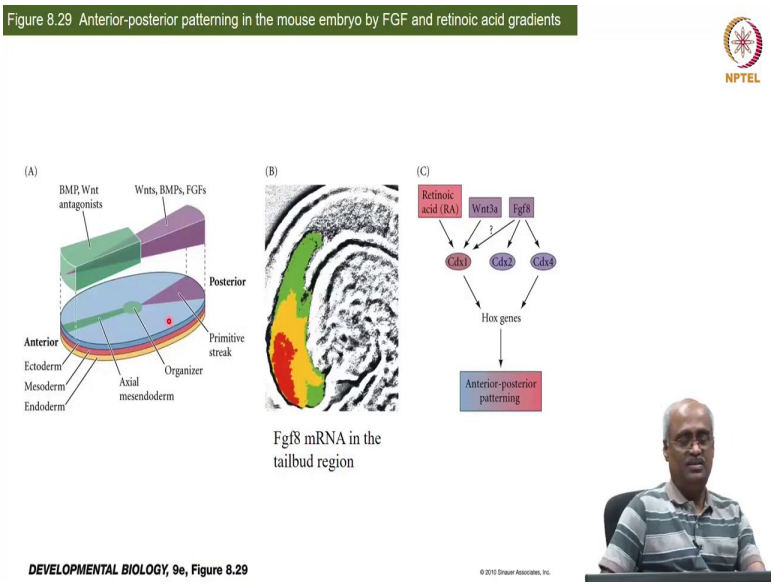

This cartoon representation is flattened disk of the embryo. This is the anterior and that is the posterior most at the end of gastrulation stage. And starting from the organizer, the anterior structures produce antagonists of these signaling molecules Wnt, BMP and FGF.

As I already said, chordin and noggin are antagonists of BMP. So as a result, these molecules are concentrated in the posterior. So FGF mRNA which is transcribed here, gets degraded as it moves anteriorly and forms a posterior to anterior gradient and the antagonists form the opposite gradient because they are produced by organizer and the anterior structures. So you have two gradients, this FGF, BMP, Wnt forming a positive anterior gradient and then their antagonists in the opposite and so these molecules specify the posterior.

So this is the tailbud region of mouse embryo where in situ hybridization shows the level of FGF8 mRNA. So towards the tip you have the highest concentration of FGF8 mRNA and it progressively decreases as you go towards the anterior part. And so the retinoic acid forms another gradient. Retinoic acid producing enzymes are in the posterior while retinoic acid degrading enzymes are in the anterior.

Retinoic acid, in addition to these three signaling proteins, also forms a posterior-anterior gradient. So signaling from these molecules, as well as retinoic acid, all converge in the activation of the Cdx proteins, which by acting on Hox genes coordinate all these signals into determining the anterior-posterior patterning. So now we move on to hox genes.

And the AVE produces signals that restrict the posterior signals to the posterior and I enable the head acne formation by the activity of cardinal noggin at a later stage. And that ends up setting up these two gradients that I just said and in addition we have a retinoic acid gradient as well. So all of these gradients of converge on hox gene expression and why which hox gene is expressed in which part along this anterior posterior axis provides the identity for those specific areas what kind of organs are going to form there. So that is our next goal.

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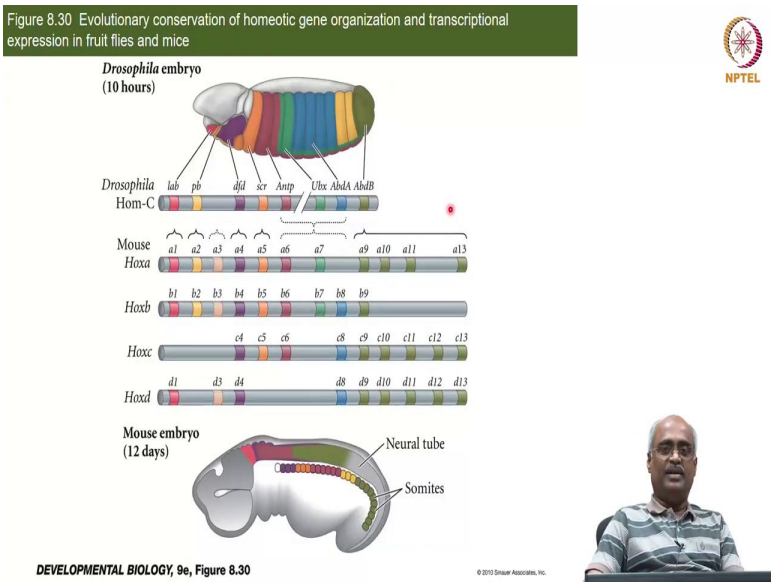

So let us revisit what we have already learnt about hoxs genes when we discussed drosophila anterior-posterior axis formation. We saw that hox genes in the drosophila are all present on one chromosome in two clusters: the anterior antennapedia cluster and the posterior thorax cluster and I told you that they are arranged on the chromosome from the 5 prime end to 3 prime end. They keep the same sequence i.e. collinearity in the way organs form from anterior to posterior. It means that hox genes present near the 5 prime most part of the chromosome specify the anterior most structures in the anterior posterior axis.

And similarly the posterior most structures are specified by hox genes that are located near the three prime most part of the chromosome. So there is collinearity in their presence in the chromosome and their action along the anterior-posterior axis. Initially their expression is in that pattern, the 5 prime most ones are expressed in the anterior most and the 3 prime ones expressed in the posterior most part of the embryo leading to those structure specifications in that order.

Remarkably, this sort of a collinearity is conserved even in all vertebrates, except that vertebrates have 4 copies of each of these clusters. There is only one cluster in drosophila corresponding to these body parts and the very extreme anterior head region is determined by two separate transcription factors.

So the 5 prime end anterior most and 3 prime end posterior most in the anterior posterior axis is exactly the same order in the vertebrate chromosomes except that there are 4 copies on 4 different chromosomes. For example in a mouse, you have Hoxa set in one chromosome, Hoxb set in another one, Hoxc in third and Hoxd in the fourth one. So this a1, b1 d1 etc are paralogs, i.e. the same sequence is present in multiple copies on the same organism.

So these are sequence-wise, the most closely related to each other than to the other Hox genes. For example, a9 is an ortholog of the drosophila AbdB and that has 4 copies, meaning 4 paralogs. So AbdB is ortholog, meaning similar sequence in another organism and; as similar sequence in the same organism there are four paralogs. For some of them, you have 4. Some you have 3 and for some you have only two. So it depends on the duplication events and the loss of the duplicates during the course of evolution.

Here again, the order is maintained. In the dorsal part of the anterior-posterior axis, you have the neural tube as well as the somites that form from the mesoderm as shown here.

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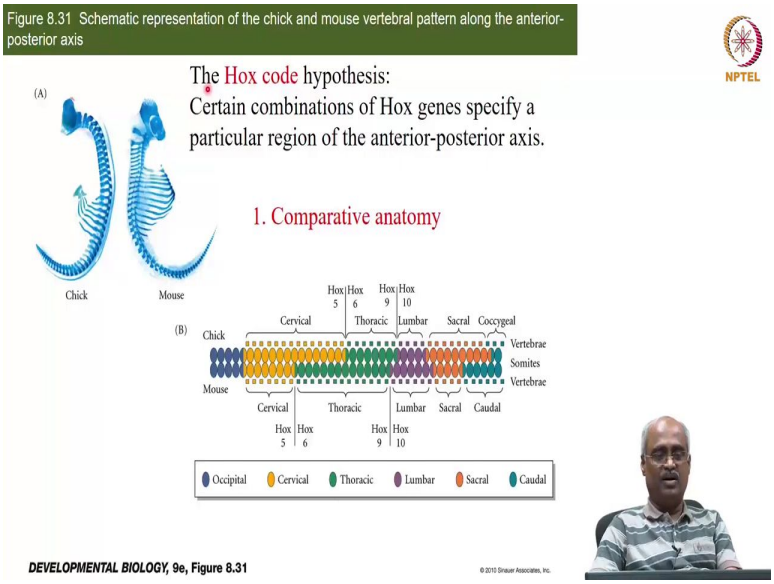

Now we move on to understanding a concept called the Hox code hypothesis. What the Hox code hypothesis says is that along the head to tail axis, what structure is going to form where is determined by the combination of hox genes expressed in that place. The combination of hox genes expressed in a given

position along the anterior posterior axis specifies what structures are going to form there. They provide the segmental identity for a given position along this axis.

The evidence for this comes from three different kinds of independent observations; one of them is comparative anatomy. So you look at the skeletal structure of a chick. Its neck is a lot longer than a mouse. Between the ribcage and the head, chicks have long necks and when we look at the Hox gene expression pattern, Hox5 expression extends more posteriorly in chicks.

As a result, Hox6 is restricted and you have a long region of cervical vertebrae. The total length remains the same; the number of vertebrae are the same. It is just that either you have a situation where the cervical vertebrae are converted into thoracic or the other way depending on which one is your starting reference, whether it is mouse or chick. So this supports the idea that which Hox gene is expressed, determines what kind of vertebra is going to form.

The anterior most head region is determined by the orthologs of orthodenticle and empty spiracles gene (seen in drosophila) These orthologs are the ones that determine the midbrain and forebrain. So the Hox gene expression extends only up to the anterior most part of the hindbrain.

So the head region in an organism, though separated by 500 million years, is determined by very similar genes. Another important point is that a given segment's identity is determined by the posterior most gene that is expressed there. For example Hox6 is what determines whether it is going to become thoracic or cervical. And this is because the anterior genes expression is restricted by the posterior gene expression. So in other words if I remove Hox6, Hox5 will extend and the anterior structures shift posteriorly. This is exactly what we saw when we were discussing about antennapedia in drosophila. So the posterior most gene expressed in a given position in the anterior-posterior axis determines what segmental identity that portion is going to get.

So this is comparative anatomy. The expression pattern of what hox gene is expressed seems to correspond to what kind of vertebra form in these two species.

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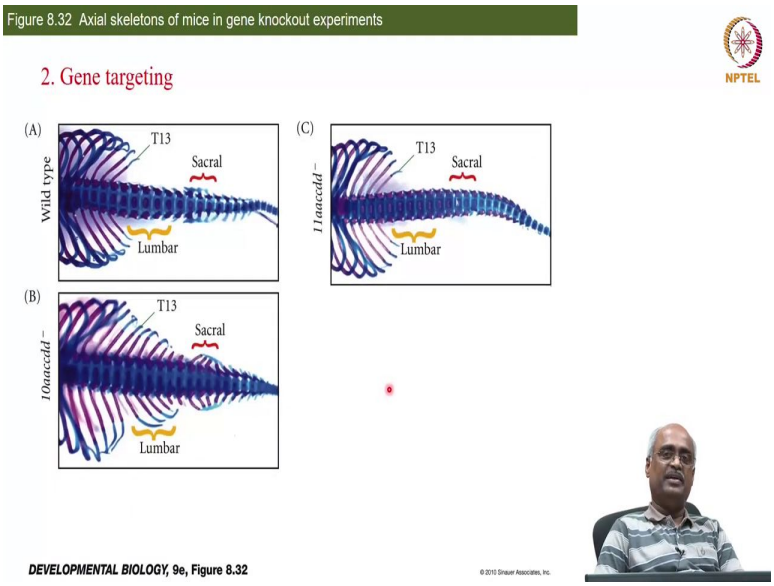

Next we are going to look at the gene knockout experiments. This is the wild type where you have the thoracic vertebra forming the ribcage and then this is the lumbar between the chest and the hip region. So remember, in a mouse it is not enough to delete one ultrabithorax or one antennapedia. Here, you have four copies and therefore all four copies need to be knocked off to get a phenotype otherwise they are redundant among themselves and they will lead to normal development. So to knockdown Hox10 you need to delete two alleles of A, two alleles of C and two alleles of D.

Then, you have the expression of Hox9 extending posteriorly, which ends up producing ribcage-like vertebra here in the lumbar region, again supporting the Hox code hypothesis. The combination of Hox gene specify the segmental identity for any given position along this axis. And in a similar experiment when all paralogs of Hox 11 are deleted, you have the sacral region getting converted into lumbar-like structures. So sacral forms this little extension and lumbar does not normally and that is what now sacral also forms. So this is the second line of evidence that supports the Hox gene code.

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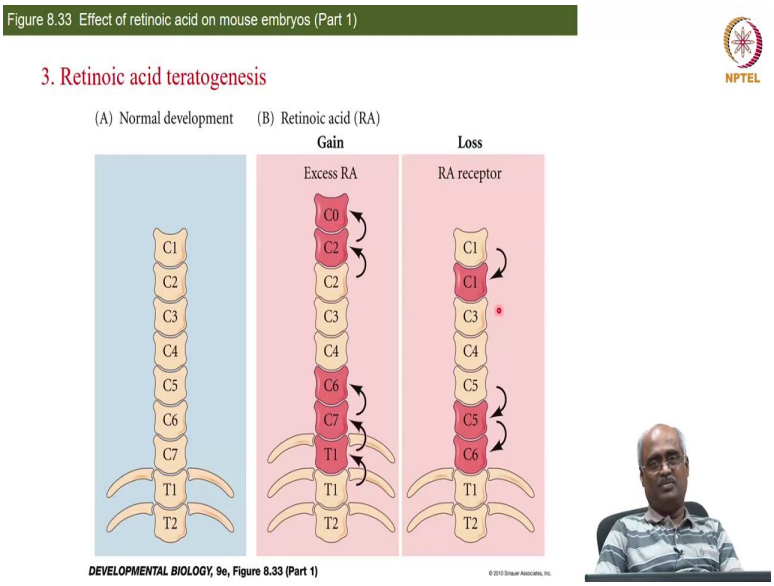

The third evidence involves retinoic acid. So remember, retinoic acid will mess up the anterior-posterior axis and we just saw that retinoic acid is expressed highest at the posterior where its synthesizing enzymes are present and it is degraded as you move anteriorly. And then we also saw that retinoic acid via Cdx genes impacts the Hox gene expression so as shown in this cartoon. So higher concentration of retinoic acid means the most posterior gene gets expressed.

So in an anterior portion, if you increase the retinoic acid then, you are going to have posterior Hox genes get expressed there and then the segmental identity also shifts the posterior structures to anterior structures. So in the wild type, you see the thoracic and cervical region. When retinoic acid concentration is increased by incubating the embryo at the appropriate stage, you have an additional shifting happening anteriorly.

So, cervical vertebra gets converted into thoracic forming these ribs here. Hence, C6 becomes C7 and so on. On the other hand if you reduce/deplete retinoic acid, you have the opposite effect i.e. C2 becomes C1 and similarly here at this junction you will see T1 becoming C6 and therefore it is cervical and it does not form the ribs and this is also seen when you mess up with the Hox gene expression.

So here Hox gene overexpression shifts anteriorly and Hox gene mutations shift posteriorly because the posterior hox genes try to suppress the expression of the adjacent anterior hox genes. As a result when you increase a positive gene expression, it shifts anteriorly because it suppresses the anterior gene expression and therefore the posterior expression domains shifts anteriorly, leading to posterior structures

developing anteriorly. That is how this becomes converted into additional rib forming thoracic vertebral column and the opposite causes the loss.

These three evidences include comparative anatomy where morphology of the different vertebrae along the anterior-posterior axis in two organisms is slightly different because of difference in gene expression. Second evidence is when we knock out all paralogs of a given hox gene, we see the structures shifting posteriorly and the third evidence is how increasing retinoic acid or decreasing retinoic acid is similar to Hox gene over-expression and under-expression. So these three lines of evidence support the Hox code hypothesis. And what is really remarkable is that this pattern of Hox gene expression along the anterior-posterior axis and the specification of segmental identity is well-conserved in all organisms starting from flatworms up to homo sapiens.

So today I will stop here and we will continue on to a new chapter, where we are going to look at the evolutionary implications of developmental mechanisms in what we call as the evolutionary developmental biology. So that will be our next topic.