Anuran Metamorphosis - an Attractive Biological Process to Study the Mechanism of the Larval to Adult Tissue Remodeling and to Detect Thyroid Disrupters -

Katsutoshi Yoshizato
Hiroshima University, Japan

I think the 5th International Symposium on Environmental Endocrine Disrupters in Hiroshima is very unique in one thing: that the symposium organizers officially select the effect of disrupters on the thyroid axis.

In this afternoon session, we have three meetings. First, we have a frog group meeting because the thyroid hormone, as you know, plays a very important role in the metamorphosis from tadpole to frog. In the second session, we have the mammalian group. The thyroid hormone also plays a great role in the development of mammals. After these two sessions, we have a get-together session, frog people and mammalian people. So please stay here through these very interesting and important sessions. As a startup, I will briefly introduce to you about amphibian metamorphosis.

This is the metamorphosis of (*Xenopus laevis)* from Africa. Very young tadpoles have no forelimbs, and you notice a very large tail and body structure here in front. At stage 60, tadpoles get into the so-called climax of metamorphosis. You can see the hind legs now actively developing and you can see forelimbs, and still this tadpole is keeping its nicely developed tail. So, this is in some sense frog in the front, but in the posterior part, it is still a tadpole.

At stage 63, already the hind legs are well developed and nicely developed forelimbs, but still a small tail is remaining. So this is the rough process of metamorphosis of anurans. This process is very interesting, and it is now generally accepted that thyroid hormone controls this process. Triiodothyronine, T_3 with this structure, you see before stage 55 tadpole, we cannot detect thyroid hormone in their plasma, but from this stage the level of thyroid hormone rapidly increases and after the completion of metamorphosis, again we cannot detect thyroid hormone, suggesting that thyroid hormone controls the metamorphic process.

This suggestion can be easily verified. If we pick up some young tadpoles and treat them with thyroid hormone, then these young tadpoles start metamorphosis. Now I would like to give you some of our recent data with attractive biological process to study the mechanism of larval to adult tissue remodeling and to detect thyroid disrupters. Due to the short time, I will give this topic, detection of thyroid disrupters utilizing frogs. I will speak about this topic in the third session as I said. My name is Yoshizato.

Now you understand that thyroid hormone and thyroid hormone receptor are key molecules, which control the process of metamorphosis. Scientists generally believe this scheme about the relationship with thyroid hormone and thyroid hormone receptors. We have thyroid hormone responsive genes here, and in the promoter region we have thyroid hormone response element, TRE. To this TRE, we have heterodimer of retinoid X receptor and thyroid hormone receptor waiting for ligand T_3.

Without T_3, this element exhibits inhibitory action on the expression of downstream gene. However, if thyroid hormone binds to this element, opposite effect will take place like this. Now the downstream gene's activity is transcribed. This is a generally believed scheme, currently.

This relationship can be easily visualized utilizing transgenic flog recently developed by an American group. We have female with eggs, male with sperm to these sperm nuclei we introduce appropriate plasmid DNA. You see in that TRE thyroid hormone response element, and as a reporter gene, usually we utilize EGFP, now we have F_0 transgenic frogs.

This is the promoter region of (*Xenopus)* thyroid hormone receptor, βA1 gene here with this structure, you notice the red area with reporter gene as I said. Now we have transgenic tadpoles at stage 49 with a bright field microscope. With fluorescent microscope, no fluorescence can be seen. At a little bit advanced stage, there is still no fluorescence. Now, tadpoles are almost becoming frogs, with short tail here. By fluorescent microscope, you can see from the dorsal view and the ventral view, now you can see fluorescence.

We have very young tadpoles at stage 56. These tadpoles are far away from metamorphosis. We kept these tadpoles in thyroid hormone containing water, with this concentration. Zero days: no fluorescence. Three days: maybe no fluorescence at all; after five days now, here is hindlimb and tail region. The tadpoles have become fluorescent. Here are ventral and dorsal views.

Let me ask this question about the tadpole metamorphosis. Now we have here complete tadpoles. Body parts will transform into frog; however, the tail region cannot transform to adult structure. Instead, all the cells in the tail region are subjected to apoptosis. So, this is region-dependent metamorphosis. My question is; why cannot the tail region transform into a frog structure? For this question, the study utilizing skin is very useful.

Here you can see arrows in the left side. We have already adult skin, or correctly speaking, pre-adult skin. In the right side, still the skin is larval type because you see a thick collagen layer. Between the collagen layer and the epidermis we have a small region of connective tissue. However, there is no connective tissue in the larval region, pre-adult region and larval region.

We found that the upper surface contains thick crystals of calcium phosphate, which can be easily stained by alizarin red S. This staining is very useful to identify if this region is the pre-adult region of the skin, or if this region is still the larval region. If the region can be stained red with this dye, then this region is a pre-adult region.

With this easy method, you see adult region and larval region. We postulate that between the adult region and larval region, some active center is functioning, called the skin transformation center. So, we have second connective tissue, thick collagen layer, first connective tissue. In contrast, in the tail region no second connective tissue, only primary connective tissue.

With this staining, we can follow the development of pre-adult skin regions. At stage 1, this is the *(Rana catesbiana)* staging, not *(Xenopus) ,* the body is uniformly covered by larval skin, no calcium deposition at all at this stage. Now in some lateral regions, we have the first red staining. Here we have the appearance of the pre-adult region in these lateral sides.

This pre-adult region spreads during development, first dorsally, then ventrally, but it never spreads to the tail region. So now we can ask the question regarding region-specific transformation of skin in this way. Why cannot skin transformation center invade this tail region?

For this study, we have a very useful molecular probe. First, I would like to introduce to you (*Xenopus)* larval keratin (XLK), which is specifically expressed in the larval epidermal cells. Now, in the immunohistochemical staining of larval skin, just focus your attention on the basal cell; dotted line represents basement membrane. On the basement membrane, you see XLK expressing larval cells, still larval cells you see at stage 54.

Now just before the metamorphosis still we have many larval cells. Stage 60 in the middle of metamorphosis climax, we have histologically identifiable adult basal cells; you see no staining against this antibody. On the other hand, (*Xenopus)* adult keratin (XAK) is very useful to identify adult epidermal cells.

I show you these are young tadpoles with no staining with (*Xenopus)* adult keratin C, in short, XAK-C at stage 58 already we have some XAK-C positive cells on the basement membrane. The number of these positive cells increases as the tadpole develops, and in adult skin you have typical XAK-C

positive basal cells on the basement membrane.

We have a second transgenic tadpoles utilizing plasmid containing XAK-C promoter and GFP. In the adult we have very strong fluorescence through the body as expected: the front, middle and posterior end of the body are nicely fluorescent, and immunohistochemical staining shows red XAK-C positive cells on the basement membrane.

From our study and other scientific literatures, we can draw this scheme about the epidermal development. Now we have embryonic basal cells positive for XAK-A1 keratin, larval epidermal cells are as I said XLK-positive, and during metamorphosis XAK-C-positive cells appear, as well as typical adult basal cells.

From literatures and from our own studies, we can say the factors which control this transition of embryonic to adult skin; first, BMP4 plays important role in changing the endoderm cells to embryonic epidermis, and FGF-10 plays an important role from the transition of the embryonic epidermis to larval epidermis.

Now, the question is; what factor controls the transition from larval epidermis to adult epidermis. We noticed that the pre-adult basal cells express platelet derived growth factor A intensively, and beneath these basal cells we have now already immature secondary connective tissues where we have fibroblasts that intensively express the receptor of this molecule, PDGFR-α, suggesting PDGF signaling plays an important role for the transition of larval to adult epidermis.

To test this hypothesis, we removed pieces of skin from the back and cultured them for 9 days with and without T_3. Now on zero day of culture, all the cells are positive for XLK and negative for XAK-C. With T_3 in nine days, all the cells are XAK-C positive, indicating larval epidermis is successfully transforming to adult basal cells, no XLK positive cells. In contrast, without T_3, still we have XLK positive cells and a small number of XAK-C positive cells. These are pre-adult basal cells, because these cells are also positive to XLK.

Now to make the function of PDGF signaling in this process clear, we utilized inhibitors of the signaling. One is tyrphostin AG1296 and the other is an extracellular domain (ECD) of this receptor. We made recombinant domain of PDGFR. We add these two inhibitors into culture media.

Now, this is with T_3 control with inhibitor AG1296: no transformation to adult skin epidermis and the same thing for recombinant ECD of receptors. This is immunostaining evidence for this transition. Also, these inhibitors effectively inhibit the activation of fibroblasts such as migration, the same thing for AG and recombinant ECD.

These experiments gave us this skin, we have larval epidermis, adult epidermis, now larval basal cells, and skin transformation center. Now in this center, larval basal cells require some mesenchymal factor to transform into pre-adult basal cells, and as we showed in the previous slide, PDGF-A is created by activated pre-adult basal cells, and with receptors, fibroblasts are activated with this PDGF-A; and probably our preliminary study suggests FGF is another mesenchymal factor which stimulates pre-adult basal cells together with thyroid hormone. Pre-adult basal cells successfully and finally transform to adult basal cells, and produce their offspring of differentiated cells like glandular cells, spinal cells, and cornified cells as in mammals.

Coming back to the first question. We have body region in red and tail region in white. The skin transformation center you are now familiar with. The question is: this center cannot invade the tail region. Why?

There is a very strong barrier between the body and tail region. We think, at present, Hox gene plays some role for making the barrier. For example, if we remove skin from three parts, body part, anterior tail part, posterior tail part, and measure the expression level of Hox-D11 among these areas by RT-PCR, this intensity represented by this color, from body part to tail part, weak, middle, and strong expression of

Hox-D11. How about Hox-C12? This regional intensity is much clearer, no signal, weak and strong expression in the tail. And A13, only the posterior tail region is very positive.

In situ hybridization experiments show that fibroblasts in the body region are not expressing Hox gene-A13. However, in the tail region, we have positive cells for Hox-A13. Now we postulate that Hox gene, posterior Hox and anterior Hox, has some role to make this barrier.

We have now very young tadpole skin, body region, tail region. All the cells on the basement membrane are represented by yellow: larval cells, no differences among them. However, only one difference we can see in the mesenchymal compartment. These cells express different Hox genes, pink cells and blue cells.

Now, at some time in the development of tadpoles, the skin transformation center emerges, and in this center active interactions between the epidermis and mesenchym takes place, and this center spread over the body region as far as we have pink cells. However, at this point, no pink cells are visible, so center cannot move further. This is our current hypothesis. Thank you.