

Dual Function of Thyroid Hormone Receptor during Amphibian Development

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Thank you for inviting me to participate in this important symposium. As you know and as you have just heard, amphibians have been and will probably continue to be one of the first indicators of endocrine disruption.

However, as you know, in the past or up to now, it has been difficult or slow to apply the findings from amphibians towards mammals, especially humans, and toward policy changes. I believe part of the reason for this is because very often we do not know what is the underlying mechanism or the molecular pathway that is being affected by endocrine disruptors *in vivo* in live animals.

What I would like to do today is to describe to you our studies to investigate the functions and underlying mechanisms of thyroid hormone receptors in vertebrate development by using (*Xenopus laevis*) as a model.

The two major questions we would like to ask are; first, what are the roles for liganded and unliganded thyroid hormone receptors or TRs in vertebrate development, and how do TRs regulate target gene expression *in vivo*?

As I said, we use (*Xenopus*) as a model. To begin with, what we really did was to use a simple hypothesis, that is, we consider thyroid hormone as like a virus. Thyroid hormone induces metamorphosis or vertebrate development through a cascading of gene regulation, like a viral induction of early genes, late genes, etc.

From many studies by many speakers, especially those you will hear later today, we know that thyroid hormone receptor functions as a heterodimer formed with RXR or 9-cis retinoic acid receptor. The TR/RXR heterodimers are believed to be constitutively in the nucleus and are bound to chromatin or to the target *in vivo*.

So, when thyroid hormone comes into the nucleus it binds to the receptor, which activates the receptor complex. That, in turn, leads to the activation of early thyroid hormone response genes, and then, in turn, late thyroid hormone response genes, eventually lead to tissue specific changes.

Therefore, to understand the molecular pathway that causes amphibian metamorphosis by thyroid hormone, there are really two major questions we need to address. The first is; what kind of function does thyroid hormone receptor, both unliganded and liganded (receptor) play during development, and what are the underlying mechanisms? The second question is; what are the genes induced by thyroid hormone and how do they in turn regulate downstream events? What I would like to do today is to address the (first) question here, that is, what is the function of the receptors, liganded versus unliganded, during development and what is the underlying mechanism?

Now, the first hint of thyroid hormone receptor function came about over 10 years ago when Dr. Yoshio Yaoita and Don Brown cloned the thyroid hormone receptor in (*Xenopus laevis*) and analyzed their expression. What they found is that the thyroid hormone receptor α genes, there are two α genes and two β genes in (*Xenopus laevis*) because (*Xenopus*) is a pseudo-tetraploid organism, the α genes are activated shortly after tadpole hatching at stage 35, 36.

The level of the mRNA is high when tadpole feeding begins at stage 45, and it remains high throughout development until the end of metamorphosis. In contrast, the TR β genes are not expressed or expressed at very low levels until metamorphosis, when endogenous thyroid hormone T3 or T4 levels rise; in fact, TR β genes are direct thyroid hormone response genes.

Now when the RXR genes were cloned, we analyzed that the RXR gene expression. Shown here is the expression or mRNA levels for the RXR α . As you can see, like TR α , it is highly expressed shortly after hatching and by the time tadpole feeding begins, and it stays high throughout development.

This kind of expression profile immediately raises the possibility that maybe the receptors have dual functions in development. That is, TR/RXR in the absence of ligand might form a complex that represses thyroid hormone response genes during pro-metamorphic tadpole growth period. That is, the unliganded receptors will allow the animals to grow until it is ready to metamorphose.

Then, during metamorphosis, when T3 or T4 are present, the receptor becomes liganded, and those same genes that were repressed here, would become activated, therefore allowing the animal to transform into an adult frog. So the question is: can we test this dual function?

What we did was actually to make use of this window period, the first several days of development. In (*Xenopus laevis*), the development from here (fertilization) to (stage) 35 is two days, (and) to (stage) 45 is about one week. So, we want to ask the question, what happens if we introduce the receptor during this window period? Can we reproduce the molecular pathway that normally occurs much later?

The way we do this is actually fairly easy. We take the fertilized egg at stage 1 and we simply microinject mRNAs encoding TR or RXR or both into the fertilized egg and then allow the animals to develop in either the absence or presence of thyroid hormone.

What we found here is, when you do not add TR/RXR and in the absence of thyroid hormone, the control animals that develop normally. When you give thyroid hormone alone, it does not matter in the first several days, (as shown here) for two-day old embryos. (This) is because there is no receptor in the system.

When you give low levels of the overexpression of TR/RXR together, without hormone the animals are normal; with hormone they are abnormal. When you add high levels of TR/RXR to the animals, what you see is that without hormone you get abnormality as well. You know that the morphology here is quite different from here.

That is consistent with the idea that unliganded receptors are transcription repressors, whereas ligand bound receptors are transcription activators. When you give high levels of the receptor and hormone, you see most embryos died during development, indicating that too much hormone (and) too much receptor at the wrong stages will affect development.

More importantly, when we use this kind of assay as well as molecular analysis of the thyroid hormone response genes, we found that RXR is critical for TR function in developing animals. That is, *in vivo* TR will not function optimally without RXR. And we found TR/RXR heterodimers can repress endogenous thyroid hormone response genes in the absence of ligand.

Finally, in the presence of ligand, TR/RXR can activate those same endogenous thyroid hormone response genes. So this is actually the overexpression analysis of early embryos. The next question we will ask is; can we actually demonstrate that thyroid hormone receptor is indeed important for amphibian metamorphosis? That is, can we show the receptor function much later (in development).

The way we did this was to use the transgenic method that Dr. Yoshizato mentioned earlier, and I will not go into detail, but suffice to say that here are five (*Xenopus laevis*) tadpoles that have different levels of transgene expression. That is, we overexpress dominant negative TR at different levels, and this is the lowest and in fact there is no receptor detectable, and this is the highest dominant negative (overexpression).

What we found is that when we take these animals and add thyroid hormone for a week, you can see that without thyroid hormone this animal is induced to undergo metamorphosis. You can see the morphology of the mouth to become pointy instead of square shape that is without thyroid hormone or in the presence of highest levels of dominant negative receptor.

We found that morphological change is inversely correlated with levels of overexpression of the dominant negative, and more importantly, when we analyzed the thyroid hormone response gene expression, we found that when you express high levels of a dominant negative thyroid hormone receptor, the endogenous thyroid hormone response genes will not be induced by the hormone. This kind of analysis demonstrates that indeed thyroid hormone receptor is important for amphibian metamorphosis both at a morphological and a molecular level.

The next question then is; how does it work? Based on the *in vitro* studies and the tissue culture cell studies from many laboratories, we would like to hypothesize, or we predict that for thyroid hormone response genes, that is those genes that are important for metamorphosis, they would be expressed first during embryogenesis at a low basal level, because there is no receptor and no hormone.

During pre-metamorphic stages, we would predict that TR/RXR would be expressed, and those receptors then should be bound to the target site even though there is no thyroid hormone. This binding then should be repressing transcription. To demonstrate that indeed the receptor is capable of binding to the target in tadpoles, we made use of a relatively new method, which is a few years old, the so-called chromatin immunoprecipitation assay, or ChIP assay.

Suffice it to say that, what we do is (to) isolate the nuclei from animal tissues or from whole embryos. Then, we crosslink DNA to protein and then sonicate them to small pieces. Then, we use antibody against thyroid hormone receptor, or any protein we are interested in, to immunoprecipitate receptor protein. If the receptor is bound to the target *in vivo*, the DNA should be coming down with the antibody and receptor. Then we can purify the DNA and analyze what genes are bound by the receptor.

When we carried out such an assay, what we found is using antibody against TR or RXR and we analyzed two promoters, the TR β gene promoter and the TH/bZip gene promoter, both genes containing thyroid hormone response element and we analyzed the thyroid hormone binding region of the promoter.

What we found is that in embryos at stage 20, for both TR and RXR there is very little binding, and when you look at the tadpoles at stage 47 when TR α and RXR α are highly expressed, what we see is TR is bound to both promoters and you also see RXR binding to the promoters. Having shown the receptor is bound, we then asked how it represses transcription.

Again, borrowing studies from mammals and tissue culture cells and *in vitro*, we predicted that corepressor complexes would be recruited. One of the best-studied corepressor complexes is the so-called N-CoR corepressor complex. N-CoR complexes are known to be histone deacetylase complexes. We cloned (*Xenopus laevis*) N-CoR, and the question we asked is (whether) N-CoR is recruited by TR/RXR to target in these pre-metamorphic tadpoles.

The result is yes, indeed. When we use ChIP assay using antibody against (*Xenopus laevis*) N-CoR both in the tail and intestine, in tadpoles at stage 45-55, what you see is that in the control, in the tail you see both promoters are bound by N-CoR, and when you add thyroid hormone, the corepressor complex is supposed to come off, and indeed we found that corepressor complexes are released in both the tail and intestine.

As I mentioned earlier, corepressor complexes contain histone deacetylase. Therefore, we would predict when we add thyroid hormone to tadpoles, the corepressor complex should come off, and therefore deacetylase would be removed. The consequences would be that histone acetylation level at the promoter would be increased.

Again, we used ChIP assay by using antibody against acetylated histone H4 to analyze the acetylation level at the promoter. What you see here is that in both TR β and TH/bZip promoters, the acetylation level is increased with increasing dose of thyroid hormone treatment.

As a positive control, we used a drug trichostatin A (TSA), which is a non-specific inhibitor of histone deacetylase, it was first discovered by a Japanese group. What you see is that TSA treatment of

tadpoles induces acetylation at both promoters. As a negative control we analyzed the intestinal fatty acid binding protein gene promoter. This promoter we know is not regulated by thyroid hormone. What we found is that the acetylation level remains unchanged by thyroid hormone treatment, even though TSA induces higher levels of acetylation.

Based on these kinds of studies, we would then reason that histone acetylation plays a role in transcription regulation of thyroid hormone response genes. That is, when we increase histone acetylation levels, the transcription from the promoters should go up. Since trichostatin A can induce acetylation levels artificially, we then asked the question. If we treat tadpoles with trichostatin A, can we prematurely induce thyroid hormone response genes just like T3?

The answer is, indeed, yes. Again, we analyzed the intestine, but for simplicity I should point out for completeness, that if you are analyzing whole animals, the regulation is more complex. I will not go into the details for time's sake.

If you look at the expression by RT-PCR of the TR β gene and TH/bZip genes, in the control animals, there is very little expression. When you treat the tadpoles with thyroid hormone T3, you see both genes are induced. Interestingly and consistent with our prediction, when you treat tadpoles with trichostatin A, which will induce artificial histone acetylation, you see that both genes are also induced.

From this result and what we predicted, since trichostatin A seems to behave like a thyroid hormone by inducing transcription of thyroid hormone response genes, can we then induce metamorphosis with this drug instead of T3?

When we carried out such an experiment, surprisingly we found just the opposite: that is, TSA actually blocks metamorphosis. As you can see here, if you look at natural metamorphosis, at day zero these are pre-metamorphic tadpoles. If you allow the animal to grow for 10 more days, you see they change to a small froglet, with no tail and with fore and hind limbs.

When you add TSA, trichostatin A, day zero is fine, but at day 10 there is no morphological change, there is very little change that has occurred. We can also carry out this kind of analysis in thyroid hormone induced metamorphosis.

As we can see here, if you take control animals, no treatment, and you add thyroid hormone for about five days, you will see that TSA will induce the limb formation and the remodeling of the body and head region. But if you add TSA together with T3, you see all the morphological changes are blocked.

This was somewhat surprising, so we went back to investigate and make sure that TSA does not block thyroid hormone response genes. What you see here is that in control animals TR β is not regulated by thyroid hormone; there is no change under any condition. TR β is known to be induced by thyroid hormone. This is a control, what we see is that T3 induced TR β , TSA as I mentioned earlier also induces TR β gene.

If you add T3 and TSA together, there is no inhibition of the induction (by T3). If you look at several other thyroid hormone inducible genes, you get the same result. That is, TSA does not inhibit thyroid hormone induced transcription of thyroid hormone response genes.

Then the question is; how does it block metamorphosis? We then analyzed genes which are much further downstream. As I mentioned earlier, thyroid hormone induces a gene regulation cascade. You have early genes and then you have late genes. The late genes are indirectly regulated by thyroid hormone. We analyzed first two genes, which are down-regulated by thyroid hormone.

That is, if you look at IFABP at day 2, intestinal fatty acid binding protein gene, there is no change under any kind of treatment. But after three days of treatment, T3 will induce the repression of this gene indirectly, and TSA does not do anything. But if you add TSA with T3 together, you see this down-regulation is prevented.

The sodium phosphate cotransporter gene, however, is first a thyroid hormone inducible gene, an early gene. As you can see here, T3 induces it after the first one or two days. But if you treat an animal for a longer period, this gene is then repressed by thyroid hormone. As you can see here after the 3-day treatment this gene is repressed, whereas in the control animal there is no induction and no repression, so T3 treatment after 3-day represses (it).

Now, if you add TSA, TSA first behaves like T3 to induce this gene because it blocks histone deacetylase. And then after three days, you see this gene (is) still expressed. However, if you add T3 and TSA together, it does not prevent the induction, but it prevents the repression. That is, the downstream late events are blocked by TSA.

In addition, we analyzed two genes Sin3 and Rpd3, (which is a histone deacetylase). Both genes are induced by thyroid hormone after several days' treatment; they are late induced genes. What we see here is that in TSA plus T3, TSA blocks the induction. So this result suggests that histone deacetylase complexes are also employed by the animal at a late stage of metamorphosis for adult organ development.

I would like to conclude my talk on the receptor function by pointing out that again thyroid hormone response genes are normally expressed at a basal level, and many of those thyroid hormone response genes are actually genes involved in morphogenesis and organogenesis. So, their initial expression, although at a basal level, is important for embryogenesis to allow the organs of the tadpole to develop.

Once the tadpole has developed, those genes are no longer needed, so they have to be turned off. To turn it off, the animal uses the unliganded receptor as a developmental switch. I believe this is important because those same genes will be re-used again during metamorphosis for adult organ development. Therefore, you cannot permanently shut off those genes. You have to use a developmental switch, and unliganded receptor functions as the switch.

So the receptor binds to the target in pre-metamorphic tadpoles and this will repress those genes, allowing the animals to grow. Without this growth period, the animals will not develop properly. Then when thyroid hormone comes up, the corepressor complexes go off and the coactivator or histone acetyltransferase complexes are recruited, and this then will activate those genes to allow metamorphosis to proceed.

However, this activation is only the first step. There are many more genes that have to be regulated downstream, and those regulations also require deacetylase complex at the downstream events.

Now, this kind of dual function model may not be unique to amphibians. In fact, I borrowed this panel from a review by Dr. J. R. Tata a few years ago that shows that if you look at thyroid hormone concentration in the human fetal plasma, what we found was a pattern that mimics what we found in amphibians.

You see that during metamorphosis the thyroid hormone level peaks. But if you look at the thyroid hormone level in human fetuses, in the first five months of pregnancy and fetal development, there is very little or no detectable thyroid hormone, and this period might be similar to the pre-metamorphic tadpole period where the receptor functions as a repressor.

Then, during metamorphosis or around birth, thyroid hormone level rises. I will not go into the details, but there are many similarities in the developmental process between mammalian development and metamorphosis. I think whatever we find from amphibian studies may at least to some extent be applicable to mammalian development, any drugs or endocrine disruptors that alter hormone levels either here or later in amphibians or mammals should have similar effects in terms of altering development.

Finally, I would like to acknowledge all the people involved in the work. The early embryo injection studies were done by former post-docs Monika Puzianowska-Kuznicki and Sash Damjanovski. The ChIP assay and TSA study were done almost exclusively by former post-doc Laurent Sachs, and the cloning of N-CoR was done by Laurent Sachs and Peter Jones. The transgenic work, I showed you only one slide

was done by Dan Buchholz, and there are two other post-docs involved in some of the co-activator and co-repressor studies right now. Thank you.

Q&A

Yoshizato: We have some time for discussions; please feel free to have questions or discussions for Dr. Shi.

Q: Did you try pulse treatment with TSA?

Shi: Pulse treatment? No, we have not.

Q: With ChIP assay, you studied TR β (and TH/bZip) promoters. Did you have any other genes with this ChIP assay?

Shi: At the time we tried, there were really only two promoters, TH/bZip and TR β , and both behaved the same. I should point out that we have now found a TRE in the stromelysin-3 promoter. Laurent (Sachs) now is back in France with Barbara (Demeneix), and I think Barbara might know more about it. She is trying to analyze the TR binding to the stromelysin-3 gene. But I do not know yet.

Q: You showed the expression of the thyroid hormone receptor. Actually, which part were you talking about?

Shi: The expression profile, the chart I showed you at the very beginning, those were published by Dr. Yaoita and Don Brown. Those were whole animal studies. Now if you look at the messenger level in the individual organs as Dr. Yaoita published, they do vary. It depends on which organ you are talking about. However, in general they correlated quite well. During metamorphosis the messenger does go up; however, the expression is present even in pre-metamorphic animals, although the level might vary.

Q: So you are saying that there is basically no difference between the tissues in terms of the regulation of thyroid hormone receptor and expression.

Shi: There are. Different tissues do have different levels.

Q: But different regulations?

Shi: In terms of the induction of TR gene, TR β gene is activated by thyroid hormone in every single organ that we and others have analyzed. In fact, that is one of the advantages of using amphibians as a model to study mechanisms *in vivo*, because the whole animal is like a tissue culture cell, because every cell has a receptor. So you can do ChIP assay or any assay you want.

Q: You mentioned that some of the genes have been turned on in the beginning and three days later get shut off, and you implied that in the absence of hormone, the response will shut down; but remember T3 is still there, so how do you envision how it shuts down itself?

Shi: Many genes including TR β , stromelysin-3, or TH/bZip and this is in the intestine, in the presence of T3 after about three or four days, the messenger gets repressed again. We think actually that repression is reflective of a more permanent change in the cell type and is not receptor mediated. It is partially due to either the activation or repression of the repressor genes or other activators. As you know, each gene or each promoter often involve multiple transcription factors. So, it is possible that some other transcription factor has been altered, which leads to the repression. I should point out that in the case of sodium phosphate cotransporter as one example, just to point out one potential mechanism, sodium phosphate cotransporter is expressed exclusively in the epithelial cells of the intestine, and after three days the larval epithelial cells undergo apoptosis. The gene gets turned off because the cells are dying. So this is one potential mechanism. The gene is actually reactivated later on when adult epithelial cells differentiate, and that may or

may not depend on thyroid hormone. It may involve a different mechanism.

Q: Did you know that any coactivator in the gene using a ChIP assay? Is any one recruited?

Shi: We actually have not done the ChIP assay with co-activators. We have cloned or made antibodies against two coactivators, one is the SRC-3 and the other one is p300. However, we

have not done the ChIP assays. We have analyzed the expression of both of them. They are expressed during metamorphosis, and SRC-3, not SRC-2, is actually induced by thyroid hormone, but it is a delayed response after three days. SRC-2 has no change, and SRC-1 is not yet cloned in (*Xenopus laevis*.)

Yoshizato: Thank you Dr. Shi for a nice presentation.