Somatic and Germinal Transgenesis for Assessing Thyroid Hormone Disrupting Activity in Amphibians

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Thank you. First of all I would like to thank the organizers for inviting me to present the work of my team and giving me the opportunity to visit Japan and the inspiring city of Hiroshima once more. My talk today is entitled "Somatic and Germinal Transgenesis for Assessing Thyroid Hormone Disrupting Activity in Amphibians.

I will try to go a bit fast through my introduction, being as we have had such excellent introductions on the mechanism of thyroid action in amphibians both by Prof. Yoshizato and by Yun-Bo Shi and again an introduction to somatic gene transfer by Dr. Yaoita, so hopefully some of the concepts I am going to present are already quite familiar to you.

In my lab over the last 10 years we have been developing somatic gene transfer methods to look at gene regulation during amphibian metamorphosis. More recently we have introduced the technique of germinal transgenesis that was developed by Drs Kroll and Amaya that other people have already referred to.

This work was carried out in the Natural History Museum in Paris, and in the short time that I have available I would like to address five questions very rapidly. First of all, I want to define the problem. Then, I will try and describe why we need to use a gene reporter test *in vivo*, and if we are doing so whether we need to use somatic or germinal transgenesis. Then, I would like to quickly address the question of which (*Xenopus*) species we should be looking to use, and which sort of genetic constructs and testing protocols would be optimal for looking at thyroid hormone disrupting activity.

Now, if we are talking about thyroid hormone disrupting activity, we are talking about setting up tests to define whether a xenobiotic can either block mimic, or modulate thyroid hormone activity. If we are talking about thyroid hormone activity as physiologists we can either think as most of us are today, in terms of tadpole metamorphosis in (*Xenopus*), which previous speakers have well defined as being the archetypical thyroid hormone dependent developmental process. Jacques Leloup from the Natural History Museum in Paris showed this process to be orchestrated by tri-iodothyronine by many years ago.

But also, we must also remember that without a minimum of thyroid hormone at the right time, not only will a tadpole fail to become a frog, but also a human baby becomes a cretin, and luckily today we see very few examples of this once unfortunately common disease. If we are trying to define whether a chemical is affecting either of these developmental systems, whether it be mammalian or amphibian, I would argue that we need to use an *in vivo* reporter gene test.

Even though we know today that many thyroid hormone disrupters will interfere at the level of the transport of thyroid hormone in the serum, it could be argued that wherever the level of disruption occurs, at some point, one is going to pick up these changes in free T4 or free T3 that might be caused by changes in equilibrium at the level of transporters.

Or, if there are more direct actions, one is always going to see the effect at the level of the transcription of target genes, either with a decrease or with an increase in transcriptional activity that will be played at the level of these thyroid hormone response elements that the three previous speakers have referred to.

I would say that using a transgenic animal with fluorescent proteins as the reporter gene will provide us with all the characteristics that one can expect of an *in vitro* system, sensitivity, specificity, low cost, requirements of only small amounts of material, and the possibility of going through the high-throughput database screening methods, with *in vivo* advantages such as the way that one can physiologically account for absorption, distribution, metabolism, excretion of the substance. In fact, they have better defined tests *in vivo*,

but if we are using a transgenic with fluorescent proteins, we get a comprehensive study of all of these effects with all the advantages of an *in vitro* system.

Now, if we are going to go for an *in vivo* system, should we be looking at a somatic or a germinal transgenic method. As I mentioned, in our laboratory we have recently applied the technique of Enrique Amaya and we have used this technique to study apoptosis in the central nervous system.

We overexpressed the same protein that Dr. Yaoita referred to, Bcl-2. We either expressed it in the whole of the animal, using a promoter that is expressed in all of the cells of the animal, or we used a promoter that limits expression to the central nervous system, as seen here, in the brain or the caudal nerves in the tail. So one should be able to use this type of approach to get on-off signals for tissue specific or hormone specific responses with this technique.

But a more simple technique is to use somatic gene transfer, whereby one actually injects into the tissue one wants to study the genes that one wants to look at and its regulation, and thus one can get temporally and spatially defined expression, either in the muscle or in the nervous system.

And according to the type of gene construct one uses, one can either analyze the promoter and its regulation to look at transcriptional regulation and here we have a reporter gene such as GFP which I have just shown you that would light up when there is transcription and not be seen when there is no transcription or another approach would be to use a strong promoter to force the expression of a gene or a protein that we want to study, and this would allow us to look at the function of the protein within a given cell or tissue.

To do this, we can inject naked DNA right into the tail, as Dr. Yaoita has just told you, or we can vectorize the DNA into the brain. Very quickly just a few pictures, beta-galactose expression following gene transfer in the tail, or GFP and a close-up view in the tail, and these are some brains of (*Xenopus*) showing different cells of the nervous system expressing GFP and this is red FP in cells surrounding the ventricle of the brain of the (*Xenopus*). If we can do that in (*Xenopus laevis)*, can we actually apply it to the more sensitive (*Xenopus tropicalis*)? The answer is yes.

In white we have the levels found in (*laevis*), and in pink the levels we get in (*tropicalis*). When we injected luciferase, a luciferase reporter gene into the brain, we get equivalent levels whether we are talking in relative light units or whether we normalize against mg of protein. Similarly in the tail we get equivalent levels in relative light units and slightly lower levels when we normalize in (*tropicalis*). But these are still very high levels of expression.

What would be the arguments for using somatic gene transfer? With this method, there is no need to maintain lots of different cell lines and it is a good way of pre-testing the functionality of constructs before one goes into germinal transgenesis.

But the limitation is that it is difficult to scale up beyond 100 tadpoles per experiment. In opposition, with germinal transgenesis, once the lines are established, you get lots and lots of animals, 1000s of embryos per batch, but the problem is often obtaining low background levels with no leakage, though Dr. Yoshizato showed us some nice examples of animals in which there was absolutely no leakage.

Now to the question as to which species is better? I have just shown you that it does work, that somatic gene transfer can be used in both (*Xenopus laevis*) and (*tropicalis*). There is not to this date very much information coming out on germinal transgenesis in (*tropicalis*), though we know it works.

What I did not mention is that in (*tropicalis*), when you are doing somatic gene transfer, you actually have to anesthetize the animals whereas with (*laevis*) you do not. But there is one nice advantage of (*tropicalis*), that it does have the advantage of a more regular and faster metamorphosis kinetics.

Just briefly to sum up the data: (*X. tropicalis*) goes from this stage 58, the beginning of metamorphosis, to full metamorphosis in exactly 9.5 days, whereas (*Xenopus laevis*) is highly variable, and in our laboratory conditions it can vary from 13.5-18 days. So if you are using a metamorphic test to look at whether a xenobiotic actually disrupts metamorphosis, the very regular, precise and rapid kinetics of (*X. tropicali*) will

certainly be an advantage.

On the left here we have got the advantages of (*X. tropicalis*): it is diploid, so it would be better for comparing results if you are going to use DNA arrays and other aspects of functional genomics. It has also got a shorter time for reproduction, and with its smaller size makes it very useful for problems of housing.

But, to date, we do not have much information as to its use in germinal transgenesis, and it is definitely a more fragile species, whereas (*X. laevis*) is much more robust, it has a larger egg size, and it is easier for transgenesis, but with (*X. leavis)* there is longer time to maturation and the pseudo-tetraploid means that it is more difficult to combine with functional genomic approaches.

Finally, which constructs and testing protocols can be used to look for thyroid hormone disruptions with these two species. If we are looking for a protocol where we can see the activity of a thyroid-like substance, we need a short-term transcriptional test, and to do so, we are going to have to block endogenous thyroid hormone production.

If we are looking at tadpoles, thyroid hormone production is going to start at very low levels, between stages 44-50. The high levels that we saw in the classical chart start about 58, but tadpoles are going to be competent; their receptors are present as Yun-Bo showed us from stage 44 on.

But we must make sure that there is no endogenous hormone, so we have to use sodium perchlorate to block production of thyroid hormone. But this will have the disadvantage that we will also reduce the T3-dependent induction of the receptors that we are going to need to see the response.

So, we have been testing different protocols to see how we could block endogenous production and yet still apply T3 and get a significant response. If we just block tadpoles from stage 55 on with perchlorate and then a month later inject them by somatic transgenesis with a T3 reporter gene, we are going to need five days of treatment to see a T3 response, and it is not that large.

What we have been doing is giving animals a very, very low dose of T3, 10-13 and a short pulse, rinsing it out over several days, and then we can see that within 48 hours 10-8 T3 will give us a much better, a more robust response to thyroid hormone, and this reduces the time of testing if you have got a potential thyroid hormone disrupter to 48 hours, rather than the full 5 days, and you have got no interference from endogenous hormones. We have done this test now with T4, T3, and also with a thyroid analog TRIAC in. In all cases you get a three- to four-fold increase over basal levels of transcription.

Similarly, one can actually in some cases, this is in the brain, injection into the brain, a myc-luciferase construct, c-myc being a thyroid hormone responsive gene, but in this time we have inhibition of transcription. I would propose that an ideal protocol would include no doubt two such constructs. One perhaps linked to red fluorescent protein and the other one to green fluorescent protein, and we would have a double test to insure that you are seeing a genuine thyroid hormone-like activity. What is more, you can actually get dose response curves. This is in the muscle following transfection with aTH/b Zip reporter construct. You get good dose response curves with this type of pretreatment protocol.

So in conclusion, I would say that if you use a gene reporter test *in vivo*, you are going to combine the advantages of both *in vivo* and *in vitro* systems. As to the question of somatic or germinal transgenesis, I would argue that both are useful, but somatic is particularly useful for pre-testing constructs in the protocols but that germinal allows scaling up. So I am not saying that one is better than the other.

As to which species, (*tropicalis*) or (*laevis*), both are amenable to somatic and germinal transgenesis, but for lineage establishment, genomics, and kinetics of metamorphosis, (*Xenopus tropicalis*) has the most advantages, and it could be a challenge to get these lines set up.

As for constructs, I have shown you that both positive and negative regulatory elements can be used in these tests, and I would further insist that it would be useful to include them together in the same animal.

I did not have the time to go into this, but I think that for good germinal transgenesis we will need insulators to protect the transgenes from interference from genes next to them once they are integrated into the

chromatin, and that one can use luciferase and or fluorescent proteins.

As to which testing protocol is optimal, I think it would be interesting to combine positive and negative elements within the same protocol. If you are working with competent tadpoles, for agonists you have to render them competent if you have given them perchlorate by giving them a pulse treatment, but for antagonists one could work with pre-competent tadpoles.

Finally, I would like to thank my collaborators, Laurent Sachs who set up the somatic gene transfer technique in (*Xenopus*) before he went on to do his post-doc with Yon-Bo Shi. Laurent Coen who set up the germinal transgenesis in the lab, and then Nathalie Turque, Caroline Alliot, Karima Palmier, and Sebastien Le Mevet who were doing the work on the protocols and the constructs for endocrine disruption, and the Natural History Museum and the CNRS who supported our work. Thank you very much for your attention.

Q&A

Yoshizato: We have some time for discussions. Please.

Q: The thyroid hormone response element also responds to quite a few other members of the nuclear receptor superfamily such as some orphan receptors. How are you going to eliminate disrupter effects through such mechanisms? Whatever chemical you use is going to induce the thyroid hormone response or induce the other receptors response.

Demeneix: If I understand you correctly you are suggesting that the thyroid hormone response element could also be responding to other receptors?

Q That is correct.

Demeneix: Then the most elegant way would be to use the GAL4 system with the UAS response element. That would be certainly the system. I think one could also say that using this type of straightforward response element and combining it with a metamorphic assay to see the speed at which the animals actually go through metamorphosis would give you a physiological argument to support the fact that the regulations you see played out at the level of the TRE are in fact played out at the level of other thyroid response genes as well. But that is a very important point.

Yoshizato: Thank you for your nice presentation.