Thyroid Hormones: Multiple Roles through Multiple Receptors

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I would like first to present my deep appreciation to the organizers of this meeting, which gives me the opportunity to present to you some data and also to listen to quite interesting presentations yesterday and today.

Dr. Seo introduced the diversity of thyroid hormone receptors, and I want to emphasize that mostly in the mammals each of the two genes, TR α and TR β, are including multiple isoforms of the receptors, and we have been interested for several years now to dissect the role of these receptors by making knockout approaches.

What I would like to do today is first to show you a summary of what we learned from these knockout studies, and in the second part of my talk I will show you how some of these receptors might have a dual function *in vivo* according to the presence or absence of thyroid hormone in the mouse. Knockout of most of the isoforms were performed mostly by three laboratories in the world, so the lab of Bjorn Vennström at the Karolinska Institute (Sweden), of Douglas Forrest at New York, and our lab.

The two major conclusions which came out from these studies are first that neither TR α and TR β are not necessary for embryonic and post-embryonic development which means that for the mice with a double knockout, these mice can be viable and can reach the adult state. I should not say that they are in good shape, but they are alive at least in the laboratory environment.

The second lesson of that is that both types of receptors have different roles in the development, which strongly suggests that early on after the divergence of these two receptors, they acquire very specific functions.

If we just summarize what might be the involvement of each receptor in the development and physiology of the mouse, we can say that TR α is mostly involved in body growth, in bone maturation, so animals with knockout of TR α show a strong delay in bone maturation and they have a defect in B cell development.

But also I should add they have some defect in the spleen, hematopoiesis, soon after birth, they have strong alteration in the development of intestine, they have a decrease in the basal heart rate, and also they have some alteration in the glucose metabolism and interestingly these animals show some diabetic phenotype.

Animals which are devoid of TR β show some alteration in the development of auditory functions, also in the development of color visions, they do not respond to thyroid hormone in terms of heart stimulation and also they have altered cholesterol metabolism.

There are some functions, which are altered only in the double knockout, and this is the production of growth hormone in the pituitary, which is abrogated only in the double knockout and there is a strong deregulation of body temperature. Also, the double knockout animals show some strong alteration in the central nervous system, mostly the development of the cerebellum during the postnatal period with an alteration in the development of the external granular layer of the cerebellum which provides mature neurons.

Also, they are deficient in oligodendrocyte differentiation in the optic nerve, which could lead to some degeneration of the retina. Also, they have a strong alteration in the regulation of the production of TSH. TR β, and especially TR β-2, is the receptor which is mostly involved in the control of TSH production. But from the double knockout animals, we also have to conclude that TR α might also work in connection with TR β in this feedback regulatory process.

As was mentioned earlier, TRs are controlling basal transcription of target genes in two ways; in the

presence of thyroid hormone the liganded receptors work as strong activators of these genes, and in the absence of thyroid hormone, the so-called aporeceptors are working as repressors. So, clearly these receptors are reversible switches against target genes.

This repressive effect has been highly documented in *in vitro* studies in mammals and in transfections on cells, and we were interested to know whether it has some physiological meaning in the development of the mouse. To address this question, we developed a genetic approach to understand what might be the function of this aporeceptor *in vivo*.

For that purpose we use a model of mouse which is congenitally hypothyroid, and this is the pax8-/- model. The pax8 gene encodes a thyroid specific transcription factor and mutation in humans have been shown to lead to some congenial hypothyroidism.

Pax8 knockout mice were developed by the group of Peter Gruss, and these mice show no development of the follicular cells in the thyroid, so as a consequence of that they have only hypothyroid and they usually die at weaning time, but the mice can be rescued by injection of T4 soon after birth.

This hypothyroid model is a quite interesting because you do not need to inject any chemical and also the mice have been suffering hypothyroidism during all their lives starting at the birth time.

We were interested in comparing the phenotypes of these congenital hypothyroid mice, which do not produce any thyroid hormone but which still produce the receptors, with that of double knockout mice which have no receptor but which still produce the hormone. For that comparison we looked at three different tissues which are strongly affected by hormone treatment: bone, spleen, and intestine.

First, if we look at the bone development, this is the wild-type and we can concentrate on this panel here. If you look at the congenital hypothyroid mice, you can see there is a strong alteration in ossification. For example, here at the knee joint where you do not have any sign of ossification as compared to the wild-type. If you look now at the double receptor knockout, you can see that you have an intermediate situation, with delayed but some remnant ossification.

If we look at spleen development as you can see in the pax8 mutant, the spleen is almost completely aplastic and if you make a smear from these tissues you will not see any hematopoietic cells. Here again you can see that the double receptor mutant shows an intermediate phenotype and there is some hematopoiesis in this spleen, but it is strongly reduced as compared to the wild-type, but it is not so drastic as in the congenital hypothyroid animal.

Intestine: these are sections of the intestine, and we show here the dividing cells within the crypt at the base of the villi, and the dividing cells are stained with a specific antibody. As you can see, in these pax8 mutants the number of dividing cells has been strongly reduced, and if you compare with the situation in the double receptor knockout, here again you can see that this double receptor knockout has an intermediate phenotype between the wild-type and the congenital hypothyroid mutant.

I want to just show here that if you inject thyroid hormone in these mutants, 48 hours after injection you can rescue the proliferation of these progenitor cells in the crypt.

This is just a numbering of the cells, a quantification of what I just showed. If you just concentrate on this panel, here you have the number of proliferating cells in the wild-type. As you can see there is a strong decrease in the hypothyroid mutant. Injection of thyroid hormone can restore some proliferation. Here you have the double receptor mutant, which shows that this is an intermediate phenotype between the hypothyroid mutant and the wild-type.

All these data show that the complete lack of thyroid hormone is much more deleterious than the total absence of thyroid hormone receptors. How could we explain that?

According to the aporeceptor model, we could propose that in the absence of thyroid hormone receptor, the target genes still have some basal transcription level, which would lead to basal expression of some essential genes and then to the development of a mild phenotype.

In contrast, in the absence of thyroid hormone, the receptor would work as aporeceptors and would strongly repress the transcription of target genes, leading to the complete suppression of essential gene expression and then to a very strong phenotype.

If this model is valid, we should be able to rescue partly the phenotype if we abrogate the expression of the receptors in this condition. Therefore, we introduce the deletion of each of the receptors within the pax8-/- genetic background, and what we expect is to restore some basal transcription of essential genes and then to rescue some mild phenotypes.

Let us look first at the survival of the compound animals. As I mentioned earlier, all of the pax8-/- animals die within the full first week after birth, in contrast to the double receptor knockout, which are all alive. If we make a transient treatment with thyroid hormone to these hypothyroid mutants we can rescue nearly 50% of the animals, which can survive.

If in this background we introduce the deletion of the β receptor, we do not improve the viability of the animal. In contrast, if we introduce the deletion of the α receptor, as you can see we can recover strong viability of at least 60% of the animals. So clearly, the viability of this hypothyroid mutant can be rescued by deletion of the TRα gene.

If we look now to the phenotype of all the tissues, here again this is the aplastic spleen of the hypothyroid animals. If we introduce into these animals the deletion of β receptor, we do not see any improvement, but if we introduce the deletion of α, you can see we have a significant recovery of the hematopoietic function.

Bone: here again absence of ossification in these pax8 mutants. If we introduce the β deletion, there is no improvement. If now we introduce the deletion of α receptor, we have a strong recovery in bone formation.

Intestine: just concentrate on this panel here. This is the number of proliferating cells in the intestine crypts. This is the hypothyroid mutant. If we introduce the deletion of the β receptor, there is no significant improvement in terms of proliferation of the cells. If we introduce the deletion of the α receptor, we have a strong recovery in the number of proliferating cells.

All these data show that if we inactivate the TRα gene but not the TRβ gene we induce a strong recovery of essential function in this congenital hypothyroid genetic background. What might be the molecular basis for that?

Of course, one explanation is that the TR aporeceptors might abrogate expression of essential genes in the hypothyroid mutant. To address this question, we looked at some genes which might be targets of the TRα receptor.

Unfortunately, we do not know yet of any such target genes in all three tissues that I mentioned. So we addressed another gene, which is the HCN2 gene, which is encoded specifically in the heart, and which has been shown recently by the group of Wolfgang Dillmann to be a gene specifically regulated by TRα. So this gene encodes a protein which is a component of the pacemaker machinery.

We looked at the expression of the gene in the heart by quantifying the mRNA. This is the level of mRNA in the wild-type. In the double receptor knockout, there is not much difference, and presumably this level represents some basal level in the steady state condition of the animals.

In contrast in the pax8 hypothyroid mutant, there is an almost complete abrogation of the expression of this gene. If we inject this animal with thyroid hormone, within 48 hours there is a strong activation of expression of this gene.

If in this background we introduce the deletion of the β receptor, there is no restoration of gene expression. If now we introduce the deletion of α, there is a significant recovery of some basal level of expression of the gene. Clearly this strongly suggests that the TRα aporeceptors could work as transcription repressors on some essential genes *in vivo*.

If we summarize all this, what we observe is that during the early period of development after birth, the pax8 congenital hypothyroid mutants are all dying at weaning time. If we make transient injection of thyroid hormone during this period, we can induce the recovery of these animals. If in these animals we delete the TRβ genes we do not bring any improvement. In contrast, if we delete the α receptor in these animals we can induce their survival.

Clearly, this demonstrates that in this congenital hypothyroid environment, it is the α receptors that are strongly deleterious, which strongly suggests that during this period the aporeceptor is blocking expression of genes which are essential for the neonatal development.

It is interesting to notice that during normal development of the mouse there is a strong peak of circulating thyroid hormone during this postnatal period and presumably what is the function of this circulating hormone is to turn this aporeceptor into an active receptor which now would be able to induce expression of genes in many organs which should develop during this period, so namely bone, intestine, hematopoiesis, but presumably also central nervous system.

What we would propose is that TRα is the master switch for turning on the expression of specific genes which are involved in post natal development. The question now is; what are these genes that are turned on by thyroid hormone during this early postnatal period?

To address this question we set up these experimental plans in collaboration with the group of Paul Meltzer and Paul Yen at NIH. We used all the mutant mice which were submitted to different treatment, either euthyroid, hyperthyroid, PTU, PTU+T3. Tissues were isolated from these mice and were screened for gene expression using a microarray approach.

Just some preliminary data show that for example in the liver at least 60 genes are activated during this early period and almost 30 genes are repressed. In the cerebellum, for example, here are a series of genes which have been activated. Interestingly, one, cyclin D2, is known to play an essential role in the maturation of neurons, and also a series of genes is specifically repressed in these tissues.

What we want to do now from this model is to set up a complete pattern of gene expression in almost all tissues of the animals during this early period after birth in response to T3 and also to try to identify the genes which are a specific targets for TRα and TRβ.

The take home message is that what we show here is that the apo-TRα work as repressors *in vivo* in the mouse and TRα is the master switch in activating T3-mediated developmental process during the neonatal period.

We would propose that TRα similarly to what has been described in the frog should control some metamorphosis-like process in mammals, and I think, we should not be ashamed to say that presumably we have undergone some metamorphosis process a few years ago.

Also, because of this aporeceptor function we have now to take into account local concentration of T3 and presumably the availability or local delivery of T3 is one very important mechanism for regulation of gene expression, and presumably local concentrations of T3 are determinant for some developmental and physiological regulation in the tissues.

As a consequence also of this TR aporeceptor effect, this could explain why so far we have not yet found some dominant negative mutation of TRα in humans.

Clearly TRα and TRβ are playing different functions in the development of mammals. TRα is being switched from aporeceptor to holo receptor during the development. TRα is controlling the switch to post natal development and some metamorphosis in these tissues, whereas TRβ should be playing later on some much more specialized functions in various tissues.

These models also provide some different targets for endocrine disrupters. For example, at this level we might imagine some endocrine disrupter could either abrogate the binding of thyroid hormone to TRα and then keep TRα in an aporeceptor configuration.

Also, we might imagine that the binding of such chemicals, constitutive binding to TRα, might transform this receptor into a dominant negative receptor. Also, all these other mechanisms should be putative targets for disruption by some endocrine disrupters, either by overactivating the function of these receptors or abrogating the function of these receptors for these essential developmental processes.

To acknowledge the contribution of some people: the work I presented today was mostly performed by Frederic Flamant, Anne-Lise Poguet and two Japanese post docs in the lab, and also part of this work was developed in collaboration with the group of Prof. Hashizume at Shinshu University. Thank you for your attention.

Q&A

Seo: Thank you very much Dr. Samarut. Because of the time limitation, maybe we can accept only one question for discussion. Yes.

Q: Thyroid hormone receptor α and β have different roles, you said. But I think it is possible to explain a different role by organ specific expression. Maybe thyroid hormone receptor α is expressed everywhere, so knockout of thyroid hormone receptor α causes reduced effect. So expression pattern might explain your result. Is it possible?

Samarut: It might be possible for some tissues. But as you said, TR α is expressed almost everywhere. We have many tissues where both α and β are expressed simultaneously. So I think the coexpression of the two receptors does not explain everything.

Q How about protein? Did you check the protein level by Western blotting?

Samarut: No. We checked in terms of RNA. We have no experience ourselves in protein, because especially for TR α, we have no good antibodies for that , and the level of expression of the protein is presumably quite low.

Seo: Thank you very much.