Session 2 Frogs

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

Katsutoshi Yoshizato

Hiroshima University, Japan

Anuran larvae drastically remodel their tissues into the adult counterparts to meet their aquatic to terrestrial habitation change. This remodeling is called metamorphosis and triggered by thyroid hormone (TH). We have been engaged in the study to identify cells, and genes and proteins that play major roles in the remodeling process, focussing on the skin. We have also developed the study to reveal the action of TH on the changes in biological characteristics of the identified cells and the expression of these molecules, and the mechanism of its action. The larvae at the early developmental stage are covered with the homogenous skin (larval skin) over their entire body. At a specific stage of development that differs among anuran species the skin conversion center emerges in the trunk skin (hereafter, body skin) where the larval skin transforms into the pre-adult skin. The emergence of this specific center was histologically discernible as the site where the secondary connective tissues that develop to the dermis after the completion of metamorphosis was newly formed between the basement membrane and the collagen layer. The skin conversion center is the front boundary between the larval and pre-adult skin. The center spread over the body region as larvae develop, but not to the tail region.

This transformation proceeds requiring the epidermal-mesenchymal interaction. Some unknown mesenchymal factor induces the differentiation of larval basal epidermal cells into pre-adult basal cells. The pre-adult basal cells secrete PDGF-A that activates subepidermal PDGFR-α-expressing mesenchymal cells. The activated mesenchymal cells construct the secondary connective tissues. We developed an in vitro model of larval skin that is remodeled to the adult skin under the influence of TH. When either AG1296, a specific inhibitor of PDGFR tyrosine kinase, or an excess of recombinant proteins of a soluble extracellular domain of PDGFR-α was added to the model, TH failed to induce the proliferation and the terminal differentiation of adult basal cells, and the development of the secondary connective tissue. At the climax stage of metamorphosis the expression of FGF-10 mRNA was enhanced in the skin and the pre-adult basal cells strongly expressed FGFR2IIIb, which suggested that PDGF-activated mesenchymal cells secrete FGF and accelerate the differentiation of pre-adult basal cells into adult basal cells. Our currently available data strongly suggest that TH regulates the cellular and molecular events taking place in the center.

As described above TH not only triggers the metamorphic tissue remodeling of anurans, but also regulates its whole process thereafter. Thus, it is clear that anuran metamorphosis provides a unique and simple biological system to detect thyroid disruptors. When given substances influences the process of metamorphosis to some extent, stimulation or inhibition, these substances could be candidate thyroid disruptors. We identified as such a candidate, bisphenol. This substance suppressed dose-dependently the tail resorption.

Dual Function of Thyroid Hormone Receptor during Amphibian Development

Yun-Bo Shi
National Institute of Health, USA

Dual function of thyroid hormone receptor during amphibian development Thyroid hormone (T3) affects a wide variety of biological processes, from embryogenesis, postnatal development, to organ function and metabolism in adult. It is the causative agent of amphibian metamorphosis. T3 is believed to do so through T3 receptors (TRs). Earlier studies have led us to suggest a dual function model for the heterodimers TR and RXR (9-cis retinoic acid receptor) in frog development. That is, TR/RXR acts as a unliganded repressor in premetamorphic tadpoles to facilitate animal growth while functions as a transcriptional activator when bound by T3 later to promote metamorphic tissue transformations. We have undertaken several different yet complementary approaches to test this model and investigate the underlying mechanisms.

By introducing TRs and/or RXRs into Xenopus embryos, we have shown that TR/RXR heterodimers plus T3, but not TRs or RXRs alone, can precociously activate endogenous genes that are known to response to T3 during metamorphosis, while repressing these genes in the absence of T3. To investigate the function of TR during metamorphosis, we have adapted sperm-mediated transgenic method to generate transgenic animals expressing a dominant negative TR. Phenotypic analysis indicate that overexpression of the dominant negative TR inhibits TH-induced metamorphosis. More importantly, we have shown that the dominant negative TR specifically blocks the expression of TH response genes that we and others have identified previously.

To determine the mechanisms of the gene regulation in developing animals, we have demonstrated by chromatin immunoprecipitation (ChIP) assay that TR/RXR heterodimers are constitutively bound to T3 response genes in premetamorphic tadpoles. We have further shown that T3 binding leads to the release of the TR-binding corepressor N-CoR, a histone deacetylase known to associate with N-CoR, and an increase in the histone acetylation levels of T3 target genes. These results, for the first time in any vertebrate developmental system, provide strong support for the involvement of both unliganded and T3-bound TRs in post-embryonic development. We further demonstrate that chromatin remodeling involving alterations in histone acetylation levels as a likely mechanism for TR to regulate gene expression *in vivo*.

To directly investigate the role of histone acetylation in gene regulation during metamorphosis, we blocked the endogenous histone deacetylases by treating tadpoles with the drug trichostatin A (TSA). This led to precocious induction of T3 target genes, confirming that histone deactylation is important for gene repression by unliganded TRs in premetamorphic tadpoles. We have further shown that deacetylase(s) is also required for metamorphosis as continued treatment with TSA blocks both natural and T3-induced metamorphosis even though it has no effect on the activation of T3 target genes. These data thus suggest that deacetylase(s) participates in two steps in development, one in transcriptional repression by unliganded TR in premetamorphic tadpoles and the other in mediating the TH signal downstream of transcriptional regulation by T3-bound TR during postembryonic tissue remodeling.

Molecular Mechanism of Muscle Cell Death by Thyroid Hormone in Amphibian Tadpole Tail

Yoshio Yaoita
Hiroshima University, Japan

As thyroid hormone (TH) rises in plasma, almost every organ undergoes the modification including the limb development and loss of the tail and gills during the amphibian metamorphosis. Little is known regarding the molecular mechanism of TH-induced cell death in the regressing tail. *De novo* protein synthesis is necessary for TH-induced tail resorption, suggesting that TH regulates some genes responsible for cell death in the regressing tail. Based on the observation that TH induces the increase of the collagenase activity in explants of tadpole tailfin and gills and the concomitant decrease of their sizes, it has been proposed that TH-induced collagenase production is involved in the remodeling of collagen in these organs. Many genes that are up- and down-regulated by TH in the regressing tail have been isolated by PCR-based subtractive hybridization and characterized by *in situ* hybridization. Extracellular matrix (ECM)-degrading enzymes such as stromelysin-3 and collagenase-3 are highly expressed in the subepidermal fibroblasts encircling the entire muscle flank, but not in tail muscle. These enzymes are also up-regulated in the myotendinous junctions to which the muscle fibers are attached. These observations lead to the idea that the increase of secreted matrix metalloproteinases induced by TH results in the degeneration of the myotendinous junctions, which detaches muscle cells from ECM and causes their death (a murder model). This is supported by the phenomenon "*anoikis*" where *apoptosis* is induced by disruption of the interactions between normal epithelial cells and ECM.

On the other hand, we have reported that TH induces apoptosis of a myoblastic cell line derived from a tadpole tail, suggesting that tail muscle cells die autonomously (a suicide model). However, it is possible that this is an *in vitro* artifact and does not reflect the physiological death in matrix-interacting cells *in vivo*. Furthermore, it does not exclude a possibility that TH-treated myoblastic cells kill each other by secreting soluble factors.

The dominant-negative thyroid hormone receptor (DNTR) binds to TH response elements but not TH, and prevents transcription of TH-responsive genes by the wild type thyroid hormone receptor. To investigate the underlying mechanism, we undertook two approaches. First, we examined the effect of DNTR on muscle cell death *in vitro*. The overexpression of DNTR suppressed TH-induced death of a tail-derived myoblastic cell line. Secondly, tadpole tails were injected with a reporter gene and the DNTR expression construct, and the reporter gene expression in muscle cells was followed during the spontaneous metamorphosis. DNTR overexpression inhibited a decrease of the reporter gene expression which began at stage 57 in the control tadpoles, but only delayed massive muscle cell death at stage 63 when tails shrink very rapidly and muscle cells round. Some DNTR-overexpressing cells remained a few weeks after the metamorphosis. These results let us to propose that TH induces the suicide of muscle cells (the cell-autonomous death) in the tail between stage 57 and 62, and that both the murder and suicide mechanisms execute muscle cell death in stage 62-64 to remove muscle promptly and completely.

Design and Testing of Thyroid Hormone Responsive Reporter Gene Constructs for Generating Transgenic Xenopus Models for Detecting Endocrine Disrupters

Barbara Demeneix, Nathalie Turque, Caroline Alliot, Karima Palmier

Muséum National d'Histoire Naturelle, France

It is now well documented that many chemicals, of natural or synthetic origin, are involved in exerting adverse effects on reproduction and general metabolism in both wildlife and humans. In the first place, most attention and many studies were focused on compounds with oestrogenic action. However, more recently, several results show that " endocrine disrupters " may also affect the whole endocrine system affecting, for instance, androgenic or thyroid functions.

Of the several thousands of chemicals produced by human activities, unfortunately few have been tested for their potential " endocrine disrupter " activity. Thus, regulatory agencies such as the OECD are examining different testing strategies, both *in vitro* and *in vivo*. We are developing in our laboratory an *in vivo* testing method based on amphibian metamorphosis using transgenic Xenopus leavis (see Coen et al. 2001 Proc. Natl Acad. Sci USA). Before metamorphosis into adult frogs, Xenopus tadpoles are aquatic, thus chemicals can be easily added to aquarium water. Also, tadpoles have a fairly translucent skin so hey can be screened for expression of fluorescent proteins without resorting to invasive techniques. Moreover an another advantage is that each brood can provide thousands of eggs. Thus, this *in vivo* approach combines the sensitivity and scale-up potential of *in vitro* high throughput screening techniques with the advantages of a physiological, *in vivo* system that takes into account uptake, metabolism and excretion of the substance to be tested. The approach thus provides a powerful system to screen chemicals with thyroid disrupter activity.

Our models are based on the basic design of composite reporter gene constructs with a hormone sensitive regulatory region upstream of a fluorescent protein. These constructs are first tested in an *in vitro* culture using a Xenopus tadpole derived cell line and then in a somatic gene transfer assay (de Luze et al. 1993; Proc. Natl Acad.

Sci USA). The best constructs can then be used for generation of different transgenic Xenopus lines. A number of promoters are being testing including some promoters that could show tissue or cell specific expression patterns. These promoters are fused to different fluorescent genes (ds RFP or GFP) giving us the possibility to combine two constructs in one transgenic line. A suitably sensitive construct should enable us to perform dose effects experiments on a known compound in a relatively short period of time (<48h). The same tadpoles can also be used to study the impact of longer term exposure to given chemicals by following the effects of the substances on metamorphic progress.

Impact of Polychlorinated Biphenyls on Amphibian Development and Endocrine Physiology

Robert J. Denver
University of Michigan, USA

Polychlorinated biphenyls (PCBs) are known to disrupt thyroid function in humans and wildlife. At least two sites of action have been identified for thyroid disruption by PCBs in mammals. First, PCBs alter thyroid hormone (TH) excretion/metabolism by altering T^4 conjugation via UDP-glucuronoyl transferases. Second, PCBs disrupt blood thyroxine (T^4) transport by competing for binding to transthyretin (TTR). In mammals, TTR is a T^4-specific binding protein. However, in nonmammalian species, TTR binds 3,5,3'-triiodothyronine (T^3), the biologically active form of TH, with much higher affinity than T^4. We conducted competitive binding assays with several PCB congeners or hydroxylated PCBs to determine whether they exhibit competitive binding activity with [^{125}I]-T^3 for recombinant bullfrog TTR (rbTTR). One goal was to evaluate predictions (based both on empirical studies with human TTR and molecular modeling) of the T^4 or T^3-like properties of the various PCB congeners. Our results show that 8 of the 12 PCBs that we tested exhibit moderate to high affinity for rbTTR. The following conclusions can be drawn from the findings: 1) Predictions of T^3- and T^4-like properties of the various PCBs based on molecular modeling were largely borne out by competitive binding assays with rbTTR. PCBs that do not exhibit potency in the mammalian TTR binding assay (predicted T^3-like compounds) are potent competitors for [^{125}I]-T^3 binding to rbTTR. None of these T^3-like PCBs have been tested for their potential to interact with the nuclear TH receptors in any species. We hypothesize that, owing to their T^3-like properties, these and perhaps other PCBs could act as agonists (or perhaps antagonists) for the TH receptors. 2) There are important species differences between mammals and amphibians in the types of PCBs that can interact with TTR. This suggests that predictions of endocrine disruptive properties of specific PCBs are highly dependent on the species under investigation. 3) PCBs can interact with amphibian TTR and this presents the potential for disruption of TH transport and consequently hormone metabolism and action in tadpoles. We found that treatment of bullfrog tadpoles with PCBs slowed development and altered plasma [^{125}]-T^3 binding capacity, and brain T^3 content. Such alterations could negatively impact TH-dependent development in amphibian species, where TH is the primary morphogen controlling metamorphosis. (Supported by Michigan Great Lakes Protection Fund grant GL00-019 to R.J.D.)