Review

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Mechanisms of tail resorption during anuran metamorphosis

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Abstract: Amphibian metamorphosis has historically attracted a good deal of scientific attention owing to its dramatic nature and easy observability. However, the genetic mechanisms of amphibian metamorphosis have not been thoroughly examined using modern techniques such as gene cloning, DNA sequencing, polymerase chain reaction or genomic editing. Here, we review the current state of knowledge regarding molecular mechanisms underlying tadpole tail resorption.

Keywords: genomic editing; metamorphosis; *ouro*; tail resorption; TALEN; targeted gene knockout; *Xenopus*.

Introduction

Metamorphosis of amphibian tadpoles can be induced by the administration of fresh horse thyroid glands (1) and can be inhibited by the removal of a tadpole's thyroid glands (2). This discovery is refined by the inhibition of metamorphosis via treatment with a thyroid hormone (TH) synthesis inhibitor and by acceleration via treatment with purified TH, thyroxine (3). These studies demonstrate that thyroid hormones are essential and sufficient to induce the drastic morphological changes during anuran metamorphosis, including tadpole tail resorption.

Phagocytosis and autolysis: A historical overview

In *Xenopus tropicalis*, the tadpole tail is twice as long as the trunk and is resorbed in around 3 days once it starts during the climax of metamorphosis. Many scientists have taken much interest in the loss of the tail with the development of limbs to adapt from an aquatic to a terrestrial life. Because phagocytes were known to be involved in morphogenesis and wound repair, researchers at the end of the 19th century believed that these cells also devoured tadpole tails during metamorphosis (4, 5). However, subsequent histological analyses showed that autolysis, rather than phagocytosis, was the primary factor in tail dissolution (6). Specifically, an occlusion of blood vessels at the tail base was hypothesized to cause a buildup of carbon dioxide and acids, in turn, blood pH decreases to trigger autolysis (6). Subsequently, degenerative changes were observed in capillaries after the dissolution of tail muscles (7), indicating that muscle autolysis precedes reduced blood flow. Detailed electron microscopic analyses support descriptions of such early changes in tail muscle cells. As early as 3 days post forelimb eruption and before any detectable reduction in tail length, researchers observed a zig-zag-like folding of the myofibrils, fading of cross striations and the structural disintegration of mitochondria. Furthermore, autolysis likely occurred without any involvement from lysosomal enzymes because active macrophages with primary lysosomes are scarce during the initial phase of tail resorption (8). Instead, changes occur that are typical of apoptosis (9). First, tail muscle dissolution begins with the appearance of longitudinal clefts between myofibrils and widespread dilation of the sarcoplasmic reticulum, followed by muscle fiber fragmentation into many apoptotic bodies exhibiting well-preserved myofibril cross striations. Macrophages then consume these apoptotic bodies. Condensed chromatin exhibits peripheral aggregation in the nuclei of striated muscle cells.

Biochemical studies on tail tissue regression suggest that acid hydrolases participate in tail autolysis, due to a concurrent increase in their activities [e.g. DNase II (10), β-glucuronidase (11, 12) and acid phosphatase (13)]. As acid hydrolases are lysosomal enzymes, their increased activity is probably linked to their release from preformed lysosomes. The hypothesis that tail autolysis occurs via lysosomal enzymes is supported by the inhibition of tail atrophy in metamorphosing tadpoles when treated with

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the RNA synthesis inhibitor actinomycin D (14). Additional support is found through showing that the presence of RNA and protein synthesis inhibitors prevent TH-induced reduction of isolated tails and concomitant increase in hydrolase activity (15). These results imply that TH stimulates RNA and protein synthesis of proteolytic enzymes to induce tail resorption. The process of tail resorption can be divided into three phases (16). The first phase involves a progressive reduction in the rate of protein synthesis and a slight increase in plasma TH levels. The second phase is characterized by the enhanced degeneration of tail tissues by autolysis via lysosomal and non-lysosomal hydrolases and by heterolysis via release of hydrolytic enzymes to extracellular milieu. In the third phase, macrophages are activated to remove and digest tissue debris.

Murder model and suicide model

Reductions in the size of isolated tail tips under TH treatment indicate the occurrence of TH-dependent tail resorption (17). The resorption of tadpole tail fin explants under TH occurs concomitantly with an increase in collagenase activity (18). Brown et al. succeeded in the enrichment of genes that were upregulated in isolated tail tips at 24 and 48 h after TH treatment (19, 20). The protein synthesis inhibitor had no effect on the TH-induced resorption of tail tips at 48 h after TH treatment, indicating that TH-induced genes enriched from mRNA 48 h after TH treatment should include executor genes involved in tail resorption. The enriched genes included *stromelysin-3* (*MMP11*) and *collagenase-3* (*MMP13*), both part of the matrix metalloproteinase (MMP) family. MMP13 cleaves collagen, a major component of extracellular matrix (ECM) proteins. Under TH treatment for 2 days, both *MMP11* and *MMP13* mRNA increased, and localized to a layer of subepidermal fibroblasts and notochord. These data led to the development of a 'murder' model for tadpole metamorphosis, wherein TH-induced expression and secretion of MMPs from subepidermal fibroblasts drive ECM degradation and separate muscle cells from the matrix, causing cell death (21, 22) (Figure 1). At the peak of this process, the notochord appears to dissolve itself through the elevated synthesis of *MMP13* mRNA (21). During notochord dissolution, tail regression is thought to occur through the contraction of 'cords' comprising two dorsal and two ventral parallel rows of slow muscle bundles, joined by collagen fibers that run along the tail's length (23).

A study of myoblastic cell lines established from an *X. laevis* tadpole tail found that physiological TH concentrations (0.1–10 nM T3) triggered apoptosis, along with positive terminal deoxynucleotidyl transferase-mediated dUTP nick end labeling and internucleosomal DNA cleavage (24). As the cell line was a homogenous population originating from a single cell and did not include subepidermal fibroblasts, these results suggested TH-dependent cell-autonomous death, or a 'suicide' model (23, 24) (Figure 1). Two-day incubation of the cells in a condition

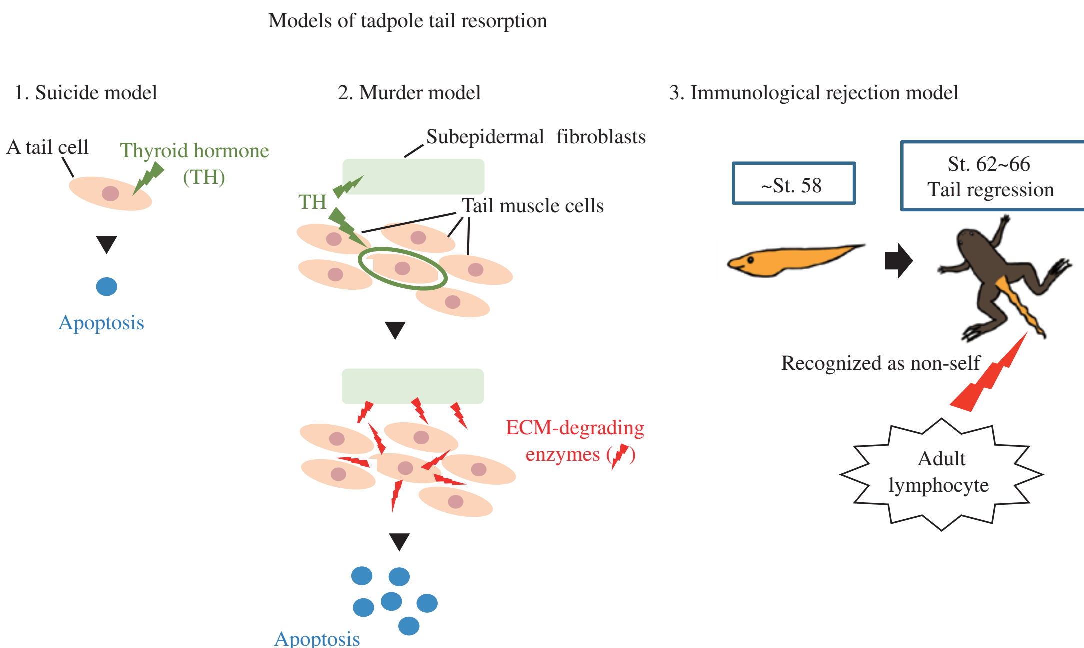

Figure 1: Models of tail resorption in amphibian tadpoles.

medium incubated with T3 did not accelerate apoptosis, suggesting that a paracrine mechanism (i.e. cells releasing proteins that kill neighboring cells) was not involved. The suicide model is also supported by the observation that tadpole skin cells died autonomously in response to TH during the metamorphosis climax (25).

T3-treated myoblastic cells synthesized mRNA encoding ECM-degrading proteinases, such as MMP9TH, MMP11, MMP13, MMP14 and fibroblast activation protein α (FAPα) (26). MMP14 is cleaved by furin within a cell into an active form that activates MMP13; in turn, MMP13 changes MMP9 into an active proteinase for digesting collagen fibers (27, 28). MMP9TH is a product of a duplicated *MMP9* gene in *X. tropicalis* and *X. laevis* that was present abundantly as an inactive precursor protein in the culture medium (29). Its presence suggests that MMP14 and MMP13 activity might be stopped by tissue metalloproteinase inhibitors, found in the bovine calf serum of the culture medium (30).

To modulate gene expression, TH binds to a heterodimer receptor composed of the TH receptor (TR) and the 9-cis retinoic acid receptor. When bound to TH, this heterodimer stimulates TH-responsive genes through the formation of a complex with the transcriptional activators, whereas when free from TH, it inhibits those genes via binding to co-repressors. A dominant negative form of thyroid hormone receptor (DNTR) can bind TH-responsive DNA elements, but not TH, due to a mutation in its TH-binding domain. This DNTR thus maintains a complex with transcriptional co-repressors even when TH is present, preventing the expression of TH-responsive genes (31).

A DNTR expression construct with a reporter gene was introduced (via electroporation) into tadpole tail muscle cells to impair TH signaling and examine the contributions of suicide/murder mechanisms during tail resorption (32). Under the suicide model, TH should direct intracellular death-inducing protein synthesis in non-transfected muscle cells, whereas DNTR expression should prevent transfected cells from producing those proteins. However, if tail resorption is induced through the murder model, subepidermal fibroblasts and non-transfected muscle cells should secrete death-inducing proteins (e.g. ECM-degrading proteinases) in response to TH, causing the deaths of their transfected and non-transfected neighbors. In fact, the death of transfected muscle cells was almost completely inhibited when TH signaling was interrupted between stages 58 and 61 (before tail resorption began), but was only partially prevented when TH signaling was interrupted during tail shortening. Therefore, a more modern hypothesis of metamorphosis mechanisms suggests that muscle cells commit suicide before tail shortening, but die according to both the suicide and murder models during tail shortening (32). This hypothesis is consistent with the observation that mRNAs of ECM-degrading enzymes increase immediately at stage 62 when tail shortening starts (26).

Immunological rejection through recognition of epidermal Ouro proteins as non-self targets in *X. laevis*

Immunological studies have shown that juvenile frogs reject tail skin grafts from syngeneic larva, with subsequent grafts rejected more rapidly as a secondary response (33). This phenomenon occurs irrespective of the donor metamorphosis stage. In contrast, body skin grafts become more acceptable as the metamorphosis of the donor tadpole proceeds. To identify the antigen involved in larval skin rejection, adult frogs were immunized with syngeneic larval skin grafts to generate anti-larval skin antibodies. Peptide sequences were determined via two-dimensional (2D) gel electrophoresis and Western blotting using anti-larval skin antibodies (34). Next, DNA fragments were amplified using degenerative primers encoding larval-specific peptide sequences and used as probes to identify the *ouro1* and *ouro2* genes in a cDNA library. *Ouro1* and *ouro2* gene expressions were induced through a heat shock promoter at the early stages of metamorphosis climax (stages 57–59) in transgenic frogs, eliciting precocious tail degeneration and T-cell accumulation. Furthermore, anti-*ouro* RNA-induced knockdown of *ouro1* or *ouro2* gene expression through a heat shock promoter considerably delayed tail resorption, leading to post-metamorphosis tail retention. These results strongly suggest that the tadpole immune system recognizes tail-skin-specific Ouro1/2 proteins as larval antigens during the metamorphosis climax, and the recognition is pivotal to tail resorption. This immunological rejection model suggests that newly differentiated adult-type immune cells recognize Ouro proteins as non-self targets for tail dissolution, allowing the elimination of larval cells during metamorphosis (35).

Ouro proteins are not necessary for tail resorption in *X. tropicalis* metamorphosis

Xenopus tropicalis models with *ouro1* and *ouro2* knockouts were used to examine the immunological rejection model (36). Spatiotemporal expression of *ouro* mRNA and proteins were the same as in *X. laevis* experiments. *Ouro1* mRNA expression in the tail of an *ouro1* knockout decreased from 1/26 to 1/50, perhaps through the nonsense-mediated

RNA decay (NMD) pathway, whereas *ouro2* mRNA was expressed similarly to wild-type expression. Additionally, Ouro1 protein was undetected and Ouro2 protein was barely detectable. In contrast, both *ouro1* and *ouro2* mRNAs were expressed at very low levels in the tail of an *ouro2* knockout; Ouro1 protein expression was slightly detectable and Ouro2 expression was undetectable. Under the immunological rejection model, these conditions should yield delayed tail resorption and tailed frogs in *X. tropicalis*. However, *ouro*-knockout tadpoles underwent metamorphosis, including tail resorption, exhibiting no morphological differences from the wild type.

A separate experiment used genome editing to mutate *Foxn1*, creating athymic frogs akin to nude mice. As athymic frogs lack splenic $CD8^+$ T cells that play a major role in cytotoxic reaction, they were capable of accepting major histocompatibility-disparate skin grafts, which should be rejected by wild-type frogs. These mutant tadpoles had shortened tails that appeared without delay during the metamorphosis climax, and tails were not retained post-metamorphosis (36).

Together, these data demonstrate that Ouro proteins are not essential for tail resorption in *X. tropicalis*. Furthermore, cytotoxic T cells are not indispensable for this species' tail regression.

Endogenous TH causes degeneration of larval skin graft on recipient syngeneic *X. tropicalis*

We observed the invasion of recipient-derived white pigment cells and a reduced number of melanocytes in wild-type larval skin grafts on *X. tropicalis* albino syngeneic frogs a few weeks after transplantation, compatible with the rejection of the larval skin grafts on *X. laevis* syngeneic frogs (33). All melanocytes disappeared within 2 months. Similarly, TH treatment causes autonomous death in a tail-derived myoblastic cell line and larval epidermal cells (24, 25), implying that larval skin cells undergo apoptosis in response to endogenous TH produced by recipient frogs. If this hypothesis is correct, larval skin grafts should survive longer with the addition of a TH synthesis inhibitor (methimazole) into breeding water. Indeed, syngeneic frogs in the continuous treatment of methimazole accepted larval skin grafts for over 150 days after transplantation (37). When wild-type skin grafts from the Nigerian-H *X. tropicalis* line were transplanted as major histocompatibility-disparate skin to the backs of methimazole-treated albino frogs from the Ivory Coast line, we observed the invasion of white pigment cells and a decrease in graft-derived melanocytes within a few weeks post-transplantation, confirming that methimazole treatment did not impede immunological rejection. We detected 6.3 nM ± 1.6 nM of T4 and 1.1 nM ± 0.86 nM of T3 in these albino frogs (37), comparable to T4 and T3 concentrations at stage 63 of *X. laevis* metamorphosis, when the tail is regressing and is equal in length to the trunk (38). Together, these data demonstrate that TH synthesized in the recipient frog drives the degeneration of syngeneic larval skin grafts.

Conclusions

Numerous models have been proposed to describe tadpole tail resorption mechanisms. Existing data fail to exclude the immunological rejection model in *X. laevis*, but clearly do not support the model in *X. tropicalis*. This discrepancy could be ascribed to species differences between *X. laevis* and *X. tropicalis*. However, we should consider the possibility that heat shock treatments to knockdown *ouro* expression may impair the active apoptotic pathway in a regressing tail. We also note that current research seems to support both the suicide and murder models. Although the exact molecular mechanisms in both the suicide and murder models should be identified more clearly in the future, our present knowledge indicates that TH induces tail cell suicide early in the metamorphosis climax, then promotes murder through activating MMPs to degenerate the ECM and triggering phagocytosis to remove cellular debris.

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