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Apoptosis in amphibian organs during metamorphosis

Atsuko Ishizuya-Oka^{1,*}, Takashi Hasebe¹, and Yun-Bo Shi^{2,*}

¹ Department of Biology, Nippon Medical School, Kawasaki, Kanagawa 211-0063, Japan

² Laboratory of Gene Regulation and Development, National Institute for Child Health and Human Development, NIH, Bethesda, Maryland, MD 20892-5431, USA

Abstract

During amphibian metamorphosis, the larval tissues/organs rapidly degenerate to adapt from the aquatic to the terrestrial life. At the cellular level, a large quantity of apoptosis occurs in a spatiotemporally-regulated fashion in different organs to ensure timely removal of larval organs/tissues and the development of adult ones for the survival of the individuals. Thus, amphibian metamorphosis provides us a good opportunity to understand the mechanisms regulating apoptosis. To investigate this process at the molecular level, a number of thyroid hormone (TH) response genes have been isolated from several organs of *Xenopus laevis* tadpoles and their expression and functional analyses are now in progress using modern molecular and genetic technologies. In this review, we will first summarize when and where apoptosis occurs in typical larva-specific and larval-to-adult remodeling amphibian organs to highlight that the timing of apoptosis is different in different tissues/organs, even though all are induced by the same circulating TH. Next, to discuss how TH spatiotemporally regulates the apoptosis, we will focus on apoptosis of the *X. laevis* small intestine, one of the best characterized remodeling organs. Functional studies of TH response genes using transgenic frogs and culture techniques have shown that apoptosis of larval epithelial cells can be induced by TH either cell-autonomously or indirectly through interactions with extracellular matrix (ECM) components of the underlying basal lamina. Here, we propose that multiple intra- and extracellular apoptotic pathways are coordinately controlled by TH to ensure massive but well-organized apoptosis, which is essential for the proper progression of amphibian metamorphosis.

Keywords

Amphibian metamorphosis; Apoptosis; Thyroid hormone; Organ remodeling; Cell-ECM interactions

Introduction

The animal body undergoes rapid and dramatic changes during amphibian (anuran) metamorphosis to adapt from the aquatic to the terrestrial life. The larva-specific organs such as the tail and the gill degenerate, while the adult-specific organs such as forelimbs develop. Numerous other organs, which exist both before and after metamorphosis, remodel from the larval to the adult form. Cell death occurs widely in not only the larva-specific organs but also the larva-to-adult remodeling organs, and thus is a key phenomenon in amphibian metamorphosis. The problem how larval cell death proceeds in balance with the

*Correspondence: A. Ishizuya-Oka, Department of Biology, Nippon Medical School, Kawasaki, Kanagawa 211-0063, Japan, a-oka@nms.ac.jp; tel: 81-44-733-3580; fax: 81-722-1231/ Or Y.-B. Shi, Laboratory of Gene Regulation and Development, National Institute for Child Health and Human Development, NIH, Bethesda, Maryland, MD 20892-5431, USA, shi@helix.nih.gov; tel: 301-402-1004; fax: 301-402-1323.

development of adult tissues/organs has attracted the attention of many biologists in this field, but the challenge to address this problem has just been started at the molecular level [1-3].

Apoptosis is an evolutionary conserved phenomenon essential for both development and tissue/organ homeostasis in all multi-cellular organisms. Cell death during amphibian metamorphosis is mainly, if not exclusively, through apoptosis. This has been shown by numerous previous studies with electron microscopy (EM) and TUNEL, which observed “apoptosis”, characterized ultrastructurally by condensation of chromatin on the nuclear membrane and subsequent fragmentation of nuclei and cytoplasm into apoptotic bodies [4], in amphibian organs including the tail [5], brain [6], and the intestine [7]. As expected, apoptosis during amphibian metamorphosis occurs in a well-organized fashion, depending on the cell-type and/or the kind of tissues/organs. This is essential for animals to overcome metamorphosis and survive. In this sense, the amphibian metamorphosis provides us a nice unique opportunity to understand regulatory mechanisms of apoptosis as a whole body/organ. It should be noted that the apoptosis of amphibian metamorphosis is triggered by a single hormone, thyroid hormone (TH) [8, 9]. In addition, previous culture studies showed that TH can organ-autonomously induce the degeneration of the larva-specific tail [10] and gill [11], and epithelial apoptosis of the remodeling small intestine [7]. These suggest that the molecular mechanisms of apoptosis during amphibian metamorphosis are controlled by a cascade of TH-dependent gene expressions in this animal model, even though the apoptotic process may involve complex tissue interactions as suggested in the tail [12-14]. Since the 1990s, toward determining the molecular mechanisms of amphibian metamorphosis, a number of TH response genes have been isolated from several organs of the African clawed frog, *Xenopus laevis*, including the tail and intestine by subtractive hybridization [15, 16] and more recently by cDNA microarray [17, 18]. Their expression and functional analyses, which are still ongoing, have already shed light on the molecular mechanisms regulating apoptosis during amphibian metamorphosis. Here we will first survey apoptosis occurring in various amphibian organs during metamorphosis and then review the current knowledge on the apoptotic factors encoded by TH response genes in these organs. Next, we will focus on the *X. laevis* intestine, one of the best-studied remodeling organs and summarize how TH regulates apoptosis of the larval epithelium both directly and indirectly through cell-ECM and/or cell-cell interactions to enable massive and cell type-dependent apoptosis during intestinal remodeling.

Apoptosis in larva-specific and larval-to-adult remodeling organs

In this section, we will begin with the description of when and where apoptosis occurs in the larva-specific and larval-to-adult remodeling organs during amphibian metamorphosis. Although apoptosis mainly occurs during metamorphic climax when TH level in the plasma is high (Fig. 1), the stage when apoptosis most frequently occurs is differently programmed in different organs and even in different tissues or cell-types of a single organ (Table 1). In addition, the timing of apoptosis in each organ sometimes varies with among different frog species, probably due to their different habitats and life histories. While we will mainly focus on the metamorphic changes in *X. laevis*, we will also describe available information in other anurans such as *Rana* and *Bufo* for comparison. The developmental stages of frogs have been assigned independently according to the genus: Nieuwkoop and Faber (NF) stage for *Xenopus* species [19], Taylor and Kollros (TK) stage for *Rana* species [20] and Rossi's stage for *Bufo bufo* [21].

Larva-specific organs

Tail—One of the most dramatic events during amphibian metamorphosis is the regression of the tail which occupies a large proportion of the tadpole. Thus, the mechanisms of tail

resorption have been extensively studied for many years [5, 16, 22-31]. The tadpole tail consists of the epidermis, muscles, connective tissue (dermis), nervous tissue (spinal cord), and notochord. By the end of metamorphosis, the tail is totally removed through apoptosis of essentially all of the tissues (Fig. 1), although only limited cell types have been shown to undergo apoptosis. In the tail of *X. laevis* tadpoles, muscles begin to undergo apoptosis at NF stage 57 (prometamorphosis) long before the start of the tail shortening at stage 62 [28], and the number of apoptotic cells in the muscles peaks at stages 63/64, when the tail drastically shortens [29]. On the other hand, in the caudal spinal cord, the number of apoptotic cells reaches its peak at stage 58 (the onset of metamorphic climax) [24], when the forelimbs protrude. Similarly, in Japanese brown frog, *Rana japonica*, the epidermis undergoes cell death at TK stages XIX-XX (around NF stage 61 for *X. laevis* [8], Fig. 1), when forelimbs extend away from the body [32]. Although TH organ-autonomously induces the regression of the tail [10], previous culture studies indicated that interactions between the epidermis and dermis are necessary for the tail regression of the bullfrog, *R. catesbeiana* [12, 13, 33].

Gill—The gill is also a larva-specific organ that degenerates nearly completely [34] or perhaps leaves a small remnant [35] during metamorphosis. As metamorphosis proceeds, the gill decreases in weight and becomes less vascularized. In the *R. catesbeiana* gill, most of the epithelial cells are closely associated with blood vessels during premetamorphosis, but are no longer in close proximity to the blood vessels at TK stage XXII (metamorphic climax, NF stage 63 for *X. laevis*) [34]. In the *X. laevis* gill, the filter plates, which occupy more than 3/4 of the buccal floor area's rostrocaudal extension during prometamorphosis, become very small and are confined to the space bilateral to the glottis at NF stages 61/62 (metamorphic climax). By using microvascular casting, it has been shown that the branching of filter row veins reaches its maximum at stage 58 and then gradually decreases in number and frequency until stage 62, when many apoptotic cells are observed in the gills. This indicates that vascular regression is organized by progressive fusion of opposite vessels [36].

Larval-to-adult remodeling organs

Brain—As metamorphosis involves a habitat shift from an aquatic to a terrestrial environment, where adult frogs exhibit behaviors and other life history characteristics dramatically different from those in tadpoles, gross morphology of the amphibian brain changes from larval to adult types during metamorphosis, e.g., the widening and shortening of the diencephalons and the narrowing and shortening of the fossa rhomboidalis of the medulla [37]. Although apoptosis has not been reported with EM or TUNEL analysis in this organ, caspase-9, one of the key apoptotic factors, is active in the remodeling of the *X. laevis* brain during NF stages 55-63, peaking at stage 60. Its mRNA is expressed in the ventricular zone of the brain. Cells expressing caspase-9 are localized in the ventral part of the anterior brain and the subpallium area at stage 61 [38]. As for other larval neurons, Mauthner neurons in the medulla and the sensory and motoneurons supplying the tail are eliminated during remodeling of the central nervous system [39]. Massive cell death in the mesencephalic fifth nucleus of the trigeminal nerve also occurs at NF stage 61 in *X. laevis* [40] and at TK stage XXI (around NF stage 62/63 for *X. laevis*) in the leopard frog, *R. pipiens* [41].

Intestine—As an herbivorous tadpole metamorphoses into a carnivorous frog, the intestine is drastically remodeled from the larval to the adult form [1, 42-44]. In the *X. laevis* small intestine, apoptotic cells are very few until NF stage 59 and then rapidly increase in number at stage 60 [45] (Fig. 1), when stem cells of the adult epithelium appear. The larval epithelial cells are totally removed through apoptosis by stage 63, while the adult epithelial cells replacing the larval ones finally differentiate by the end of metamorphosis. Although

massive apoptosis occurs only in the larval epithelium throughout metamorphosis, a small number of fibroblasts also undergo apoptosis at stages 61/62 [7, 45].

Pancreas—Pancreas, which mainly consists of the endocrine tissue producing hormones and exocrine tissue producing digestive enzymes, is also one of the digestive organs that are remodeled during metamorphosis [46]. The *X. laevis* pancreas loses about 40% of its cells by NF stage 62 (metamorphic climax), when many cells, though their cell-type is not specified, undergo apoptosis. It is suggested that some, if not all, larval acinar cells dedifferentiate during metamorphic climax and then redifferentiate into the adult type after metamorphosis [47]. In both the *R. pipiens* [48] and *R. catesbeiana* pancreas [49], β -cells producing insulin decrease in number at TK stages XX or XXI (metamorphic climax, around NF stage 62 for *X. laevis*). Similarly, the common European toad, *Bufo bufo*, shows the reduction of pancreatic volume during Rossi's stages IX (late prometamorphic period, around NF stage 56) - XV (the end of metamorphosis, NF stage 66). This degeneration of pancreas is caused in part by apoptosis of cells around the blood vessels and pancreatic ducts. The number of apoptotic cells reaches a peak at Rossi's stage XIII (around NF stage 58-62), when forelimbs emerge [50].

Spleen—During metamorphosis, the immune system is also remodeled. Although the larval immune system is capable of defending against potential pathogens, it is not equivalent to the adult immune system acquired during metamorphosis. Remodeling of the immune system may serve to eliminate unnecessary larval lymphocytes, which could be destructive if they recognize newly emerging adult-specific antigens on the adult type tissues [51]. The spleen is known to be a major organ that is responsible for the immune system. In the *X. laevis* spleen, which becomes lymphoid at NF stage 50 [52], apoptotic cells are very few during premetamorphosis and then become detectable mostly in the red pulp, but not in the white pulp, throughout metamorphosis. The number of apoptotic lymphocytes in the red pulp reaches its peak at stage 59 [53].

Thymus—The thymus is another organ responsible for the immune system and a periodic removal of thymocytes as well as apoptosis of lymphocytes during metamorphosis has been reported. In the thymus of *X. laevis*, apoptotic levels are high during premetamorphosis (NF stages 49-54), low from prometamorphosis to early climax (NF stages 56-61) and high again at middle climax (NF stages 62, 63). While apoptotic cells are distributed nearly uniformly between cortex and medulla of the thymus from premetamorphosis to early climax, the dying lymphocytes are localized in the medulla at middle climax [53].

Red blood cells (RBCs)—The circulating red blood cells (RBCs) also change from larval RBCs containing larval-type hemoglobin (Hb) to adult RBCs containing adult-type Hb (so-called Hb switching) during metamorphosis [54]. The major difference in these two types of Hb is their affinity to oxygen. Thus, the Hb switching is physiologically important to adapt from aquatic to terrestrial habitat. Several lines of evidence have shown that Hb switching in anuran amphibians is due to an overall replacement of the RBC population rather than an alteration of globin-gene expression in the single RBC population [55-57]. In *R. catesbeiana*, adult RBCs first appear in the systemic circulation at TK stage XX (the onset of metamorphic climax, around NF stage 62 for *X. laevis*) and become predominant as larval RBCs totally disappear by stage XXIV just before the end of metamorphosis (around NF stage 65 for *X. laevis*) [54]. On the other hand, in *X. laevis*, adult RBCs become detectable at NF stage 53 (premetamorphosis) and increase in number as metamorphosis proceeds. Surprisingly, approximately 10% of total RBCs are still larval-type at 3 weeks after the completion of metamorphosis, and it takes another 5 months to complete Hb switching [57]. This delayed removal of larval cells may be related to the fact that

postmetamorphic *X. laevis* froglets remain aquatic. Larval RBCs, but not co-existing adult RBCs, are selectively sequestered from the systemic circulation by macrophages in the liver and spleen during metamorphic climax [56]. Interestingly, in *R. catesbeiana* during the metamorphic climax, circulating adult RBCs, but not larval RBCs, contain fragmented genome (DNA ladder) that is typical to apoptotic cells, suggesting that some of the adult RBCs undergo apoptosis just when they appear as in the mature adult frog [55, 58] and that larval RBCs do not undergo typical apoptosis at least in the circulating blood [55]. In *X. laevis*, larval RBCs trapped in the spleen also exhibit the apoptotic feature, and the number of apoptotic cells reaches its peak at NF stage 60 [57].

Body Skin—The larval skin is remodeled into the adult one to acquire critical features for the terrestrial life, e.g., different permeability to ions, production of antimicrobial peptides, etc [51, 59, 60]. Although the skin remodeling occurs during metamorphosis in *R. japonica* and *X. laevis* [61, 62], the remodeling begins much earlier in *R. catesbeiana* [61, 63]. The epidermis of the body skin during premetamorphosis consists of apical, skein and basal cells [14]. In the *X. laevis* skin, apical and skein cells of the epidermis undergo apoptosis at the onset of metamorphic climax, whereas a population of basal cells dedifferentiates into adult stem cells and reconstitute the adult epidermis by the end of metamorphosis [14]. The adult epidermis, which is localized in the limbs at NF stage 56 (prometamorphosis), expands to the trunk region at stage 60 and then, by stage 63, almost all of the regions except for the tail and some part of the head [62].

Apoptotic factors regulated by TH

In general, two major apoptotic pathways have been characterized; the extrinsic pathway triggered by membrane death receptors and the intrinsic pathway initiated in mitochondria [64]. These implicate conserved mechanisms involving proteases called caspases [65, 66], and regulatory mitochondrial factors [67, 68] such as the Bcl-2 family including both proapoptotic (e.g. Bax) and antiapoptotic (e.g. Bcl-2) factors. The relative levels of each protein ultimately determine the cell fate [69]. Numerous investigations have demonstrated that such factors are also involved in apoptosis during amphibian metamorphosis. Although the progression of apoptosis during metamorphosis is dependent on tissues or cell-types as mentioned above, the entire process, like all other events during metamorphosis, is controlled by TH. Here we will summarize how TH initiates apoptotic pathways and what factors are involved in the amphibian apoptosis, focusing on TH response genes (Table 2).

Thyroid hormones and deiodinases

The amphibian thyroid gland secretes not only 3,5,3'-triiodothyronine (T3) but also thyroxine (T4). T4 is the precursor of T3, which is a biologically more active hormone, and can be converted to T3 by type I and II deiodinase (D1 and D2) [70-72], and T3 is finally inactivated by type III deiodinase (D3) [70, 73]. Since the metamorphosing tissues are exposed to the same plasma concentration of TH, local activities of both enzymes are proposed to play important roles in regulating TH action in peripheral tissues, e.g., growth of the limbs versus resorption of the tail [70]. Indeed, D2 expression becomes very high in the tail and the intestine during metamorphic climax [70, 74], and overexpression of D3 has been shown to inhibit metamorphosis [75].

Thyroid hormone receptors and TH response genes

TH exerts its effects via TH receptor (TR), which belongs to the superfamily of nuclear hormone receptors [76]. TR has been shown to be both necessary and sufficient to mediate the metamorphic effects of TH by using transgenic *X. laevis* tadpoles. For example, overexpression of dominant-positive mutant of TR in the premetamorphic tadpoles in the

absence of TH led to precocious metamorphosis including apoptosis of the larval intestinal epithelium [77], while overexpression of dominant-negative mutant of TR in the presence of TH inhibits metamorphosis including the intestinal remodeling, gill resorption, tail shortening and epidermal cell death [28, 78-80].

There is a pair of TR subtypes encoded by separate genes, TR α and TR β [81]. TR α is expressed early in development, long before the functional thyroid gland develops [82]. Just prior to metamorphosis, TR α is ubiquitously expressed, but its expression level is particularly high in the brain, limb buds, skin and other tissues that are destined to proliferate and differentiate into adult organs in the response to TH [83, 84]. In contrast, much lower levels of TR β mRNA are detectable prior to metamorphosis [82, 85]. During metamorphosis, while TR β levels remain very low in the growing limbs, TR β expression is strongly up-regulated by TH in other organs such as the tail and intestine. It has been shown that the treatment of premetamorphic tadpoles with the TR β -specific agonist, GC-1, induces the gill resorption and tail shortening in vivo [86]. Those data suggest that apoptosis of larval cells in the metamorphosing organs is mediated predominantly by TR β rather than TR α .

As a transcription factor, TR binds to thyroid hormone response elements located in the direct TH response genes in the genome, thereby regulating their expression. Subsequently, the products of these genes often control the expression of other so-called indirect or late TH response genes. For simplicity, we will designate both groups of genes as “TH response genes” unless otherwise specified. To date, a number of TH response genes including proapoptotic and antiapoptotic genes have been identified in several organs of *X. laevis* tadpoles [15-18, 87-90] as summarized below.

Factors involved in the mitochondrial pathway

The expression of Bax is highly upregulated in the tail during natural and TH-induced metamorphosis [31]. It has been demonstrated that overexpression of Bax induces apoptosis in the tail muscle in vivo [31, 91]. On the other hand, xR11, a homologue of mammalian Bcl-X_L, is strongly expressed in the brain during metamorphic climax [92]. Its antiapoptotic role is evident, since overexpression of xR11 significantly inhibits Bax-induced apoptosis of the muscle and brain in prometamorphic tadpoles as well as naturally occurring apoptosis of Rohon-Beard neurons during metamorphic climax [93]. In addition, xR11 exhibits a protective effect against muscular apoptosis induced by the truncated (activated) form of BH3-interacting death agonist (BID) [94], which acts upstream of the Bax/Bcl-2 members [95] and accelerates TH-induced tail shortening in vitro [94]. Furthermore, overexpression of xR11 in the brain promotes cell proliferation but suppresses apoptosis where caspase-9 is involved, indicating that xR11 together with caspase-9 controls apoptosis/proliferation balance during the brain remodeling [38]. These studies suggest a role of Bax and xR11 in regulating permeability of the mitochondrial outer membrane, while the function of other members of Bcl-2 family such as Bak remain unknown during amphibian metamorphosis.

A number of proteins released from mitochondria also play a central role in the execution of the apoptotic pathway [96]. In *X. laevis*, mitochondrial apoptotic regulators such as apoptosis-inducing factor (AIF), endonuclease G and HtrA/Omi have been identified in the expressed sequence tag (EST) database. In addition, Smac/DIABLO is expressed during early embryogenesis and capable of inhibiting apoptosis induced in HeLa cells by γ -irradiation [97], although it is not clear whether these factors are also involved in apoptosis during metamorphosis.

Caspases and other related factors

The expression of caspase-9, one of the key apoptotic factors, is up-regulated in the brain, intestine and tail during metamorphic climax [30, 98]. Activation of caspase-9 protein is dependent on Bax, and the level of active caspase-9 is the highest in the tail muscle and brain during metamorphic climax [30, 38]. Overexpression of dominant-negative mutant of caspase-9 (dnCasp9) has been shown to inhibit Bax-induced and naturally occurring apoptosis in the metamorphosing tail and brain, indicating the involvement of caspase-9 in apoptosis [30, 38]. These findings further suggest that dnCasp9 is associated with apoptotic protease-activating factor (Apaf)-1 and cytochrome c to form the inactive apoptosome, although direct evidence has not been obtained [30]. In the tail, the expression of caspase-1 and -3 is also up-regulated during metamorphosis [30, 98, 99], suggesting their involvement in apoptosis. In fact, a specific inhibitor of caspase-3 suppresses TH-induced apoptosis of XLT-15, a myoblastic cell line derived from the tadpole tail [99] in vitro, whereas caspase-1 is capable of inducing apoptosis in XLT-15-11, a subline of XLT-15 [98]. In addition, caspase-2 and -8 are involved in apoptosis in the tail, since the specific inhibitor of either one blocks TH-induced tail shortening in vitro. These caspases are thought to function by activating BID [100]. The levels of caspase-2 and -8 are the highest in the tail during metamorphic climax, whereas the levels of other enzymes such as calpains are either undetectable or unchanged, suggesting that caspase-2 and -8 are responsible for activation of BID [94]. Other factors suggested to play a role in apoptosis of the tail are caspase-7 and inhibitor of growth (ING) family [30, 98, 101].

Among the other organs, the expression of caspase-3 has also been reported in the remodeling of skin [79]. In the *X. laevis* skin, active caspase-3 is rarely detectable before metamorphic climax, but is widespread at NF stage 60, when the larval epidermis is being replaced by the adult one [79]. In the intestine, the expression of caspase-1, -2, -3, -6, -7, -8, -9 and -10 has been shown to be up-regulated during metamorphic climax [98]. Although most of caspases identified so far are able to induce apoptosis of XLT-15-11 in vitro [98], the roles of some of them in vivo still remain to be investigated. Among the other apoptotic factors, the inhibitor of apoptosis (IAP) family of proteins containing the highly conserved baculoviral IAP repeat (BIR) are known to inhibit apoptosis, in some cases, through direct binding of caspases. XIAP and XcIAP, the *X. laevis* BIR family proteins, have been shown to inhibit the apoptotic process in the cell-free system of *X. laevis* egg extract [102, 103]. However, their involvement in metamorphosis as well as TH dependency remains to be determined.

Death receptors

The cell surface receptors that belong to the tumor necrosis factor receptor (TNFR) superfamily are the major death receptors involved in the extrinsic pathway of apoptosis. To date, several members of the TNFR superfamily and one of their ligands, xTNF α , have been reported in *X. laevis* tadpoles [104-107]. It has been shown that xTNF α binds to its cognate receptor, xTNFR1 and inhibits TH-induced apoptosis of XLgoo, a vascular endothelial cell line derived from tadpole tail. Exogenous xTNF α also partially inhibits TH-induced tail degeneration in vitro, at least partly through repressing the levels of TR β and caspase-3 [106]. It still remains unclear whether xTNF α and xTNFR1 are TH response genes, since the level of xTNFR1 mRNA does not change in XLgoo treated with T3, and xTNF α is absent in this cell line. However, when total RNAs from the entire tadpoles at different metamorphic stages are assessed, both xTNF α and xTNFR1 are expressed in a bimodal way, i.e., very weak expression at NF stage 55 occurring between stronger expression at stage 51 and at stage 57. Thereafter, the expression of both genes becomes weak again at stage 61, suggesting that these genes may be negatively regulated by TH in a tissue-specific manner [106].

Kinases

Several lines of evidence have shown the importance of phosphorylation in the tail regression [108]. Inhibition of cyclin-dependent-kinase (Cdk) 2, whose expression is down-regulated during metamorphic climax, accelerates TH-induced tail regression [109]. In contrast, inhibition of protein kinase C prevents TH-induced tail regression [110]. In addition, the activity of Cdk8 together with its regulatory unit, cyclin C, whose expression is TH dependent, is required for the tail shortening, suggesting their proapoptotic role [111].

Lessons from intestinal remodeling: Assurance of apoptosis through cell-ECM and cell-cell interactions

Larval epithelial apoptosis associated with adult epithelial development and ECM remodeling

The *X. laevis* small intestine is one of the typical remodeling organs where apoptosis has been well characterized at the cellular level. Using this organ, we delve deeper into the problem how TH successfully induces massive apoptosis in a cell type-dependent fashion. To adapt to a new food habit, the *X. laevis* intestine dramatically shortens and remodels from the larval to adult form during a short period of metamorphosis [43, 112]. Apoptotic process of the larval epithelium during the intestinal remodeling has been precisely investigated by EM and TUNEL [7, 45]. Before metamorphic climax the tadpole intestine consists of a single layer of larval epithelium and thin layers of the immature connective tissue and muscles. Apoptotic cells are very few, if any, in any tissues until NF stage 59. Around stage 60 (the onset of metamorphic climax), when a small number of adult stem cells appear and actively proliferate, apoptotic cells suddenly increase in number in the remaining larval epithelium [45] (Fig. 2). These cells share common characteristics of apoptosis; formation of apoptotic bodies following the condensation of chromatin against the nuclear envelope. The apoptotic bodies are then phagocytosed by intraepithelial macrophages which increase in number in the degenerating larval epithelium. The macrophages become enlarged by engulfed phagosomes including the apoptotic bodies and are finally extruded into the intestinal lumen [7]. In this way, almost all of the larval epithelial cells except for the adult stem cells are lost during stages 60-62 (the early period of metamorphic climax). Characteristically, apoptosis of the larval epithelium is closely related to the modifications of the underlying basal lamina [113] (Fig. 2). The basal lamina is thin before metamorphic climax and then, concomitantly with the start of apoptosis of the larval epithelium, suddenly becomes thick by vigorous folding and amorphous. Through such modified basal lamina, the macrophages and other leukocytes migrate from the connective tissue into the degenerating larval epithelium. The basal lamina underneath the larval epithelium remains amorphous until the larval epithelial cells finally disappear through apoptosis, whereas the basal lamina underneath the adult stem cells and their descendent cells becomes thin again as they differentiate into the adult epithelium analogous to the mammalian adult one. These chronological observations suggest that apoptosis of the larval epithelium, which progresses in balance with development of the adult epithelium, is strictly regulated spatiotemporally, possibly involving extracellular matrix (ECM) remodeling of the basal lamina.

TH induces epithelial apoptosis under the influences of ECM in vitro

To clarify the mechanisms of the intestinal remodeling at the molecular level, we first established an organ culture system and found that the apoptotic process during the intestinal remodeling described above could be reproduced by the inductive action of TH in vitro [7]. In organ cultures of *X. laevis* intestines at stage 57 (before metamorphic climax), apoptosis of the larval epithelial cells occurs most frequently after 3 days of TH-treatment. Apoptotic

bodies derived from the larval epithelial cells are phagocytosed by intraepithelial macrophages, which are extruded into the outside. Similar to apoptosis during natural metamorphosis, TH-induced apoptosis of the larval epithelium in vitro coincides well with the modification of the basal lamina underneath the epithelium, suggesting the involvement of the remodeling of the basal lamina ECM in the epithelial apoptosis. Thus, it seems likely that apoptosis of the larval epithelium is induced by TH organ-autonomously but under the control of cell-ECM interactions, which may function as a spatiotemporal regulator of the epithelial apoptosis.

Next, to examine whether TH can cell-autonomously induce apoptosis, epithelial cells were isolated from the small intestine of *X. laevis* premetamorphic tadpoles, scattered, and cultured on plastic dishes in vitro. TH induces some of these epithelial cells to undergo apoptosis [114], whereas TH stimulates proliferation of other cells. In addition, this TH-induced apoptosis has been shown to be inhibited by cell death inhibitors such as Cyclosporine A (Cys A), which is generally known to inhibit the mitochondrial permeability transition pore (MPTP) opening by binding with cyclophilin D [115], showing similarity between the amphibian TH-induced apoptosis and the mammalian apoptosis [116]. Although cyclophilin D has not yet been identified in amphibians, it has been shown that CsA suppresses TH-induced mitochondrial swelling, which results in the activation of caspases, in the *Rana* liver [117]. Thus, it is highly possible that MPTP also plays an important role in the amphibian TH-induced apoptosis. Moreover, to clarify whether ECM components affect apoptosis of the *X. laevis* intestinal epithelium in cell cultures, isolated epithelial cells were cultured on the dishes coated with various components of ECM including laminin, fibronectin, collagens I and III. In the dishes coated with any ECM component, the cells become resistant to TH-induced apoptosis [114]. These results indicate that TH cell-autonomously induces apoptosis of the intestinal epithelium, but this apoptotic process can be inhibited by ECM components.

Function of ST3 and other matrix metalloproteinases (MMPs)

To understand molecular aspects of the intestinal remodeling, a number of TH response genes have been isolated from the *X. laevis* intestine by using PCR-based subtractive screening [15, 87] and cDNA microarray [17]. Proteins encoded by these genes include intracellular proteins such as TR β and other transcription factors that directly regulate downstream genes, membrane-bound proteins, and secretory proteins such as signaling molecules and MMPs. Previous in situ hybridization (ISH) analysis showed that the expression of TR β mRNA is highly up-regulated in all of the larval epithelial cells just before the start of their apoptosis [118], in agreement with cell-autonomous induction of apoptosis by TH in vitro. As for other TH response genes, we paid special attentions to MMPs, which are a superfamily of Zn-dependent proteases capable of degrading various components of ECM, since the epithelial apoptosis shows a close correlation with the modification of the basal lamina both in vivo and in vitro as mentioned above. Among the TH response genes identified previously were a number of MMP genes including stromelysin-3 (ST3; MMP-11) [119], collagenase-3 (MMP-13), -4 (MMP-18) [120], membrane type-1 MMP-1 (MMP-14), and gelatinase A (MMP-2) [121]. ISH analyses have shown different expression profiles of different MMPs, suggesting their different roles in intestinal remodeling [122]. Of particular interest is ST3. This is because the expression profile of ST3 mRNA most closely correlates with the epithelial apoptosis, and ST3 expression is directly up-regulated by TH in fibroblasts through the TR-TREs binding [123].

In mammals, ST3 is known to be expressed in fibroblasts surrounding invading cancer cells [124, 125] and epithelial tissues undergoing apoptosis [126]. To date, ST3 is the only MMP whose key roles in apoptosis of the intestinal epithelium have been experimentally demonstrated both in vivo and in vitro. Since ST3 is secreted as an active protease [127], we

first performed organ-culture experiments of the *X. laevis* intestine using a function-blocking antibody against the catalytic domain of ST3. The addition of ST3 antibody to the culture medium inhibits TH-induced apoptosis of the larval epithelium in a dose-dependent manner and also inhibits the basal lamina modification [128]. Moreover, using transgenic tadpoles expressing ST3 under a heat shock-inducible promoter, we have shown that overexpression of ST3 in the premetamorphic tadpoles induces the epithelial apoptosis following the basal lamina modification even in the absence of exogenous TH [129, 130]. This indicates that ST3 alone can induce apoptosis of some of larval epithelial cells in vivo, possibly through altering interactions between epithelial cells and the basal lamina ECM. However, the number of apoptotic cells in the transgenic intestine overexpressing ST3 is smaller than that in the wild-type intestine at the onset of metamorphic climax, when both levels of endogenous TH and ST3 are high [130]. This suggests that massive apoptosis occurring during normal metamorphosis are caused by the coordinated function of ST3 with other TH response genes. Although apoptotic pathway induced by ST3 has not been fully determined, a cell-surface laminin receptor (LR) has been identified as a substrate of ST3 in the remodeling intestine [131-133] and the tail [26]. Importantly, its cleavage by ST3 during metamorphosis correlates well with apoptosis of larval intestinal epithelial cells and tail epidermal cells [26, 131-133]. Thus, one possible pathway is that ST3, whose expression is up-regulated by TH in fibroblasts, cleaves LR of larval epithelial cells and alters the interaction between them and the basal lamina laminin, which in turn signals the epithelial cells to undergo apoptosis (Fig. 3). Interestingly, tail muscle cells do not appear to express LR and transgenic expression of ST3 causes significant muscle cell apoptosis without detectable LR cleavage in the tail [26]. Consistent with a potential role of LR cleavage in epidermal cell death, little epidermal apoptosis is induced in the tail by the transgenic overexpression of ST3 in premetamorphic tadpoles under the experimental conditions [26]. Thus, ST3 appears to be capable of inducing cell death via two pathways, one involving LR cleavage for epithelial or epidermal cells which express LR for attachment to basement membrane, and another independent of LR cleavage. Clearly, identification of additional ST3 substrates is needed to determine how ST3 causes muscle cell death. As for the other MMPs, MMP-9 and MMP-9TH have also been identified as TH response genes [134]. Recently, an ISH study showed that the expression profile of MMP9-TH, one of the duplicated MMP-9 genes, is similar to that of ST3 [135]. Since mammalian MMP-9 is known to degrade type IV collagen, a major component of the basal lamina [136], MMP-9TH may be also involved in apoptosis by altering cell-ECM interactions. It is intriguing to study how ST3 cooperates with other MMPs or intracellular signals and enables the massive and cell type-dependent apoptosis during metamorphosis.

Other local cell-cell interactions

It is generally known that a transition of major histocompatibility complex antigens (MHC) occurs during amphibian metamorphosis [137, 138]. Accordingly, the immune system of *X. laevis* tadpoles is also remodeled as mentioned above. In the *X. laevis* intestine, previous morphological studies showed that, other than macrophages, lymphocytes are frequently located in both the larval and adult epithelia throughout metamorphosis [139, 140]. Although it still remains unknown when larval lymphocytes are replaced by adult ones in this organ, larval epithelial cells must be recognized as nonself by adult lymphocytes as proposed in the skin [63, 141]. This immunological reaction is biologically significant as an assurance program to remove larval epithelial cells if they remain by accident in the adult intestine after intestinal remodeling.

Amphibian metamorphosis as a model for apoptosis

Amphibian metamorphosis is a systematic phenomenon to adapt from the aquatic to the terrestrial life, which possibly mimics the evolutionary process from fish to tetrapods in the

Devonian period of the Paleozoic era. Since the entire processes of metamorphosis including apoptosis can be experimentally induced by TH, amphibian metamorphosis serves as a unique and excellent animal model to clarify mechanisms regulating apoptosis in a whole body and/or in each organ at the molecular level [142]. Although only fragmentary information is available on its mechanisms at present, comparison of recent genome-wide microarray data across multi-organs including the tail, brain, and intestine [17, 18] should help to establish a molecular framework for further clarification of the mechanisms regulating apoptosis during amphibian metamorphosis.

In the *X. laevis* small intestine, a number of TH response genes have been already characterized and their functional analyses are in progress by using transgenic and culture techniques both *in vivo* [77, 78] and *in vitro* [143]. One of the important notions obtained from such studies so far is that TH simultaneously initiates multiple apoptotic signaling pathways in the same cell. TH can induce apoptosis of the larval epithelium not only by intracellular (cell-autonomous) pathways where TH up-regulates the expression of apoptosis-related genes such as caspases directly or indirectly, but also several extracellular pathways through cell-ECM interactions or immunological reactions (Fig. 3). These pathways may cross-talk with each other to ensure apoptosis of specific cell-types occurring at appropriate time and locations. One of the major remaining questions is why adult stem cells, which are recently demonstrated by using transgenic tadpoles expressing GFP to be dedifferentiated from a small number of larval epithelial cells [144] as proposed by previous observations [112, 145, 146], escape from apoptotic pathways and begin to actively proliferate. Previous studies did not report on differences between larval epithelial cells and adult progenitor cells in underlying ECM components of the basal lamina or MMPs. To answer the question, it is worth proceeding further the expression and functional analyses of TH response genes that have been identified in the intestine but not yet been investigated.

Another major remaining question is to what extent apoptotic signaling pathways during amphibian metamorphosis are conserved in the mammalian organs. From an evolutionary standpoint, amphibian metamorphosis is often compared to mammalian postembryonic development, a period around birth in the case of humans. Amphibian organs remodeled after metamorphosis are mostly analogous to the mammalian adult ones. Furthermore, it is known that TH levels in the mammalian plasma increase transiently, and its high levels are critical for late embryonic and postnatal development such as organ maturation [2, 147], a period that bears many similarities to anuran metamorphosis [2]. Subsequently, TH levels become lower, but appropriate levels of TH continue to function in the adult mammals and amphibians as a metabolism-modulating hormone to stimulate the basic metabolic activity of cells. Interestingly, it has been shown that TH regulates both cell proliferation and apoptosis in the epithelium of adult mammalian intestine after irradiation, similar to that during amphibian intestinal remodeling. In such intestine during regeneration after DNA damage, TH not only promotes proliferation of surviving epithelial progenitor cells but also apoptosis of damaged epithelial cells for their fast elimination through TR α [148]. These findings implicate that mechanisms underlying the amphibian intestinal remodeling may be at least partly conserved in the adult mammalian intestine during organ regeneration. Currently, we do not have sufficient molecular data on the similarity and difference between amphibian metamorphosis and mammalian postnatal development and/or regeneration. On the other hand, an increasing number of TH response genes are being identified in the adult mammals [149-151]. Functional studies of the TH response genes involved in apoptosis in mammals and frogs by using modern molecular and genetic methodologies such as the transgenic frog technology [152] should help to achieve full-understanding of the complicated mechanisms controlling apoptosis and provide insights into the likely evolutionally conserved regulatory mechanisms.

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Fig. 1. Stage-dependent apoptosis in *X. laevis* organs during metamorphosis. The developmental stages are based on Nieuwkoop and Faber [19]. Thyroid hormone (T3) levels in the plasma are high during metamorphic climax [153], when the larva-specific tail becomes entirely smaller and undetectable by stage 66. In the larval-to-adult remodeling intestine, massive apoptosis occurs only in the larval epithelium (LE, dotted area) during stages 60-62. AE, adult epithelium; CT, connective tissue; M, muscles; pre- and promet, pre- and prometamorphosis, respectively.

Fig. 2.

A diagram showing the larval-to-adult intestinal remodeling of the *X. laevis* intestine. During the onset of metamorphic climax, the larval epithelium (LE) begins to undergo apoptosis, whereas stem cells of the adult epithelium (AE) appear and actively proliferate (mitotic cells, mc). At the same time, the basal lamina becomes thick and amorphous. Through the modified basal lamina, macrophages migrate from the connective tissue (CT) into LE, but not AE. They phagocytose apoptotic bodies (ab) derived from LE and are then extruded into the intestinal lumen.

Fig. 3. Model of apoptotic pathways of the larval epithelium during *X. laevis* intestinal remodeling. T3, some of which is converted from T4 by deiodinases D1 or D2, up-regulates apoptotic genes such as caspases directly by TR/RXR (9-cis retinoic acid receptor) (the blue/green columns shown in the nuclei, respectively), the heterodimer that mediate TH signal, or indirectly through other TH response genes in the larval epithelial cell, resulting in activation of intracellular apoptotic pathway (1). On the other hand, T3 also directly up-regulates ST3 expression in fibroblasts. ST3 protein is then secreted towards the epithelium and alters the cell-laminin interaction by cleaving laminin receptor (LR) of the epithelial cell surface. This extracellular pathway also leads to apoptosis of the larval epithelial cell (2). In addition, other MMPs such as MMP-9TH may be involved in apoptosis by altering cell-ECM interactions. These intra- and extracellular pathways are integrated to cause apoptosis at the appropriate time during metamorphosis. If the larval epithelial cell remains after the remodeling, the immunological reaction by the newly formed functional adult lymphocytes may cause deleterious effect on the metamorphosing animal. See text for details.

Table 1

Location and timing of apoptosis during amphibian metamorphosis

Organs	Tissues, cell types or areas that exhibit apoptotic features	Developmental stages of massive cell death	Ref
(Larva-specific organs)			
Tail	Muscle	NF62-64 (<i>X. laevis</i>)	[28]
	Spinal cord	NF58 (<i>X. laevis</i>)	[24]
	Epidermis	TK XIX-XX (<i>R. japonica</i>)	[32]
	Notochord	NF63 (<i>X. laevis</i>)	[27]
Gill	Not specified (probably vascular and parenchymal cells)	NF62 (<i>X. laevis</i>), TK XXII (<i>R. catesbeiana</i>)	[34, 36]
(Remodeling organs)			
Brain	Ventral part of the anterior brain (the subpallium area)	NF61 (<i>X. laevis</i>)	[38]
	Mesencephalic fifth nucleus of the trigeminal nerve	NF61 (<i>X. laevis</i>), TK XXI (<i>R. pipiens</i>)	[40, 41]
Intestine	Epithelium	NF60, 61 (<i>X. laevis</i>)	[45]
	Fibroblast	NF61-62 (<i>X. laevis</i>)	[7, 45]
Pancreas	Beta-cells and other cells (including parenchymal and acinar cells)	NF62 (<i>X. laevis</i>), TK XX-XXI (<i>R. catesbeiana</i> , <i>R. pipiens</i>), Rossi IX (<i>B. bufo</i>)	[47-50]
Spleen	Lymphocytes in the red pulp	NF59 (<i>X. laevis</i>)	[53]
Thymus	Lymphocytes in the medulla	NF62, 63 (<i>X. laevis</i>)	[53]
Blood	Red blood cells	NF60 (<i>X. laevis</i>)	[57]
Skin	Apical and skein cells	NF60 (<i>X. laevis</i>)	[79]

Table 2

TH-regulated genes involved in apoptosis of larval cells/tissues.

Genes	Function, role or phenotype	Ref
Type II deiodinase (D2)	T4 to T3 conversion, Metamorphosis (↑)	[70, 74]
Type III deiodinase (D3)	Degradation of T3, Metamorphosis (↓)	[70, 75]
Thyroid hormone receptors (TR)	Metamorphosis (↑)	[77-80]
Bax	Activation of caspase-9, Apoptosis in the tail muscle and probably in other tissues (↑)	[28, 91]
BH3-interacting death agonist (BID)	Apoptosis of the muscle cells (↑)	[94]
xR11 (Bcl-Xl)	Bax-induced apoptosis in the muscle and brain (↓), tBID-induced apoptosis in the muscle (↓), Apoptosis in Rohon-Beard neurons (↓)	[93, 94]
Caspase-1	Apoptosis of XLT-15-11 cell (↑), Involvement in apoptosis in the tail and intestine	[30, 98]
Caspase-2 and -8	Activation of BID (potential), Apoptosis of XLT-15-11 cell (↑), Involvement in apoptosis in the tail and intestine	[94, 98]
Caspase-3	Apoptosis of XLT-15 and XLT-15-11 cells (↑), Involvement in apoptosis in the tail and skin	[30, 79, 98, 99]
Caspase-6, -7 and -10	Apoptosis of XLT-15-11 cell (↑), Involvement in apoptosis in the tail and intestine	[30, 98]
Caspase-9	Apoptosis of XLT-15-11 cell (↑), Involvement in apoptosis in the tail, intestine and brain	[30, 38, 98]
Tumor necrosis factor (TNF) α and TNF receptor (TNFR) 1	Apoptosis of XLgoo cell (↓), Tail degradation (↓)	[106]
Cyclin-dependent-kinase (Cdk) 2	Tail regression (↓)	[109]
Protein kinase C	Tail regression (↑)	[110]
Cyclin C/Cdk8	Tail regression (↑)	[111]

(↑) induction or promotion; (↓) inhibition