

## Thyroid hormone regulation of apoptotic tissue remodeling during anuran metamorphosis

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### ABSTRACT

Anuran metamorphosis involves systematic transformations of individual organs in a thyroid hormone (TH)-dependent manner. Morphological and cellular studies have shown that the removal of larval organs/tissues such the tail and the tadpole intestinal epithelium is through programmed cell death or apoptosis. Recent molecular investigations suggest that TH regulates metamorphosis by regulating target gene expression through thyroid hormone receptors (TRs), which are DNA-binding transcription factors. Cloning and characterization of TH response genes show that diverse groups of early response genes are induced by TH. The products of these TH response genes are believed to directly or indirectly affect the expression and/or functions of cell death genes, which are conserved at both sequence and function levels in different animal species. A major challenge for future research lies at determining the signaling pathways leading to the activation of apoptotic processes and whether different death genes are involved in the regulation of apoptosis in different tissues/organs to effect tissue-specific transformations.

**Key words:** *Xenopus laevis*, thyroid hormone receptor, extracellular matrix, apoptosis.

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### INTRODUCTION

Anuran metamorphosis is a postembryonic developmental process initiated and controlled by thyroid hormone (TH)[1],[2]. TH exerts its effects on target tissues via binding to thyroid hormone receptors (TRs), which are transcription factors that belong to the nuclear receptor super-family. TR modulates gene expression by binding to specific DNA sequences in target genes, most likely by forming a heterodimer with retinoid X receptors (RXRs, or 9-cis retinoic acid receptors)[3],[4]. Thus, it is believed that TH induces a gene regulation cascade in indi-

vidual tissues/organs to effect their transformations.

Although TH controls all transformations during metamorphosis, individual tissues/organs undergo distinct changes at different developmental stage[1],[2]. Some organs, such as the limbs, develop de novo, likely from undifferentiated cells preserved in the tadpoles. Others, such as the tail, undergo complete resorption. The vast majority of the organs, such as the intestine and brain, are present both in the tadpole and frog. They undergo partial yet drastic remodeling to adapt to their function in the frog, which have different diets and living habitats. Extensive morphological, cytological, and biochemical analyses have shown that apoptosis is an essential aspect of many of the changes during metamorphosis. In this article, we will first review some of these studies. We will then provide a brief summary on

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Abbreviations: TH, Thyroid Hormone; TR, Thyroid Hormone Receptor; MMP, Matrix metalloproteinase; ECM, Extracellular matrix. Received Nov-8-2000 Revised Dec-26-2000 Accepted Jan-9-2001

the genes known to be involved in the regulation and execution of apoptosis based on studies in different animal species. Finally, we will discuss the molecular pathways induced by TH that lead to apoptotic tissue transformations during metamorphosis.

### APOPTOSIS DURING ANURAN METAMORPHOSIS

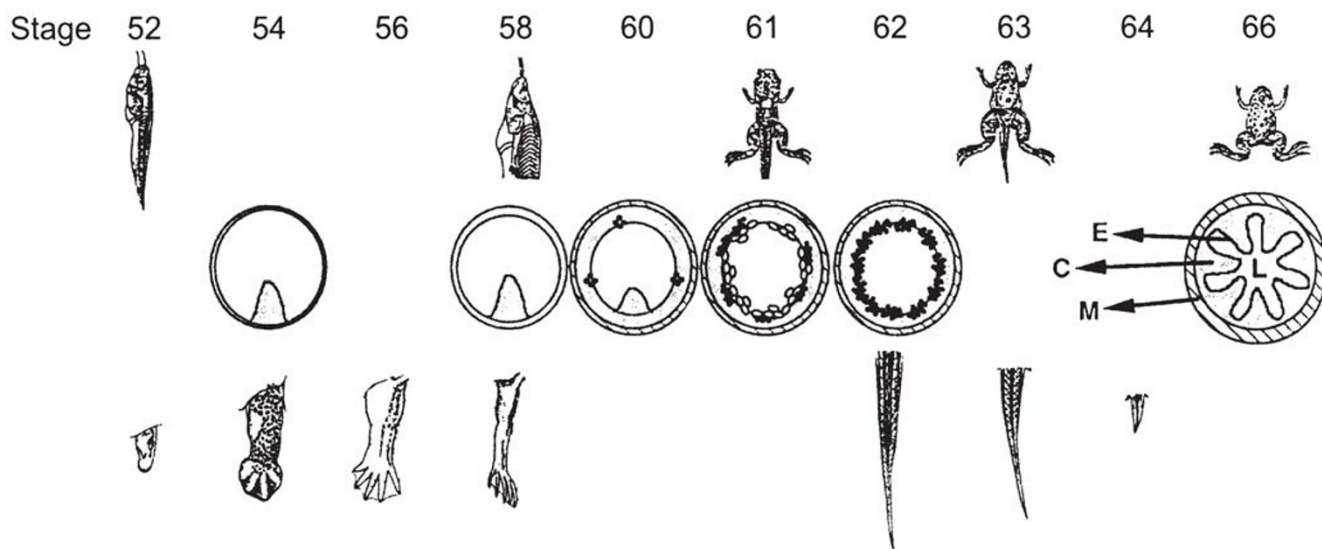
Many larval tissues are removed during metamorphosis and it is presumed that their removal is due to cell death caused by TH on these target tissues (Fig 1). Kerr et al[5] were the first to demonstrate that apoptosis occurs during anuran metamorphosis. They examined electron microscopically the resorption of the tail muscle and epidermal cells during metamorphosis of the dwarf tree frog *Litoria glauerti*. They found that the two major cell types, epidermal and muscle cells, of the tail undergo a series of well defined, sequential morphological changes of apoptosis, including the condensation of the cytoplasm and the nuclear chromatin and the subsequent formation of the apoptotic bodies. Similar findings have also been reported for *Rana japonica*[6] and *Xenopus laevis*[7].

Intestinal remodeling represents the second type

of transformations during metamorphosis. The tadpole intestine is a simple tubular structure consisting of predominantly larval epithelial cells surrounded by sparse connective tissue and muscles (Fig 1)[8]. During metamorphosis, the larval epithelium undergoes complete degeneration and is replaced by adult epithelium. Morphological and biochemical analyses indicate that the larval epithelial cells undergo apoptosis and the apoptotic bodies are removed at least in part through phagocytosis by macrophages migrating over from the connective tissue[8].

While it is expected that tissue resorption and remodeling require removal of larval cells, the de novo development of limbs is likely to involve cell death as well (Fig 1). As the limbs undergo morphogenesis, the inter-digital cells have to be selectively removed, although it remains to be shown that their death is apoptotic.

Several studies indicate that TH appears to induce cell death mainly by targeting the dying cells directly in the tail and intestine. First, isolated epidermal cells from the tail of *Rana catesbeiana* tadpoles can be induced to die in vitro when cultured in the presence of TH[9], suggesting that the TH-dependent epidermal cell death is cell autonomous,



**Fig 1.** Stage-dependent organ transformations during *Xenopus laevis* metamorphosis.

The developmental stages are from Nieuwkoop and Faber[54]. The tails at stages 62-66 are drawn to the same scale to show the resorption (no tail remains by stage 66), while the tadpoles, intestinal cross-sections (middle) and the hindlimbs at different stages are not in the same scale in order to show the morphological differences. Tadpole small intestine has a single epithelial fold, where connective tissue (C) is abundant, while a frog has a multiply folded intestinal epithelium (E), with elaborate connective tissue and muscle (M). Dots: proliferating adult intestinal epithelial cells. Open circles: apoptotic primary intestinal epithelial cells. L: intestinal lumen.

at least when cultured in isolation in vitro. Second, a cell line has been established from *Xenopus laevis* tail muscles and found to respond to TH by undergoing apoptosis[10]. Finally, we have isolated the larval epithelial cells from the intestine of *Xenopus laevis* tadpoles and cultured them in vitro in the presence or absence of TH[11],[12]. Our results indicate that the larval epithelial cells are induced to die by physiological concentrations of TH. Biochemical and morphological analyses have shown that the TH-induced epithelial cell death has all the characteristics of apoptosis, including the formation of apoptotic bodies and nucleosomal sized nuclear DNA fragments. Interestingly, when the larval intestinal epithelial cells were cultured on plastic dishes coated with various components of the ECM, such as fibronectin and laminin, etc., the TH-induced cell death was inhibited[11]. However, the ECM components failed to influence TH-stimulated cellular DNA synthesis and the downregulation of two epithelial specific genes. These results indicate that the ECM preferentially affects cell death. They further suggest that the apoptosis is not entirely cell autonomous in vivo but depends on cell-cell and/or cell-ECM interactions. Such a conclusion is also consistent with findings from organ culture studies. For example, the removal of tail epidermis prevents TH-induced tail resorption in organ cultures[13]. On the other hand, there is also evidence to suggest that adult-type non-T leukocytes may participate in the specific elimination of larval tail cells[14]. In addition, when intestinal fragments are cultured in the presence of TH, larval cell death takes place within 2-3 days, followed by the development of adult epithelium after 5-7 days[8]. When isolated epithelium is cultured with TH, only cell death occurs. The development of the adult epithelium requires the presence of the connective tissue as co-culturing the larval epithelium and connective tissue restores adult epithelial development. Thus, cell-cell interactions are likely important for both larval tissue degeneration and adult tissue development.

## CELL DEATH GENES

Many genes involved in cell death have been isolated and characterized. The first genes were identified from genetic studies in the nematode *Caenorhaditis elegans*[15], [16]. During normal de-

velopment of *C. Elegans*, 131 out of the 1090 somatic cells in the developing animal undergo programmed cell death. While the signals for the death of these 131 cells are still under investigation, analyses of mutants that are defected at various steps of the apoptotic process have led to the cloning of many genes involved at the death execution or later steps. Subsequently, the homologs of some of these genes were found in mammals and shown to have similar functions[17-20]. The best studied among them are those acting at the execution step, where three *C. Elegans* genes, *ced-3*, *ced-4*, and *ced-9*, are involved. The *ced-3* and *ced-4* are cell death promoters, while *ced-9* inhibits cell death. The mammalian homologs of these *C. Elegans* death genes have been cloned and their gene products have been characterized biochemically. The most extensively studied are the *ced-3* homologs, which encode cysteine proteases capable of cleaving after an aspartic acid residue within a substrate[17],[21]. Over 10 such proteases, now referred to as caspases, exist in mammals. Upregulation of these caspases through increased expression and/or activation of the proenzymes leads to the degradation of various cellular substrates and cell death. Gene knockout studies in mice provide direct support for different caspases in apoptosis of different tissues/organs in development[22].

The mammalian homologs of *ced-9* are the *bcl-2* family members[18], [19],[23],[24]. Interestingly, there are two subfamilies within the *bcl-2* superfamily, the *bcl-2* and *bax* subfamilies. The *bcl-2* subfamily members protect cells from apoptosis while the *bax* subfamily members promote cell death. Many members of this family can form homodimers and/or heterodimers and thus the balance of the two subfamilies can affect cell fate. The exact mechanisms by which they regulate apoptosis remain to be determined. One likely mechanism for the *bcl-2* subfamily to inhibit apoptosis was suggested by the cloning and characterization of *ced-4* and its mammalian homolog Apaf-1[20],[25]. Apaf-1 (*ced-4*) can interact directly with caspases (*ced-3*), thus participating directly in caspase activation. Interestingly, *ced-4* also interacts with *ced-9* directly, suggesting that *ced-9* (*bcl-2*) may directly inhibit caspase activation through this interaction. In mammals, in addition to Apaf-1, at least another factor, Apaf-3 or cytochrome C, is also required for caspase activation

[25]. Cytochrome C resides in the space between the inner and outer membranes of mitochondria. There is evidence to suggest that bcl-2 family members can regulate caspases activation by influencing cytochrome C release either directly or indirectly.

There are at least two other classes of proteins participating in apoptotic execution or its regulation in mammals. The first are the inhibitors of apoptosis (IAPs)[25],[26]. These proteins bind to caspases directly and thus block caspase function. Their degradation appears to be an important factor in the commitment of cells to apoptosis. The second class includes nucleases that degrade nuclear DNA during apoptosis[20],[27-29]. The best characterized is the DNA fragmentation factor (DFF), which is a heterodimer of caspase-activated DNase (CAD) or DFF40 and inhibitor of CAD (ICAD) or DFF45[27]. While CAD can cleave chromosomal DNA to generate the nucleosomal DNA ladder that is commonly observed in vertebrate apoptosis, the heterodimer is inactive. The cleavage of ICAD triggered by apoptotic signals activates CAD and leads to the cleavage of nuclear DNA, thus irreversibly committing the cell to the apoptotic pathway.

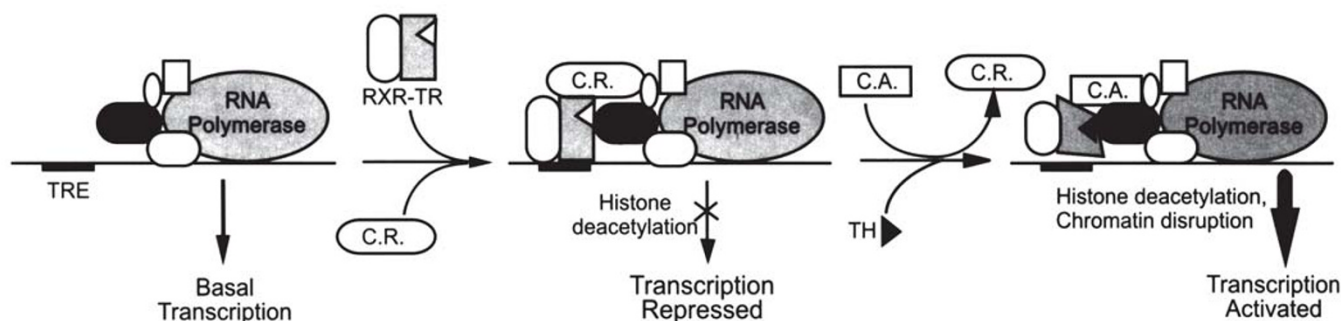
In addition to the above genes, there are also many genes involved in the subsequent steps, i.e., the engulfment and degradation of dead cells or the resulting apoptotic bodies[30], [31]. Relatively little is known about these steps in mammals. On the other hand, it is expected that like the other genes partici-

pating in execution of apoptosis as described above, these genes are likely to be conserved among different apoptotic processes.

## GENE REGULATION BY TR<sub>S</sub>

TH is believed to induce a series of gene regulation steps during metamorphosis. The first step is the transcriptional regulation of direct TH response genes by TRs (Fig 2). TRs are transcription factors that recognize specific DNA sequences, or TH response elements (TREs), in their target genes. Numerous in vitro and tissue culture cells studies as well as in vivo functional studies in developing *Xenopus laevis* indicate that TRs are constitutively bound to TREs in chromatin as heterodimers formed with RXRs[3, 4, 32-34]. In the absence of TH, TR/RXR heterodimers can recruit corepressor complexes containing histone deacetylases to the target genes, which leads to histone deacetylation to facilitate gene repression by the receptors. When TH is present, the corepressor complexes are released and are replaced by coactivator complexes, which often have histone acetyl transferase activity. Thus, it is hypothesized that TH activates gene transcription in part through increasing local histone acetylation (Fig 2).

Our own studies in the reconstituted TH-dependent *Xenopus* oocyte system and our analyses on endogenous TH-response genes during *Xenopus* development have shown directly that TR/RXR heterodimers are bound to TRE in chromatin in in



**Fig 2.** A model for transcriptional regulation by TR. TR is assumed to form a heterodimer with RXR. The heterodimer can bind to the TRE in a target gene. The binding of an unliganded TR-RXR heterodimer leads to transcriptional repression possibly through the recruitment of a corepressor complex (C.R.) and/or interaction with the basal transcription machinery. Upon binding by TH, a conformational change takes place in the heterodimer, which may be responsible for the release of the corepressor and recruitment of a coactivator complex (C.A.), thus leading to transcriptional activation. In addition to RNA polymerase, many other factors are required as depicted in part in the Figure.

vivo and repress target gene expression in a process requiring histone deacetylase activity[32],[33]. Furthermore, TH treatment of premetamorphic tadpoles leads to local increase in histone acetylation specifically at the target genes at least in the tail and the intestine, both of which undergo extensive apoptotic degeneration of the larval tissues. In addition, treating the tadpoles with the histone deacetylase inhibitor trichostatin A can activate the TH responses genes and increase histone acetylation levels at TH target genes in these organs. Thus, unliganded TRs appear to recruit histone deacetylase complexes to repress gene expression at least in these two organs. In support of this, a known TR-binding corepressor, N-CoR, is expressed in premetamorphic tadpoles (Sachs and Shi, unpublished data) and at least two N-CoR containing histone deacetylase complexes exist in *Xenopus*[35]. The release of these or other corepressor complexes upon TH binding to TRs and the concurrent recruitment of coactivator complexes, which remain to be characterized in amphibians, are likely responsible for the activation of genes involved in metamorphosis. The presence of different corepressor or coactivator complexes and variation in the levels of such complexes may play an important role in determining the gene regulation programs induced by TH in individual organs/tissues.

## GENES INVOLVED IN TH-INDUCED APOPTOSIS

### *Early TH response genes*

The dependence of anuran metamorphosis on TH allows one to identify genes that are involved in mediating the effects of the TH signal in apoptosis in different tadpole tissues by using various molecular approaches. Many of the genes regulated by TH during *Xenopus laevis* metamorphosis have been isolated by using PCR-based subtractive hybridization with mRNAs from different organs of premetamorphic tadpoles with or without TH treatment[2]. Characterization of the early genes, i.e., those regulated by TH within a 24 h treatment period, shows that diverse groups of genes are induced by TH in organs such as the limb, tail, intestine, and brain. In general, these TH response genes include transcription factors, signaling molecules, matrix metalloproteinases (MMPs), and others.

Most of the transcription factors and MMPs are expressed in different organs and their spatial and temporal regulation during development and TH treatment suggests that they act upstream of the gene regulation steps that specify tissue specific changes during metamorphosis. The transcription factors are expected to regulate downstream genes directly. Currently, little is known about their target genes and their roles in larval cell death remains to be established.

We have been studying the roles of MMPs in larval tissue degeneration. MMPs are Zn<sup>2+</sup>-dependent proteinases that are extracellular or bound to plasma membrane[36-40]. They can cleave specific proteinaeous components of the extracellular matrix (ECM) and non-ECM proteins. Numerous studies in mammals have suggested that MMPs are involved in diverse developmental and pathological processes by modifying or degrading the ECM, thus influencing cell behavior[41-43]. Through gene expression analyses, we and others have shown that *Xenopus* collagenase-3, collagenase-4, and stromelysin-3 (ST3) are expressed in tissues where apoptosis occurs during metamorphosis[33]. In particular, we have demonstrated a tight spatial and temporal correlation of ST3 mRNA and protein with ECM remodeling and epithelial apoptosis during intestinal metamorphosis(Fig 3). Organ culture studies have provided direct support for a requirement of MMP activity in TH-induced epithelial cell death in the intestine (Fig 4)[45]. Thus, ST3 may modify the ECM to influence larval cell death. Further studies are needed to determine the signaling pathways mediating the effect of ST3.

### *Late TH response genes*

Noticeably absent in the original screens for early TH response genes are cell death genes described above. This may not be surprising considering that cell death is a late event and that cell death genes are unlikely regulated by TH early in the gene regulation cascade. Interestingly, a PCR-based subtractive screen for genes regulated in the *Xenopus* intestine after a 4-day TH treatment of premetamorphic tadpoles also failed to isolate any cell death genes[2],[46]. It did, however, isolate many late TH response genes. These genes again fall into several categories, including transcription factors and signaling molecules. The isolation of such genes is

consistent with the idea that multiple gene regulation steps are needed to complete the transformation of the intestine. Unfortunately, few studies have been carried out to reveal any potential roles of these genes in apoptosis.

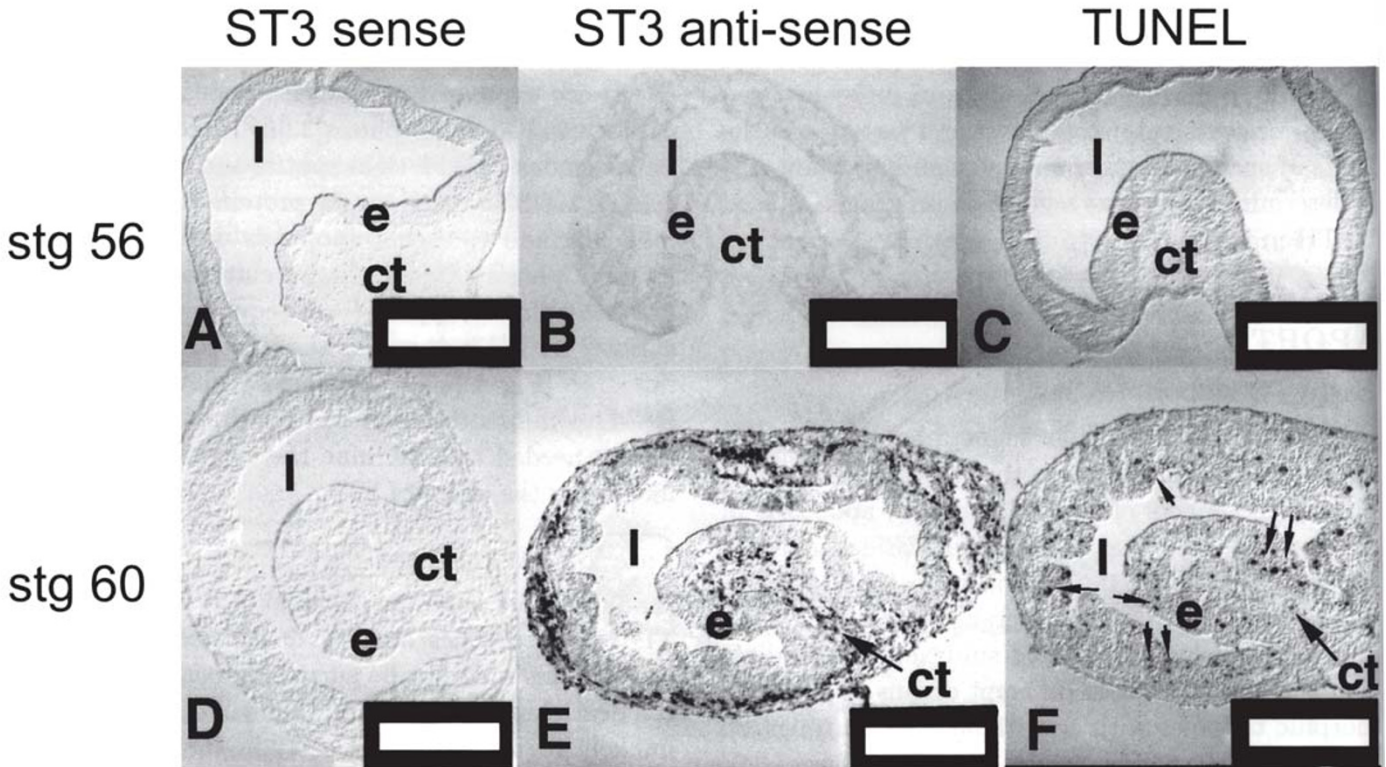
*Cell death genes during metamorphosis*

Although no cell death genes were isolated in various screens for TH response genes, the similar biochemical and morphological properties between apoptosis during anuran metamorphosis and that in other animal species suggest the involvement of similar cell death genes. This is further supported by the ability of known apoptosis inhibitors, i.e., those against caspases and nucleases that degrade proteins and DNA, respectively, to inhibit TH-induced apoptosis in primary cultures of tadpole intestinal

epithelial cells[11] or in cultures of a cell line derived from tadpole tail muscles[10].

Based on the likely conservation in sequences among anuran and mammalian cell death genes, several genes encoding caspases and bcl-2 family members have been cloned in *Xenopus laevis*[47], [48]. Sequence analyses have shown that these genes are indeed highly homologous to their mammalian counterpart and have similar molecular functions when expressed in tissue culture cells.

The *Xenopus* caspase genes are expressed in the tail during metamorphosis[10],[47]. While they are upregulated by TH treatment of premetamorphic tadpoles, their upregulation by TH is relatively small and requires more than one day of TH treatment. Thus, they are late TH response genes and their small magnitudes of regulation by TH may



**Fig 3.** Association of ST3 expression with larval epithelial cell death during intestinal remodeling. Stages 56 (A, B, C) and stage 60 (C, D, E) intestines were analyzed by in situ hybridization for ST3 expression (A, B, D, E) and by TUNEL assay for apoptotic cell detection (C, F). There was no detectable ST3 expression at stage 56 (B). ST3 expression was strong in all regions of the connective tissue at stage 60 but not in the epithelium or muscles (E). Likewise, at stage 56, there was no apoptotic signals (C), while there were many apoptotic cells, mainly within the epithelium, at stage 60 (arrows). Note that the control hybridization with the sense RNA probe did not detect any signal, as expected. ct, connective tissue; e, epithelium; l, lumen. White bars: 250  $\mu$ m. From Damjanovski et al.[55].

explain why they escaped the differential screens. Their presence during metamorphosis suggests that they participate in TH-induced apoptosis.

The two *bcl-2* family members cloned in *Xenopus* belong to the subfamily of cell death inhibitors [48],[49]. They are capable of inhibiting cell death when expressed in tissue culture cells or in *Xenopus* tadpoles. On the other hand, limited expression studies suggest that they are expressed at only low levels and/or their expression levels do not change during metamorphosis. Thus, it is unclear whether they play any roles in cell fate regulation during metamorphosis.

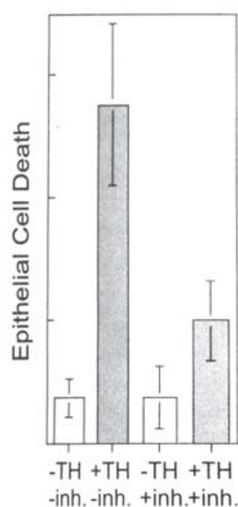
## CONCLUSION AND PROSPECTS

Programmed cell death or apoptosis plays important roles in development of animals from *C. elegans*, *Drosophila*, to human, and is critical for the physiological function of many organs, such as the immune system[50-53]. Anuran metamorphosis is a postembryonic process that is absolutely dependent upon the presence of TH. The degeneration of larval tissues through programmed cell death during this process offers a unique opportunity to study the molecular pathways that lead to apoptosis of different cell types during postembryonic development in vertebrates. Existing studies suggest that the apoptosis during anuran metamorphosis likely utilize the same cell death genes as those in mammals. The cloning and functional characterization of the cell death genes should provide insights on whether different cell death genes are involved in the apoptosis of different larval tissues. The causative

effects of TH on metamorphosis are mediated by TRs. The analyses of the TH response genes suggest that TH induces initially the expression of several diverse groups of genes. A major challenge will be to determine how these early genes in turn influence the expression and/or the function of cell death genes. The availability of a large array of molecular and genetic approaches should make such endeavors not only feasible but also highly rewarding in the years to come.

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**Fig 4.** Blocking MMP function inhibits TH-induced epithelial apoptosis. Intestinal explants were cultured for 3 days in the presence or absence of TH and/or a synthetic MMP inhibitor (inh.). The apoptosis in the epithelium was detected with the TUNEL method and the number of labeled nuclei (apoptotic cells) was quantified for each treatment. See Ishizuya-Oka et al.[45] for more details.

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