Differential Regulation of Cell Type-specific Apoptosis by Stromelysin-3

**A POTENTIAL MECHANISM VIA THE CLEAVAGE OF THE LAMININ RECEPTOR DURING TAIL RESORPTION IN XENOPUS LAEVIS**

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Matrix metalloproteinases (MMPs) have been extensively studied because of their functional attributes in development and diseases. However, relatively few in vivo functional studies have been reported on the roles of MMPs in postembryonic organ development. Amphibian metamorphosis is a unique model for studying MMP function during vertebrate development because of its dependence on thyroid hormone (T3) and the ability to easily manipulate this process with exogenous T3. The MMP stromelysin-3 (ST3) is induced by T3, and its expression correlates with cell death during metamorphosis. We have previously shown that ST3 is both necessary and sufficient for larval epithelial cell death in the remodeling intestine. To investigate the roles of ST3 in other organs and especially on different cell types, we have analyzed the effect of transgenic overexpression of ST3 in the tail of premetamorphic tadpoles. We report for the first time that ST3 expression, in the absence of T3, caused significant muscle cell death in the tail of premetamorphic transgenic tadpoles. On the other hand, only relatively low levels of epidermal cell death were induced by precocious ST3 expression in the tail, contrasting what takes place during natural and T3-induced metamorphosis when ST3 expression is high. This cell type-specific apoptotic response to ST3 in the tail suggests distinct mechanisms regulating cell death in different tissues. Furthermore, our analyses of laminin receptor, an in vivo substrate of ST3 in the intestine, suggest that laminin receptor cleavage may be an underlying mechanism for the cell type-specific effects of ST3.

The extracellular matrix (ECM), the dynamic milieu of the cell microenvironment, plays a critical role in dictating the fate of the cell. The cross-talk between the cell and ECM and the timely catabolism of the ECM are crucial for tissue remodeling during development (1). Matrix metalloproteinases (MMPs), extrinsic proteolytic regulators of the ECM, mediate this process to a large extent. MMPs are a large family of Zn$^{2+}$-dependent endopeptidases potentially capable of cleaving the extracellular as well as nonextracellular proteins (2–9). The MMP superfamily includes collagenases, gelatinases, stromelysins, and membrane-type MMPs based on substrate specificity and domain organization (2–4). MMPs have been implicated to influence a wide range of physiological and pathological processes (10–13). The roles of MMPs appear to be very complex. For example, MMPs have been suggested to play roles in both tumor promotion and suppression (13–19). Unfortunately, relatively few functional studies have been carried out in vivo, especially in relation to the mechanisms involved during vertebrate development.

Amphibian metamorphosis presents a fascinating experimental model to study MMP function during postembryonic development. A unique and salient feature of the metamorphic process is the absolute dependence on the signaling of thyroid hormone (20–23). This makes it possible to prevent metamorphosis by simply inhibiting the synthesis of endogenous T3 or to induce precocious metamorphosis by merely adding physiological levels of T3 in the rearing water of premetamorphic tadpoles. Gene expression screens have identified the MMP stromelysin-3 (ST3) as a direct T3 response gene (24–27). Expression studies have revealed a distinct spatial and temporal ST3 expression profile in correlation with metamorphic event, especially cell death (25, 28–31). Organ culture studies on intestinal remodeling have directly substantiated an essential role of ST3 in larval epithelial cell death and ECM remodeling (32). Furthermore, precocious expression of ST3 alone in premetamorphic tadpoles through transgenesis is sufficient to induce ECM remodeling and larval epithelial apoptosis in the tadpole intestine (33). Thus, ST3 appears to be necessary and sufficient for intestinal epithelial cell death during metamorphosis.

ST3 was first isolated as a breast cancer-associated gene (34), and unlike most other MMPs, ST3 is secreted as an active protease through a furin-dependent intracellular activation mechanism (35). Like many other MMPs, ST3 is expressed in a number of pathological processes, including most human carcinomas (11, 36–40), as well as in many developmental processes in mammals (10, 34, 41–43), although the physiological and pathological roles of ST3 in vivo are largely unknown in mammals. Interestingly, compared with other MMPs, ST3 has
only weak activities toward ECM proteins in vitro but stronger activities against non-ECM proteins like α1 proteinase inhibitor and IGFBP-1 (44–46). Although ST3 may cleave ECM proteins strongly in the in vivo environment, these findings suggest that the cleavage of non-ECM proteins is likely important for its biological roles. Consistently, we have recently identified a cell surface receptor, laminin receptor (LR) as an in vivo substrate of ST3 in the tadpole intestine during metamorphosis (47–49). Analyses of LR expression and cleavage suggest that LR cleavage by ST3 is likely an important mechanism by which ST3 regulates the interaction between the larval epithelial cells and the ECM to induce cell death during intestinal remodeling (47, 48).

Here, to investigate the role of ST3 in the apoptosis in other tissues during metamorphosis and whether LR cleavage serves as a mechanism for ST3 to regulate the fate of different cell types, we have analyzed the effects of precocious expression of ST3 in premetamorphic tadpole tail. The tail offers an opportunity to examine the effects of ST3 on different cell types. The epidermis, the fast and slow muscles, and the connective tissue underlying the epidermis in the myotendinous junctions and surrounding the notochord constitute the major tissue types in tail (50). Even though death is the destiny of all these cell types, it is not clear whether they all die through similar or different mechanisms. Microscopic and histochemical analyses have shown that at least the muscle and epidermal cells undergo T3-dependent apoptosis during metamorphosis (23, 29, 51, 52). To study whether ST3 regulates apoptosis of these two cell types, we have made use of the transgenic animals that express a transgenic ST3 under the control of a heat shock-inducible promoter (33). We show that whereas extensive apoptosis is present in both the epidermis and muscles during natural as well as T3-induced metamorphosis, transgenic expression of ST3 induces cell death predominantly in the muscles. Furthermore, we show that LR is expressed in the epidermis and connective tissue but not in muscles of the tadpole tail. More importantly, LR cleavage products are present in the tail during natural metamorphosis but not in transgenic tadpoles overexpressing ST3. These results suggest that ST3 has distinct effects on the epidermis and muscles in the tail, possibly because of the tissue-specific expression and function of LR.

**MATERIALS AND METHODS**

**Transgenesis and Heat Shock Treatment**—Wild type *Xenopus laevis* tadpoles were purchased from Nasco. Transgenic animals and their wild type siblings were obtained by mating parental lines generated as previously described, and the transgenic animals were identified by the expression of GFP in the lens of their eyes (33). Wild type and transgenic tadpoles were heat-shocked twice at 34 °C for 30 min, separated by 30 min at 18 °C (33, 53). The animals were maintained at room temperature throughout the experiment (about 18–21 °C). The transgenic animals were identified under a UV dissecting microscope with a chroma filter set for GFP expression in the eyes of transgenic but not wild type animals. For T3 treatment, stage 54 premetamorphic tadpoles were treated with 5 nx for 3 days. All animal studies were approved by National Institutes of Health NICHD Animal Use and Care Committee.

**Quantitative Reverse Transcription-PCR (qPCR)**—Total RNA was isolated from wild type and transgenic tadpoles as described previously (54, 55). The RNA was made DNA-free by treatment with RNase-free DNase I (Ambion) to remove any DNA contamination and repurified with TRIzol reagent (Invitrogen). For cDNA synthesis, 100 ng of total RNA was reverse-transcribed in a 20-μl reaction according to the manufacturer’s instructions for the High Capacity cDNA Archive Kit (Applied Biosystems), and 4 μl of each cDNA was used for qPCR analysis. The standard samples to produce a standard curve for qPCR analysis was made from mixed total RNA isolated from tadpoles of all stages encompassing the entire metamorphosing period and was reverse-transcribed at a higher concentration (1 μg of total RNA in a 20-μl reaction). The cDNA was diluted at series of 1:2 or 1:3 to cover the whole range of the tested samples.

Quantitative reverse transcription-PCR was carried out to quantify gene expression with ABI 7000 (Applied Biosciences) as described previously (33). For detection of both the endogenous ST3 and the ST3-GFP transgene, forward primer 5′-GGTGTCG-AAATCGTCAGAAGG-3′, reverse primer 5′-CGGATGATC-TTGTACGTAGGTT-3′, and probe 5′-ACGTGGGACAAG-ACA-3′ (Assay-by-Design, Applied Biosystems) in the ST3 coding region were used. A set of primer/probe specific for rpL8 was used as a control for RNA input of each sample, and the expression level of the gene of interest within each sample was normalized to that of rpL8 (33).

**Terminal Deoxynucleotidyltransferase-mediated Biotin-dUTP Nick End Labeling (TUNEL) Assay**—Tadpole tails were isolated and fixed in 4% paraformaldehyde in 1× phosphate-buffered saline for 4 h at room temperature. The tissues were then rinsed in PBS and transferred to 70% ethanol and processed in a tissue processor (Thermo Shandon) for histological analysis. Paraffin sections were cut at 7 μm and placed on Fisher Probe-On charged slides. Sections were processed for TUNEL assay. Slides were refixed in 4% paraformaldehyde for 15 min at room temperature and then rinsed in PBS. Permeabilization of sections was done with 20 μg/ml protease K in 10 nx Tris, pH 8, for 10 min at room temperature. After rinsing the sections with PBS, endogenous peroxidase activity was quenched for 20 min with 3% H2O2 in 90% methanol. The TUNEL reaction with or without terminal deoxynucleotidyltransferase (TdT) was carried out at 37 °C for 90 min in TdT buffer (Oncogene), 0.04 mM dUTP (Roche Applied Science), and 0.3 units/μl TdT (Invitrogen). Then the sections were rinsed in PBS, blocked for 10 min with 1% bovine serum albumin, and incubated with ABC Elite Ready-To-Use solution (Vector Laboratories, Burlingame, CA). After 30 min, antibody binding was detected with a 3,3′-diaminobenzidine staining kit (Vector). The sections are counter-stained with methyl green (Sigma).

**In Situ Hybridization**—A partial cDNA encoding GFP was obtained by PCR with pCGCG (53) as the template with the primers 5′-GAACATTTTCATGGAGGGTTGCCC-3′ (forward) and 5′-GGGCCATGCACAGGAG-3′ (reverse). The PCR product was cloned into pcRII-TOPO vector (Invitrogen) and verified by sequencing. To synthesize antisense RNA probe, these plasmids were linearized with BamHI and transcribed with T7 RNA polymerase (Roche Applied Science). In
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**Ubiquitous Expression of ST3 Transgene in the Tail of Transgenic Animals after Heat Shock Treatment**—We have previously generated transgenic animals expressing a ST3-GFP fusion transgene under the control of heat shock-inducible promoter by using a double promoter construct, where the second promoter, γ-crystallin promoter, drives the expression of GFP in the animal eye for easy identification of transgenic animals (33). To determine the relative expression levels of the transgenic ST3 upon heat shock treatment, we carried out a quantitative reverse transcription-PCR analysis using a primer/probe set, specific for both the transgenic ST3-GFP and the endogenous ST3. Tail RNA was isolated from stage 54 premetamorphic (when there is little or no T3) wild type and transgenic tadpoles treated with or without heat shock treatment for the indicated number of days and analyzed by qPCR for ST3 mRNA levels. For comparison, we also analyzed tail RNA from tadpoles at the climax of natural metamorphosis (stage 62, when endogenous T3 is at peak levels) or stage 54 premetamorphic tadpoles that were induced to metamorphose with T3 treatment (Fig. 1). Endogenous ST3 mRNA was expressed only in tadpoles undergoing natural metamorphosis (stage 62) or after T3 treatment (Fig. 1). Transgenic ST3 mRNA was expressed after 1–7 days of heat shock treatment, and its expression levels were a few folds higher than those of endogenous ST3 at stage 62, the climax of metamorphosis. The transgene expression attained a peak at 4 days of heat shock treatment for unknown reasons, but this is similar to the expression pattern observed in the intestine (33). It should be pointed out that although the ST3 transgene was expressed at much higher levels compared with endogenous ST3 in premetamorphic tadpole tail (e.g. stage 54). Endogenous ST3 is expressed at high levels by stage 62 when tail resorption begins and reaches even higher levels in the tail at later stages. The endogenous ST3 expression in the tail at stage 63 is severalfold higher than that at stage 62 (see Fig. 8A) and, thus, is comparable with that observed in the transgenic animals.

The heat shock-inducible promoter is expected to drive ubiquitous gene expression. To determine the expression pro-

**RESULTS**

**Western Blot and Immunohistochemistry**—Proteins were extracted from tail and intestine at the indicated stages of tadpoles as previously described (48). The membrane was incubated with a 1/10,000 dilution of anti-Xenopus LR antibody (48). Horseradish peroxidase-conjugated secondary antibody was detected by chemiluminescence (Amersham Biosciences ECL Plus Western Blotting Detection System). For immunohistochemistry, the tail was fixed in 4% paraformaldehyde in 1× PBS at room temperature for 4 h. The tissues were then rinsed in PBS and transferred to 70% ethanol and processed in a tissue processor (Thermo Shandon) for histological analysis. Cross-sections were cut at 7 μm and placed on Fisher Probe-On charged slides. Paraffin sections (7 μm) were incubated with 1/4,000 dilution of the anti-Xenopus LR antibody in combination with ABC elite kit (Vector) and 3,3′-diaminobenzidine for detection.

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**Transgenic Expression of ST3 Induces Apoptosis Predominantly in the Muscle Region over the Epidermal Region**—Expression studies have shown that during metamorphosis, endogenous ST3 is expressed in the fibroblastic cells underneath the dying epidermis and surrounding the muscle fibers in the tail (29, 30), suggesting a role of ST3 in T3-induced apoptosis of the epidermal and muscle cells. To investigate this possibility, we heat-shocked transgenic and wild type tadpoles at premetamorphic stage 54, i.e. before any expression of endogenous ST3, to induce transgene expression in transgenic animals. Extended heat shock treatment caused no obvious external morphological changes in either the wild type or transgenic animals. After 4 days of heat shock treatment, the tail was isolated and subjected to TUNEL assay to detect apoptotic cells. In the transgenic animals, apoptotic cells were mainly detected in the muscle region, whereas rela-

**FIGURE 1. Transgenic ST3 expression is induced in premetamorphic tadpole tail after heat shock.** Transgenic (TG) and wild type animals at stage 54 (premetamorphic) were heat-shocked (HS) for 0, 1, 4, and 7 days. Total RNA was isolated from the tail of these tadpoles as well as stage 62 metamorphosing wild type animals or stage 54 wild tadpoles treated with or without 5 nm T3 for 3 days. The total RNA was subjected to reverse transcription and quantitative PCR with a primer/probe set specific for both endogenous ST3 and the transgene ST3-GFP. A primer/probe set specific for the rpl8 gene was used as the RNA control. The ST3/ST3-GFP signals were normalized to those of rpl8. *, p value < 0.05.
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respectively. The tails from these animals were subjected to detecting the transgene (endogenous ST3 had little or no expression at this stage, see Fig. 1 and Ref. (29). Connective tissue; transgenic animals.

Heat shock treatment leads to ubiquitous expression of ST3-GFP transgene in the tail of wild type ones (Fig. 3, respectively few apoptotic cells were detected in the epidermal region (Fig. 3, B and b). Few or no apoptotic cells were detected in wild type tadpoles after heat shock treatment (Fig. 3, A and a). Quantitative analysis revealed that there were significant numbers of apoptotic cells in the muscle region of transgenic tadpoles compared with the those in the wild type (Fig. 3C), although the increase in the apoptotic signal in the epidermal region was not statistically significant in the transgenic animals compared with wild type ones (Fig. 3C). These results suggest that ST3 expression leads to the precocious apoptosis in the tail as in the case of intestine (33), but cell death occurred predominantly in the muscles of the tail.

As indicated above, expression studies have suggested a role of ST3 in epidermal cell death. To investigate whether epidermal cells have a delayed response to ST3 expression, we extended the heat shock treatment to 7 days and also treated the animals with or without T3 to induce metamorphosis. Wild type and transgenic tadpoles at stage 54 were divided into four groups, group I without any treatment, i.e. without heat shock or T3 treatment (−HS−T3), group II with T3 treatment only (−HS+T3), group III with heat shock treatment alone (+HS−T3), and group IV with both heat shock and T3 treatment (+HS+T3). All groups were treated with or without daily heat shock starting at day 0 and, when indicated, began to also receive T3 treatment on day 4 for an 3 additional days. After 7 days the tail was isolated from all groups, and cell death was analyzed by TUNEL assay. There were no apoptotic cells detected in the control group (group I), which received neither heat shock nor T3 treatment (Fig. 4A, a and B, b). As expected, T3 treatment alone (group II) resulted in apoptotic cells in both the muscle and epidermis of the tail of both wild type and transgenic tadpoles (Fig. 4, C, c and D, d). Heat shock induction of transgenic ST3 alone for 7 days induced apoptosis in both the muscle and epidermis of the tail of transgenic (Fig. 4F, f) but not wild type tadpoles (Fig. 4E, e) (group III). The group IV tadpoles which received both T3 and heat shock treatment had apoptotic cells in muscle and epidermis of both wild type and transgenic animals (Fig. 4, G, g and H, h). Quantitative analysis confirmed that there were significantly more apoptotic cells in the muscle region of transgenic tadpoles after heat shock treatment alone for 7 days compared with the wild type ones under the same conditions (Fig. 5A). Again, like the 4-day heat shock treatment, although apoptotic cells in the epidermal regions were more in the tail of transgenic animals compared with the wild type ones after heat shock treatment alone, the difference was not statistically significant under our experimental conditions (Fig. 5B). When the animals were treated with both heat shock and T3, cell death in the muscle regions was similar in the wild type and transgenic animals (Fig. 5A), whereas slightly more apoptotic cells were found in the epidermal regions of the transgenic animals compared with the wild type ones (Fig. 5B). These results revealed differential effects of ST3 on different cells in the tail, with the muscle cells much more sensitive to ST3 than the epidermal cells.

FIGURE 2. Heat shock treatment leads to ubiquitous expression of ST3-GFP transgene in the tail of transgenic animals. Transgenic (TG) tadpoles at stage 54 were treated without (−HS) (A) or with (+HS) (B, C, and D) heat shock. C and D correspond to the boxed regions in B, magnified under 10× and 40× objectives, respectively. The tails from these animals were subjected to in situ hybridization with a GFP antisense probe for detecting the transgene (endogenous ST3 had little or no expression at this stage, see Fig. 1 and Ref. (29). Ct, connective tissue; Ep, epidermis; M, muscle; Sc, spinal cord; Mc, melanocytes; Nc, Notochord. A, B, and C, scale bar = 100 μm; D, scale bar = 10 μm.
remodeling intestine during metamorphosis (48). To investigate whether LR plays a similar role in the tail, we heat-shocked transgenic and wild type tadpoles at stage 54 for 4 days. For comparison, naturally metamorphosing tadpoles (stage 61, when there is yet little reduction in the tail length, and stage 63, when the tail has resorbed to half of the original length) and stage 54 tadpoles treated with T3 (5 nM) for 3 days were included in the analysis. Total proteins were isolated from the tail of each group of tadpoles and subjected to Western blot analysis. Indeed, we observed LR fragments of expected sizes from ST3 cleavage in the tail of stage 63 tadpoles when rapid tail resorption takes place (Fig. 6A). On the other hand, no cleavage products were detected in the tail of stage 61-metamorphosing tadpoles, T3-treated stage 54 tadpoles, or stage 54 transgenic and wild type tadpoles treated with only heat shock (Fig. 6A). It is interesting to note that other than tadpoles at stage 63, there was no obvious reduction in tail length for any of the tadpoles, including those at stage 61 or treated with T3, suggesting that LR cleavage is correlated with rapid tail resorption, which is likely caused by massive cell death in the epidermis and muscle regions (23, 29, 51, 52).

Because heat shock treatment for 7 days led to more cell death in the transgenic animals than 4-day heat shock treatment, we investigated whether such prolonged ST3 expression led to LR cleavage in the tail. We isolated proteins from the tail of both wild type and transgenic premetamorphic tadpoles subjected to 7 days of heat shock treatment. For a positive control we also isolated proteins from the intestine of wild and transgenic premetamorphic tadpoles treated with heat shock for 3 days, which was known to cause LR cleavage in the intestine (48). Tail and intestinal protein samples from naturally metamorphosing tadpoles were also included for comparison. Western blot analysis of these protein samples showed that the two LR bands of sizes expected from ST3 cleavage and comparable with the sizes of in vitro cleavage products were found in the intestine of stage 62 metamorphosing tadpoles and heat-shocked premetamorphic tadpoles treated with heat shock for 3 days.

**Tissue-specific Cleavage of LR in the Tail during Metamorphosis**—As reviewed in the introduction, ST3 has relatively weak activities toward ECM proteins but can cleave several non-ECM proteins. In particular, we have shown that LR is a conserved substrate of ST3 and is cleaved by ST3 in the remodeling intestine during metamorphosis (47, 48). Spatiotemporal expression of LR and its cleavage suggest that LR cleavage by ST3 plays a role in T3-induced larval epithelial cell death in the tail during metamorphosis (47, 48). To investigate whether LR plays a similar role in the tail, we heat-shocked transgenic and wild type tadpoles at stage 54 for 4 days. For comparison, naturally metamorphosing tadpoles (stage 61, when there is yet little reduction in the tail length, and stage 63, when the tail has resorbed to half of the original length) and stage 54 tadpoles treated with T3 (5 nM) for 3 days were included in the analysis. Total proteins were isolated from the tail of each group of tadpoles and subjected to Western blot analysis. Indeed, we observed LR fragments of expected sizes from ST3 cleavage in the tail of stage 63 tadpoles when rapid tail resorption takes place (Fig. 6A). On the other hand, no cleavage products were detected in the tail of stage 61-metamorphosing tadpoles, T3-treated stage 54 tadpoles, or stage 54 transgenic and wild type tadpoles treated with only heat shock (Fig. 6A). It is interesting to note that other than tadpoles at stage 63, there was no obvious reduction in tail length for any of the tadpoles, including those at stage 61 or treated with T3, suggesting that LR cleavage is correlated with rapid tail resorption, which is likely caused by massive cell death in the epidermis and muscle regions (23, 29, 51, 52).
transgenic but not wild type premetamorphic tadpoles (Fig. 6B), in agreement with earlier observations (48). The two higher molecular weight products were present in both wild type and transgenic tadpole intestine samples and, thus, independent of the ST3 transgene (Fig. 6B) (48). In contrast, in the tail samples no cleavage products were detected even after 7 days of heat shock treatment of the transgenic tadpoles, although again naturally metamorphosing tadpoles at stage 63 had LR cleavage in the tail (Fig. 6B).

As extensive cell death occurs in the muscles but not the epidermis of ST3 transgenic animals that had no detectable LR cleavage, LR cleavage likely occurs only in the epidermis when massive epidermal cell death takes place. To investigate this possibility, epidermis and muscles in the tail of stage 63 tadpoles (when the tail has resorbed to half of the original length) were separated, and proteins were isolated. Western blot analysis revealed that LR cleavage products as observed in the whole tails was found in the isolated epidermis but not the muscles (Fig. 6D). Thus, even though LR is expressed in both the epidermis and muscle regions, its cleavage only occurs in the epidermal region during rapid tail resorption (stage 63 to stage 66, the end of metamorphosis when tail is completely resorbed).

High Levels of Apoptosis in the Epidermis Correlate with LR Cleavage during Natural Metamorphosis—An earlier study with TUNEL labeling of apoptotic cells suggests that at stage 62 when there is yet little reduction in the tail length, extensive cell death takes place in the epidermis just above the subepithelial fibroblasts that express high levels of ST3 (29). This cell death
continues till the end of metamorphosis when the tail epidermis is completely resorbed. This raises the possibility that high levels of ST3 expression after stage 62 may be correlated with LR cleavage and epidermal apoptosis. To investigate this possibility, we first determined the relative cell death in the epidermis and muscles at stage 61 and stage 63 (Fig. 7). At stage 61, when tail length reduction has yet to occur, there were relatively few apoptotic cells in both the epidermis and muscle (Fig. 7, A and C). By stage 63, when the tail length is roughly

FIGURE 5. Differential effects of T3 and transgenic ST3 in inducing apoptosis in the epidermis and muscles. Quantification of the apoptotic cells in the muscle (A) or epidermal (B) region per an arbitrarily defined unit area of the tail section of wild and transgenic tadpoles. The animals were treated and analyzed as in Fig. 4. The area of the tail sections was calculated using NIH Image J Software. Five randomly chosen sections/animal of three animals/treatment group were counted. Note that ST3 overexpression for 7 days led to an increase in apoptosis in both the muscle and epidermal regions but significantly only in the muscle region, just as the results from 4-day heat shock treatment (Fig. 3C). T3 treatment induced apoptosis in both the epidermis and muscles. Overexpression of ST3 had a tendency to enhance T3-induced cell death, although not very significantly. The p values for pairwise comparisons are shown.

FIGURE 6. LR is cleaved in the tail during natural metamorphosis but not after T3 treatment or ST3 overexpression. A, LR is degraded in the tail only when rapid tail resorption takes place (stage 63). Premetamorphic transgenic (TG) and wild type animals were heat-shocked (HS) daily for 4 days, and the tail was isolated. Total proteins were isolated from these samples as well as the tail of metamorphosing tadpoles at stage 54 treated with or without 5 nM T3 or naturally metamorphosing tadpoles at stage 61 (before tail length reduction) and 63 (when the tail was half its original length). The proteins were subjected to Western blotting with anti-Xenopus LR antibody. Note that one major LR cleavage product of the size expected from ST3 cleavage (closed circle) was observed at stage 63, when endogenous ST3 was highly expressed, whereas no cleavage was detected in transgenic or T3-treated tadpoles. B, LR is cleaved in the tail and intestine at the climax of metamorphosis as well as in the intestine but not the tail of metamorphosing tadpoles. Transgenic and wild type animals were heat-shocked daily. The tail was isolated after 7 days, and intestine was isolated after 3 days (as a positive control of LR cleavage by ST3 transgene as done previously (48)). Total protein was isolated from these samples as well as from the intestine or tail of naturally metamorphosing tadpoles at stage 62 or 63 (to be consistent with earlier studies (48)). The proteins were subjected to Western blotting with anti-Xenopus LR antibody. Note that LR cleavage was observed in stage 63 tail and stage 62 intestine as well as in the intestine of transgenic tadpoles overexpressing ST3. No cleavage was detected in transgenic or T3-treated tadpoles. C, LR is cleaved in the epidermis but not the muscles of the tail at the climax of metamorphosis (stage 63). Total protein was isolated from the tail of stage 63 naturally metamorphosing tadpoles or isolated epidermis and muscles of the stage 63 tail. The proteins were subjected to Western blotting with anti-Xenopus LR antibody. The arrowhead indicates full-length LR. The faint arrowheads indicate likely nonspecific cleavage products of LR (48). The open and closed circles indicate the expected cleavage products by ST3.
halved, extensive apoptosis was found in both the muscle and epidermal cells with a higher number of apoptotic cells present in the epidermal region compared with the muscle region (Fig. 7, B and C). This contrasts sharply with that observed during T3 treatment of premetamorphic tadpoles or in ST3 transgenic animals, where much more cell death was found in the muscle region (Figs. 3C and 5).

Next we analyzed ST3 expression and LR cleavage during natural metamorphosis in the tail. In agreement with earlier observations (25, 26), ST3 mRNA expression was up-regulated by stage 60 (Fig. 8A). Much higher levels were attained by stage 62, right before rapid tail length reduction occurs. The expression reached even higher levels at stage 63, when the tail is about half that of its original length (Fig. 8A). Interestingly, Western blot analysis revealed that even though the full-length LR protein was expressed constantly throughout the tail resorption, LR cleavage was observed only at stage 62 and stage 63 (Fig. 8B) when ST3 expression was very high (Fig. 8A) and extensive epidermal cell death took place (Fig. 7C). This data suggest that LR cleavage is associated with high levels of ST3 expression and epidermal apoptosis.

Cell Type-specific Expression of LR in the Tail—One implication of the above findings is that LR is expressed in the epidermal cells to help their attachment to the ECM and that its cleavage by ST3 facilitates apoptosis. To test this possibility, immunohistochemical analysis was carried out to determine the spatial expression profile of LR in the tail. In the tail of stage 63 tadpoles when apoptosis occurs in both the epidermis and muscles, strong LR expression was found in the epidermis and in the fibroblasts present in the notochordal sheath (Fig. 9A, a, b, and c). No LR signal was found in the muscles except in area where apoptotic muscle cells were present (Fig. 9c). On the
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DISCUSSION

MMPs have been implicated to participate in the regulation of multiple aspects of cell fate and behavior including cell proliferation, adhesion, migration, differentiation, senescence, and apoptosis. However, relatively few functional studies in vivo have been reported to address the roles of MMPs during postembryonic development. Amphibian metamorphosis is a unique model to study postembryonic development because of its total dependence on T3. Making use of transgenic animals expressing ST3 under an inducible promoter, we have demonstrated here for the first time that 1) precocious expression of ST3 alone is sufficient to cause muscle cell death in the tail of premetamorphic tadpoles; 2) distinct mechanisms control the apoptosis of muscles and epidermis during tail resorption; 3) cell type-specific expression and cleavage of LR by ST3 may contribute to the distinct cell death regulation of the epidermis and muscles.

The tadpole tail offers an interesting opportunity to study developmental cell death as it consists of multiple cell/tissue types that are all resorbed by the end of metamorphosis. Earlier studies have shown that the two major tissues, the epidermis and muscles, undergo apoptosis during metamorphosis, whereas it is unknown how the third major tissue, the connective tissue, is resorbed (23, 29, 51, 52). Expression studies have provided strong evidence to implicate a role of ST3 in epidermal and muscle cell death. ST3 is strongly up-regulated during T3-induced and natural metamorphosis in the tail (25, 26). The kinetics of the response of ST3 to T3 in both intestine and tail is rapid compared with several other MMPs (25, 26, 58–61), consistent with the fact that ST3 is a direct response gene of T3 (24). This suggests that ST3 plays a role early during tail resorption. Furthermore, spatially, during tail resorption, strong expression of ST3 is present in the subepithelial fibroblasts, the fibroblasts in the notochordal sheath and surrounding the muscle fibers (29, 30). These findings suggest that ST3 secreted from the fibroblasts may directly or indirectly alter the ECM and/or cell-ECM interaction to regulate the fate of the nearby epidermal and muscle cells. Consistently, our developmental studies during natural metamorphosis revealed a strong temporal correlation between high levels of ST3 expression in the tail and high levels of apoptosis in the epidermis and muscle regions (Figs. 7 and 8). Furthermore, electron microscopic studies have revealed specific changes suggesting the catabolism of ECM before muscle cell death (51, 62). Our studies here provide direct in vivo evidence to support a role of ST3 in muscle cell death. Although transgenic ST3 is expressed ubiquitously in the transgenic animals after heat shock treatment, the secreted nature of ST3 argues that the exact cells where ST3 is expressed should not alter its function as long as ST3 is secreted to the same ECM, which is the case here. This is also supported by the transgenic studies in the tadpole intestine (33).

Surprisingly, the second major cell type in the tail that undergoes degeneration through apoptosis, i.e. the epidermis, had relatively little apoptotic response to transgenic expression of ST3. The lack of a good antibody has prevented us from determining ST3 protein levels. It is possible that ST3 protein near the epidermis in the transgenic animals is at lower levels than that during natural metamorphosis. This is unlikely as reverse transcription-PCR analysis showed that the transgene was expressed at levels even higher than the endogenous ST3 at the climax of metamorphosis when extensive epidermal cell death takes place. Furthermore, the transgene was sufficient to induce muscle cell death. More likely, the two cell types utilize distinct mechanisms to undergo apoptosis during tail resorption. ST3 plays a major and sufficient role in muscle cell death, whereas epidermal cells likely require additional T3-induced changes in order to undergo apoptosis.

The recent isolation of LR as an in vivo substrate of ST3 (48) gives us a direction to have a closer look into the functional significance of substrate cleavage by the enzyme during metamorphosis. LR was first isolated from murine fibrosarcoma cells and can bind the ECM protein laminin with high affinity in mammals (63, 64). LR cleavage by ST3 separates the laminin.
binding domain from the transmembrane domain, thus interfering with cell-ECM interactions (47, 56, 63–65). Our earlier studies have shown that LR is expressed in the larval epithelial cells of the intestine, and its cleavage is spatiotemporally correlated with T3-induced apoptosis of these epithelial cells (48). More importantly, transgenic expression of ST3 in the premetamorphic tadpole intestine induces both larval epithelial cell death and LR cleavage (33, 48), supporting a role of LR cleavage by ST3 in disrupting epithelial cell-ECM interaction and leading to apoptosis. As in the intestine, our analysis showed that LR is indeed cleaved during rapid tail resorption (stages 62–66) when ST3 is highly expressed and cell death occurs. Surprisingly, however, there were no cleavage products detected in the transgenic tail even though transgenic expression of ST3 strongly induced muscle cell death. Although it is possible that low levels of LR cleavage escaped our detection, this lack of detectable LR cleavage correlates with the relatively little effect of the transgene on apoptosis in the epidermis of the transgenic tail. In addition, we also observed that during natural metamorphosis, high levels of ST3 expression in the tail is tightly correlated with high levels of apoptosis in the epidermal region and LR cleavage (Figs. 7 and 8). One possible interpretation is that muscle cells do not express LR, and thus, ST3 induces muscle cell death independent of LR cleavage. Indeed, immunohistochemical analysis showed that LR is not expressed in the muscles but in the surrounding fibroblasts. Furthermore, Western blot analysis on isolated epidermis and muscles showed that LR in the epidermis is cleaved at the climax of metamorphosis in the tail when epidermis undergoes apoptosis. On the other hand, the LR expressed by the fibroblasts in the isolated muscles is not cleaved during natural metamorphosis when muscles undergo apoptosis. These results support a model that ST3 causes muscle cell death independent of LR cleavage.

Our Western blot analysis on proteins isolated from the epidermis indicates that the LR cleavage products in stage 63 tail are from the epidermis. Immunohistochemical localization

**FIGURE 9. Spatial expression of LR in the tail during natural metamorphosis.** Stage 63 tail sections were immunostained with anti-Xenopus LR antibody (A, a, b, c) or preimmune serum (B). Shown in A and B were composite pictures of the photos taken for different regions of the same sections. Note that LR is strongly expressed in the epidermis (a) and connective tissue (b) but not in the muscle cells except the apoptotic muscle regions, where the signals are likely because of infiltrating fibroblasts (c). M, muscle; Ep, epidermis; Nc, notochord; Ns, notochord sheath; Sc, spinal cord; Mc, melanocytes; Ap, apoptotic bodies. Scale bar in A and B = 100 μm. Scale bar in a, b, and c = 50 μm.
showed that LR is strongly expressed in the epidermis. Thus, just like the larval epithelial cells in the intestine, the tail epithelial cells appear to utilize LR to bind to the laminin in the ECM that separate the epidermis from the underlying connective tissue. The cleavage of the LR in the epidermal cells in the tail or larval epithelial cells in the intestine by ST3 expressed in the fibroblasts of the underlying connective tissue may be a common mechanism by which ST3 induces the apoptosis of these two types of epithelial cells. The lack of LR cleavage in the transgenic tadpole tail is, thus, consistent with the relatively few apoptotic cells in the tail epidermis, contrasting the high levels of apoptosis in the tail muscles in the transgenic animals. It is also worth noting that at the metamorphic stages 61 and after T3 treatment, there was also no detectable LR cleavage. However, in these animals little tail length reduction has occurred, and ST3 expression in the tail is relatively low compared with that at stage 63 when tail is resorbed to about half of its original length (25, 26, 29, 58). These may explain the lack of detectable LR cleavage in the tail of these animals, although cell death has begun in the tail epidermis. In addition, it is possible that low levels of LR cleavage, below our detection limit, do take place when a small number of epidermal cells undergo apoptosis. Thus, in the animal tail the two major tissues undergoing apoptotic resorption make use of distinct mechanisms, with the epidermis involving LR cleavage and the muscles independent of LR cleavage. Furthermore, ST3 appears to function through at least two pathways, inducing muscle cell death independent of LR cleavage in the tail while inducing apoptosis in intestinal epithelium or tail epidermis likely through an LR-dependent process. Clearly additional in vivo functional studies are needed in the future to determine the role of LR cleavage in epidermal cell death and to isolate and characterize ST3 substrates important for muscle cell death.

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