Requirement for Matrix Metalloproteinase Stromelysin-3 in Cell Migration and Apoptosis during Tissue Remodeling in *Xenopus laevis*

Atsuko Ishizuya-Oka,* Qing Li,‡ Tosikazu Amano,‡ Sashko Damjanovski,‡ Shuichi Ueda,* and Yun-Bo Shi‡

*Department of Histology and Neurobiology, Dokkyo University School of Medicine, Mibu, Tochigi 321-02, Japan; and ‡Laboratory of Molecular Embryology, National Institute of Child Health and Human Development, National Institutes of Health, Bethesda, Maryland 20892

Abstract. The matrix metalloproteinase (MMP) stromelysin-3 (ST3) was originally discovered as a gene whose expression was associated with human breast cancer carcinomas and with apoptosis during organogenesis and tissue remodeling. It has been shown previously, in our studies as well as those by others, that ST3 mRNA is highly upregulated during apoptotic tissue remodeling during *Xenopus laevis* metamorphosis. Using a function-blocking antibody against the catalytic domain of *Xenopus* ST3, we demonstrate here that ST3 protein is specifically expressed in the cells adjacent to the remodeling extracellular matrix (ECM) that lies beneath the apoptotic larval intestinal epithelium in *X. laevis* in vivo, and during thyroid hormone–induced intestinal remodeling in organ cultures. More importantly, addition of this antibody, but not the preimmune antiserum or unrelated antibodies, to the medium of intestinal organ cultures leads to an inhibition of thyroid hormone–induced ECM remodeling, apoptosis of the larval epithelium, and the invasion of the adult intestinal primodia into the connective tissue, a process critical for adult epithelial morphogenesis. On the other hand, the antibody has little effect on adult epithelial cell proliferation. Furthermore, a known MMP inhibitor can also inhibit epithelial transformation in vitro. These results indicate that ST3 is required for cell fate determination and cell migration during morphogenesis, most likely through ECM remodeling.

Key words: apoptosis • matrix metalloproteinase • *Xenopus laevis* • thyroid hormone • metamorphosis

Introduction

Tissue remodeling and organogenesis are critical aspects of postembryonic development in vertebrates. These processes require an intricate control of cell fate (apoptosis or programmed cell death versus cell proliferation and differentiation) and cell migration (Hay, 1991; Jacobson et al., 1997; Shi et al., 1998; Werb and Chin, 1998). Numerous studies with various cell culture models have demonstrated the importance of the extracellular matrix (ECM)[1] in cell fate determination and cell migration (Ruoslahti and Reed, 1994; Boudreau et al., 1995; Frisch and Ruoslahti, 1997; Shi et al., 1998). The ECM not only provides the structural support for cells within individual organs and/or tissues, but also interacts directly with the cells that it surrounds. Such interactions are important as it has been shown that blocking cell–ECM interactions by using antibodies against the cell surface receptors for ECM, such as integrins, leads to cell death. The ECM further serves as a medium through which neighboring cells interact with each other through direct contacts and signaling molecules. Finally, the ECM also stores a large number of signaling molecules such as growth factors and morphogens and their precursors (Vukicevic et al., 1992; Werb et al., 1996). Thus, any changes in the ECM will inevitably affect the behavior of the nearby cells.

The ECM is made of numerous proteinaceous and nonproteinaceous components (Hay, 1991; Timpl and Brown, 1996). Its remodeling is accomplished largely through the action of matrix metalloproteinases (MMPs). MMPs are a family of Zn^{2+}-dependent extracellular proteases (Alexander and Werb, 1991; Woessner, 1991; Birkedal-Hansen et al., 1993; Parks and Mecham, 1998). They are synthesized as preenzymes and secreted extracellularly as inactive proenzymes, with the exception of membrane-type MMPs and stromelysin-3 (ST3), which are matured to active enzymes intracellularly and are inserted into the plasma membrane and secreted extracellularly, respec-

Q. Li, T. Amano, and S. Damjanovski contributed equally to this work.

Address correspondence to Yun-Bo Shi, Laboratory of Molecular Embryology, NICHD/NIH, Bldg. 18 T, Rm. 106, Bethesda, MD 20892. Tel.: (301) 402-1004. Fax: (301) 402-1323. E-mail: shi@helix.nih.gov

[1] *Abbreviations used in this paper:* α_2M, α_2-macroglobulin; ECM, extracellular matrix; MMP, matrix metalloproteinase; ST3, stromelysin-3; TdT, terminal deoxyribonucleotidyl transferase; TH, thyroid hormone; TUNEL, TdT-mediated dUTP-biotin nick end labeling.

tively (Pei and Weiss, 1995; Uria and Werb, 1998). The proenzymes are kept inactive through the coordination of the catalytic Zn^{2+} ion by the cysteine residue conserved in the propeptide and are activated upon the removal of the propeptide through various mechanisms (Van Wart and Birkedal-Hansen, 1990; Nagase et al., 1992; Kleiner and Stetler-Stevenson, 1993; Murphy et al., 1994; Nagase, 1998).

The activated MMPs are then capable of cleaving proteinaceous components of the ECM with distinct but overlapping substrate specificities (Sang and Douglas, 1996; Uria and Werb, 1998). Thus, differential regulation and activation of different MMPs can lead to distinct ECM remodeling to facilitate various developmental and pathological processes. In fact, several MMP genes have been found to be highly expressed during tumor invasion and tissue remodeling in processes such as wound healing (Tryggvason et al., 1987; Alexander and Werb, 1991; Matrisian, 1992; Birkedal-Hansen et al., 1993; Stetler-Stevenson et al., 1993). Of particular interest among the MMPs is ST3, which was first identified as a gene associated with human breast cancer carcinomas (Basset et al., 1990). Furthermore, ST3 mRNA is also highly expressed in mammalian tissues where cell death takes place during development (Basset et al., 1990; Lefebvre et al., 1992; Muller et al., 1993). On the other hand, direct proof for the role of ST3 in apoptosis and cell migration has been lacking.

Amphibian metamorphosis is an excellent model to study the developmental functions of MMPs. Over 30 years ago, Gross and Lapiere (1962) discovered the first MMP, collagenase, in the resorbing tadpole tail. More recently, we and others have shown that ST3 gene is upregulated during *Xenopus laevis* metamorphosis (Wang and Brown, 1993; Patterton et al., 1995), a process that is controlled by thyroid hormone (TH) (Dodd and Dodd, 1976; Gilbert and Frieden, 1981). Furthermore, its mRNA is temporally and quantitatively correlated with apoptosis in various organs and tissues (Patterton et al., 1995; Ishizuya-Oka et al., 1996; Berry et al., 1998a,b; Damjanovski et al., 1999). We demonstrate here that ST3 protein is spatially and temporally associated with not only apoptosis but also the remodeling of the basal lamina underlying the degenerating larval epithelium during both natural and TH-induced intestinal metamorphosis. Furthermore, we provide direct evidence for a role of ST3 in facilitating larval epithelial cell death and adult epithelial morphogenesis during intestinal metamorphosis.

Materials and Methods

Generation of Antibody against ST3 Catalytic Domain and Western Blot Analysis

A pAb against the ST3 catalytic domain was generated by immunizing a rabbit with two synthetic polypeptides in the catalytic domain, one (amino acids 118–123; Patterton et al., 1995) coupled to a polylysine matrix, the other (amino acids 203–218) coupled to KLH (Research Genetics). The resulting antiserum appeared to recognize only the first polypeptide (amino acids 118–123; not shown).

A cDNA fragment encoding the catalytic domain (ST3-N, amino acids 86–247; Patterton et al., 1995) or the carboxyl hemopexin domain (ST3-C, amino acids 247–477; Patterton et al., 1995) was cloned into the overexpression vector pET-28a (Novagen) that contains a T7 RNA polymerase promoter. A cDNA fragment encoding the full-length ST3 was also cloned into a pSP64-based vector that contains a SP6 RNA polymerase promoter. The respective proteins were generated by using the TNT Quick Coupled Transcription/Translation System (Promega) in vitro in the presence of [^{35}S]methionine. Equal amounts of the reaction mixture were electrophoresed on triplicate SDS-protein gels. One gel was dried and autoradiographed to detect the [^{35}S]methionine-labeled in vitro–translated proteins, the other two were subjected to Western blot analysis with a pAb against the catalytic domain of ST3 or the preimmune serum as described in Ishizuya-Oka et al. (1997). For the analysis of ST3 during intestinal development, tadpole small intestine was extracted with the lysis buffer (2% SDS, 1 mM EDTA, 50 mM Tris-HCl, pH 6.8) and 10 μg/lane of the resulting protein was subjected to Western blot analysis by using the pAb.

α_2-Macroglobulin Capture Assay

[^{35}S]Methionine-labeled ST3-N was generated through in vitro translation as above and used in the α_2-macroglobulin (α_2M) capture assay as described (Reddy et al., 1994; Pei et al., 1994). 1 μl of the in vitro translation product was mixed with 5 μl of the reaction buffer (150 mM NaCl, 1 mM $CaCl_2$, 1 mM $MgCl_2$, 0.1 mM $ZnCl_2$, 20 mM Tris-HCl, pH 6.8) and 2 μl PBS containing the indicated amount of anti-ST3 or preimmune serum to reach the final concentration of 0–1%. The mixture was incubated for 30 min at room temperature before the addition of 2 μl (1 μg) of α_2M and incubation for another 60 min. The resulting mixture was then analyzed on a native polyacrylamide gel (4/20% gradient gel). The gel was stained with Coomassie blue to identify the position of the α_2M and then dried and autoradiographed to identify the α_2M–ST3 complex. It should be pointed out that preincubation of ST3 with the serum was not important, as the anti-ST3 antibody also blocked the complex formation between ST3 and endogenous serum α_2M (see Results).

α1-Antitrypsin Cleavage

ST3 catalytic domain (ST3-N) cloned in pET-28a (alone) was overexpressed in *Escherichia coli* and purified using the His-tag at the NH_2 terminus on a Ni-NTA spin column according to the manufacturer's instructions (QIAGEN). The ST3 was eluted into 0.5 M NaCl, 10 mM $CaCl_2$, 200 mM imidazole, 0.1% Triton X-100, 20 mM Tris-HCl, pH 7.9. The purified ST3 (1 μl of 40 ng/μl) was incubated overnight with 1.5 μg of human α1-antitrypsin (Athens Research and Technology, Inc.) in 20 μl of 200 mm NaCl, 10 mM $CaCl_2$, 0.1% Triton X-100, 50 mM Tris-HCl, pH 7.5, in the presence or absence of the indicated amount of preimmune or anti-ST3 antibody. The samples were than subjected to Western blot analysis with anti–human α1-antitrypsin antibody (Calbiochem).

Tissue Preparation

Tadpoles of the South African clawed frog (*X. laevis*) from stage 55 (∼4 wk after fertilization) to 66 (∼2 mo after fertilization and the end of metamorphosis; Nieuwkoop and Faber, 1956) were purchased from a commercial source in Hamamatsu, Japan. Tissue fragments were isolated from the anterior part just behind the bile duct junction of the small intestine, unless otherwise noted.

Organ Culture

Tissue fragments isolated from tadpoles at stage 57 (before metamorphic climax) were cultured as described in our previous paper (Ishizuya-Oka and Shimozawa, 1991). In brief, the tubular fragments were slit open lengthwise with forceps and cultured in the medium containing 60% Leibovitz-15 medium supplemented with 100 IU/ml of penicillin, 100 μg/ml of streptomycin, and 10% fetal bovine serum, which had been treated with activated charcoal (Sigma-Aldrich) according to the method of Yoshizato et al. (1980) to remove endogenous thyroid hormones (CTS medium). To induce metamorphosis, 3,3′,5-triiodo-L-thyronine (T_3), insulin, and hydrocortisone (Sigma-Aldrich) were added to CTS medium at 10^{-8} M, 5 μg/ml, and 0.5 μg/ml, respectively (TH-containing medium). Where indicated, the general inhibitor of MMPs, 4-Abz-Gly-Pro-D-Leu-D-Ala-NH-OH (MMP inhibitor I; Calbiochem) was added at 100 μg/ml (Odake et al., 1994). In addition, to examine the effects of the anti–*Xenopus* ST3 antiserum on the intestinal remodeling, the antiserum was added to TH-containing medium at various concentrations. In our preliminary experiment, when high concentrations of the antiserum (5% and higher) were added to the medium, necrosis of the epithelial cells was often recognized before 3 d of cultivation (not shown). Therefore, in this study, we used antiserum at concentrations of 0–1%, where the necrosis was undetectable at least until 3 d of cultivation. As a control, the same concentration of preimmune rabbit serum was added to the medium instead of the anti-ST3 antiserum. The culture medium was changed every other day for the 5-d treatment at 26°C.

Electron Microscopy

The explants were fixed in 7.5% glutaraldehyde in 0.1 M cacodylate buffer (pH 7.4) at 4°C for 2 h, postfixed with 1% osmium tetroxide in the same buffer at 4° for 2 h, stained en bloc with uranyl acetate, and embedded in epoxy resin. Ultrathin sections were stained with lead citrate and examined with a JOEL 200CX electron microscope.

Immunohistochemistry for ST3

Tissue fragments and cultured explants were fixed with 95% ethanol, processed for paraffin embedding, and cut at 5 μm. The sections were incubated for 1 h with the polyclonal antiserum (diluted 1:500) and then visualized by sequential incubation with streptavidin-biotin–peroxidase complex and DAB/H_2O_2, as described above. There was no positive staining when preimmune rabbit serum was applied (diluted 1:500) as the specificity control (not shown). At least three fragments were examined for each experimental point.

Nick End Labeling of Apoptotic DNA

DNA fragmentation associated with apoptosis was detected according to the terminal deoxyribonucleotidyl transferase (TdT)-mediated dUTP-biotin nick end labelling (TUNEL) method of Gavrieli et al. (1992). In brief, tissue fragments were fixed in 4% paraformaldehyde at 4°C for 4 h, and embedded in paraffin. Sections cut at 5 μm were treated with 20 μg/ml proteinase K (Boehringer) at room temperature for 15 min, and immersed in 1% H_2O_2 at room temperature for 15 min to inactivate endogenous peroxidase. They were then incubated with TdT buffer (100 mM potassium cacodylate, pH 7.2, 2 mM cobalt chloride, 0.2 mM DTT; GIBCO BRL) containing 0.3 equivalent U/μl terminal deoxynucleotidyl transferase (TdT; GIBCO BRL) and 0.04 mM biotinylated-dUTP (Boehringer) at 37°C for 90 min. The reaction was terminated by placing the slides in a washing solution (300 mM sodium chloride and 30 mM sodium citrate) for 15 min at room temperature. After the treatment with 10% normal rabbit serum for 10 min, the sections were incubated with peroxidase-labeled streptavidin (Nichirei) for 30 min and then with 0.02% 3,3′-diaminobenzidine-4HCl (DAB) and 0.006% H_2O_2 for 5 min. As a positive control, some sections were pretreated with 2 U/μl DNase I (Sigma-Aldrich) in 30 mM Tris buffer, pH 7.4, for 10 min before the treatment with TdT. The omission of either TdT or dUTP gave completely negative results (not shown). The labeling indices were calculated as the ratio of clearly labeled intact nuclei to total epithelial nuclei in >10 sections randomly selected from each explant cultured for 3 d. More than three explants were examined for each experimental point. Results were statistically analyzed by Student's *t* test.

Staining with Methyl Green–Pyronin Y

To identify adult epithelial primordia, explants cultured for 5 d were stained with methyl green–pyronin Y (Muto) as described by Ishizuya-Oka and Ueda (1996). During the replacement of larval epithelial cells by adult ones, the adult epithelial cells were intensely stained red because of their RNA-rich cytoplasm, whereas the staining of larval epithelial cells became much weaker towards their death (Ishizuya-Oka et al., 1997).

Results

Correlation of ST3 Expression with ECM Remodeling and Epithelial Transformation during Intestinal Metamorphosis

The *Xenopus* tadpole intestine is structurally simple, predominantly consisting of a single tubular layer of larval epithelium (McAvoy and Dixon, 1977; Ishizuya-Oka and Shimozawa, 1987; Yoshizato, 1989; Shi and Ishizuya-Oka, 1996). During metamorphosis, the larval epithelium undergoes complete degeneration through TH-dependent apoptosis (Fig. 1 B; McAvoy and Dixon, 1977; Ishizuya-Oka and Ueda, 1996). Concurrently, cells of the adult epithelium (Fig. 1 A), connective tissue, and muscles rapidly proliferate and subsequently differentiate to form a complex organ comprised of a multiply folded epithelium with elaborate connective tissue and muscle. We have shown previously that ST3 mRNA is expressed in the fibroblasts beneath both degenerating larval epithelial cells and proliferating adult epithelial islets in the intestine (Patterton et al., 1995; Ishizuya-Oka et al., 1996). However, nothing is known about ST3 protein levels during this process. Using a pAb against the catalytic domain located in the NH_2-terminal half of the protein, we found that ST3 protein was highly expressed only during the period of larval cell death and adult cell proliferation in the intestine (Fig. 2). Consistently, immunohistochemical analysis failed to detect any ST3 in the intestine before stage 58 (Fig. 3 A). By stage 60, when larval epithelial cells began to undergo apoptosis (McAvoy and Dixon, 1977; Ishizuya-Oka and Ueda, 1996), ST3 was present in the fibroblastic cells of the connective tissue adjacent to the epithelium (Fig. 3 B). The number of ST3 expressing cells was the highest around stage 61 (Figs. 3, C–E), when massive cell death took place in the larval epithelium, and adult epithelial cells proliferated rapidly (McAvoy and

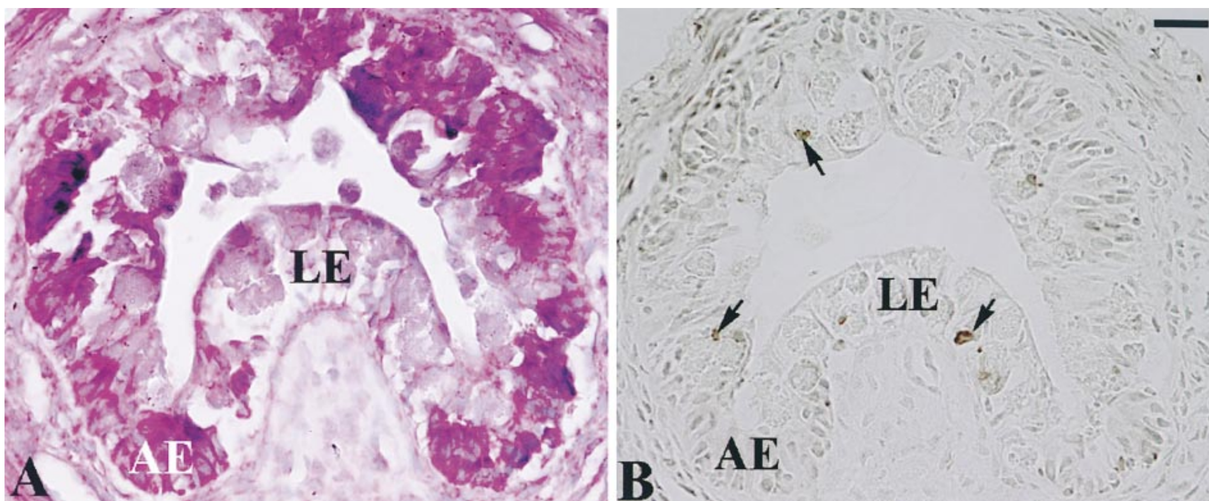


Figure 1. Larval epithelium (LE), but not adult epithelium (AE), undergoes apoptosis during intestinal metamorphosis (stage 61). (A) Methyl green–pyronin Y staining. AE primordia were strongly stained. (B) TUNEL labeling detected apoptosis only in the larval epithelium (arrows). Bar, 20 μm.

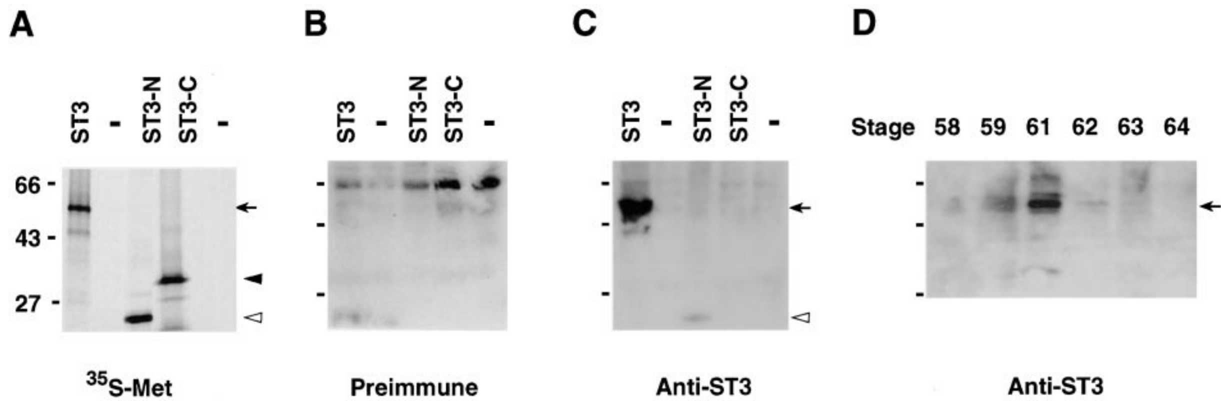


Figure 2. ST3 is expressed only during the period of metamorphic transformation in the tadpole intestine. (A–C) A pAb specifically recognizes the catalytic domain of ST3. The full-length ST3, the catalytic domain at the amino half (ST3-N), or the carboxyl half of ST3 (ST3-C) was made through coupled transcription–translation in vitro in the presence of [^{35}S]methionine (^{35}S-Met) and the reaction mixtures were electrophoresed on an SDS-protein gel (− lanes had no added cDNA clone during in vitro translation). Multiple identical gels were either dried and autoradiographed directly (A) or subjected to Western blot analysis with a pAb against the catalytic domain of ST3 (C) or the corresponding preimmune serum (B). Note that both the full-length (arrow) and the catalytic domain (ST3-N) (white arrowhead) were recognized by the antibody whereas the carboxyl half (ST3-C) (black arrowhead) was not. The preimmune serum did not recognize any of the ST3 polypeptide although an unknown protein from the in vitro translation extract was recognized. (D) Western blot analysis of the ST3 levels in the intestine during metamorphosis. 10 μg/lane of protein isolated from the small intestine of *X. laevis* tadpoles at the indicated stages was subjected to Western blot analysis with the antibody against the catalytic domain of ST3. Stage 58, stages 59–62, and stages 63–66 (end of metamorphosis) correspond to the onset of metamorphic climax, the period of larval epithelial cell death and adult epithelial cell proliferation, and the period of adult epithelial cell differentiation and epithelial morphogenesis, respectively.

Dixon, 1977; Ishizuya-Oka and Shimozawa, 1987; Ishizuya-Oka and Ueda, 1996). Subsequently, as adult epithelial cells differentiated, ST3 expression gradually disappeared, with the expression occurring last near the crest of the newly developing adult epithelial folds (Figs. 3, F and G).

Concurrent with the peak levels of ST3 expression around stages 60–61, the adjacent ECM that separates the epithelium and the connective tissue, such as the basal lamina, also underwent extensive remodeling. It changed from a thin but continuous layer in pre- and prometamorphic tadpoles (stage 58 or earlier; Fig. 3 H) to a multiply folded, zigzag structure at the metamorphic climax (stages 60–62; Fig. 3 I). Subsequently, as ST3 expression subsided, the ECM returned to a structure similar to that in premetamorphic tadpoles (data not shown). Thus, ST3 may play a direct or indirect role in this ECM remodeling process.

Concurrent Induction of ST3 Expression by TH with Intestinal Remodeling in Organ Cultures

Like other processes during amphibian metamorphosis, intestinal remodeling is under the control of TH. Furthermore, this process is organ autonomous and can be induced by simply adding physiological concentrations of TH to the culture medium of intestinal fragments from premetamorphic tadpoles (Ishizuya-Oka and Shimozawa, 1991). Immunohistochemical analysis showed that ST3 was absent in such intestinal explants from premetamorphic tadpoles cultured in the absence of TH (Fig. 4 E) or at the onset of TH treatment (Fig. 4 A). After 3 d of treatment, ST3 was expressed in the fibroblastic cells underneath the larval epithelium that had begun the degeneration process as reflected by the presence of lysosome-like granules (Fig. 4 B). By the fifth day of treatment, as larval epithelial degeneration approached completion, ST3 could only be detected weakly in a few cells in the connective tissue as epithelial cell death approached completion (Fig. 4 C). Similar to observations in vivo, high levels of TH-induced ST3 expression were accompanied by the increased folding of the basal lamina (Fig. 4 F), whereas in the absence of TH, the basal lamina remained as a continuous thin lining separating the epithelium and connective tissue (Fig. 4 G).

The most dramatic event during intestinal remodeling is the degeneration of larval epithelium through apoptosis or programmed cell death. In the intestinal explants, in situ detection of apoptosis using the TUNEL assay (Gavrieli et al., 1992) revealed numerous epithelial cells, but few other types of cells, undergoing cell death after 3–5 d of TH treatment (Fig. 5, B, C, and F). Apoptotic cells were rarely or not at all detected in explants without TH treatment (Fig. 5 E) or at early stages of TH treatment (Fig. 5 A). After 7 d of treatment, as larval epithelial degeneration progressed toward completion and the larval epithelium was replaced by the adult epithelium, few apoptotic cells were detected in the newly developed adult epithelium (Fig. 5, D and F). Thus, both larval cell death and basal lamina remodeling were temporally correlated with ST3 expression during TH-induced intestinal remodeling, again supporting a role for ST3 in ECM remodeling to facilitate epithelial transformation.

A Role for ST3 in TH-induced Larval Epithelial Degeneration

To determine if ST3 is indeed important for larval epithelial degeneration, we reasoned that the most direct approach is to block ST3 function in organ cultures. Since the anti-ST3 antibody is against the catalytic domain, it is very likely that it can interfere with ST3 activity through interference with the catalytic function or blocking substrate

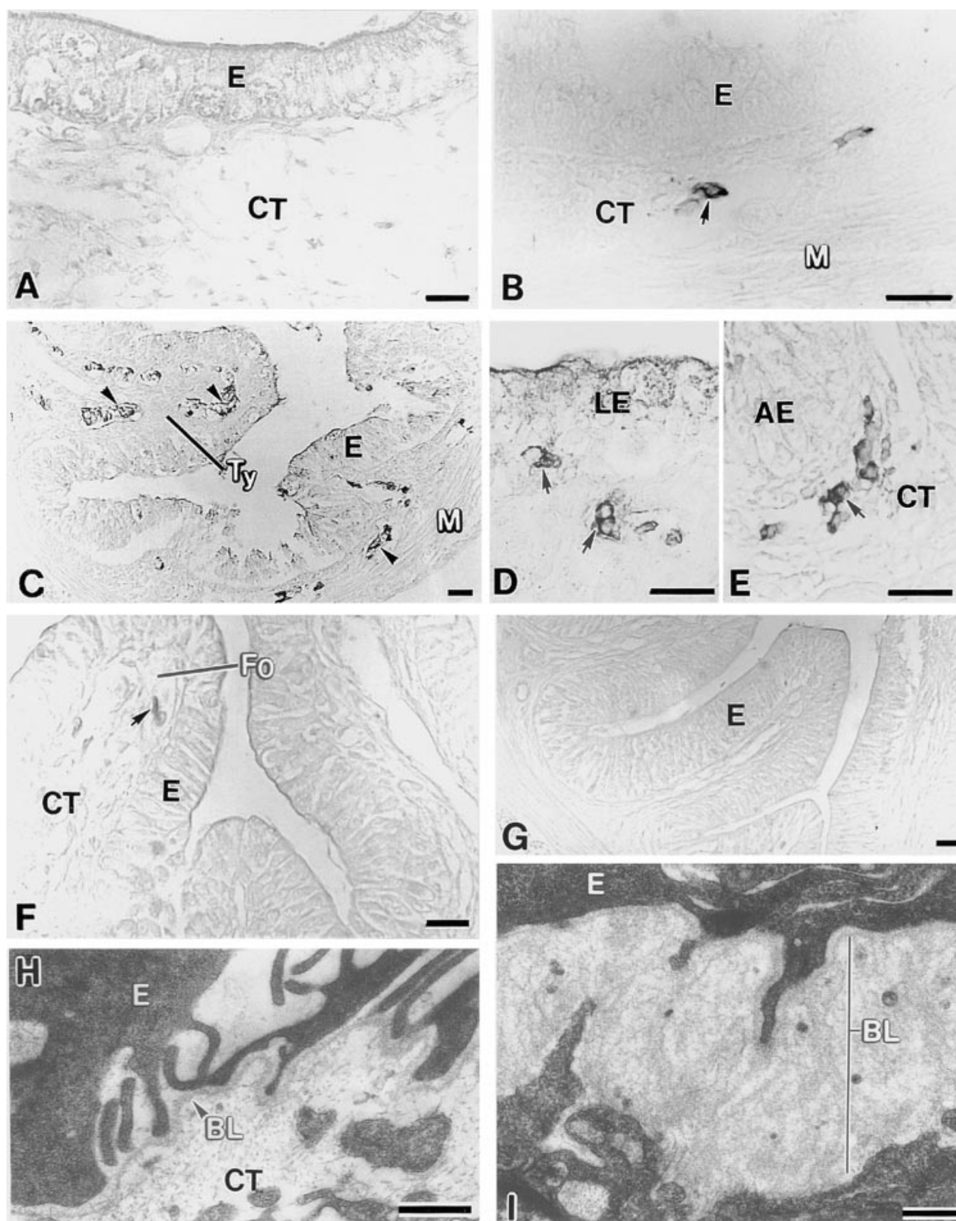


Figure 3. Immunohistochemical analysis reveals a correlation of ST3 expression with ECM remodeling in the *X. laevis* small intestine during metamorphosis. (A) No ST3 could be detected at stage 58, when the larval epithelium (indicated by E) degeneration has yet to take place. CT, connective tissue. (B) ST3 protein could be detected in fibroblasts (arrow) by stage 60. M, muscle. (C) Fibroblasts/fibroblast-like cells expressing ST3 (arrowheads) were present adjacent to the metamorphosing epithelium at stage 61. Ty, typhlosole. (D and E) ST3-expressing fibroblasts (arrows) near the degenerating larval epithelium (LE) and the adult epithelial primordia (AE) at stage 61. Note that the labeling in the larval epithelium was due to nonspecific binding by the fragments of dying cells. The adult epithelium was protruding into the connective tissue. (F) Relatively few cells with weak ST3 levels (arrow) could be detected in the crest of intestinal folds (Fo) at stage 63. (G) No ST3 expression could be detected at stage 65. (H) Electron micrograph of the epithelial–connective tissue interface at stage 58 showing a thin but continuous basal lamina (BL). (I) Electron micrograph of the epithelial–connective tissue interface showing a multiply folded basal lamina at stage 61. Bars: (A–G) 20 μm; (H and I) 0.5 μm.

access. To test this directly, we made use of the ability of active MMPs to interact with α_2M. Thus, in vitro–synthesized ^{35}S-labeled ST3 catalytic domain was incubated with α_2M in the absence or presence of anti-ST3 antiserum or preimmune serum. The resulting mixture was electrophoresed on a native polyacrylamide gel. Under the gel conditions, free ST3 failed to enter the gel due to the lack of negative charges associated with SDS binding under denaturing conditions, whereas α_2M–ST3 complex migrated at the same position as free α_2M as they were similar in size (Fig. 6 A, compare lanes 2 and 11; the bottom panel shows the staining of α_2M by Coomassie blue). The addition of anti-ST3 antiserum led to a dose-dependent inhibition of the formation of the α_2M–ST3 complex and a concurrent formation of ST3–antibody complex (Fig. 6 A, bottom band in lanes 3–6; note that this antibody complex formed regardless of whether α_2M was present, but was absent when preimmune serum was used, lanes 7–10). In contrast, the preimmune serum had no effect on α_2M–ST3 complex formation. In fact, ST3 also formed a complex with endogenous α_2M in the preimmune serum (Fig. 6 A, lane 10 where no exogenous α_2M was added; the endogenous α_2M migrated slightly slower than exogenous α_2M, compare lanes 9 and 10), which was also inhibited by the anti-ST3 antibody (compare lanes 6 and 10).

To directly test the ability of the antibody to inhibit the enzymatic activity of ST3, purified, bacterial expressed ST3 catalytic domain was incubated with α1-antitrypsin, a known substrate of mammalian ST3 (Pei et al., 1994) in the presence or absence of the antibody. Similar to mammalian ST3, the frog ST3 specifically cleaved α1-antitrypsin to produce two fragments. The larger fragment, as well as the full-length α1-antitrypsin, could be detected by a specific antibody (Fig. 6 B, lanes 1 and 2). The addition of as little as 1% of anti-ST3 antiserum completely blocked the cleavage whereas as much as 10% of the preimmune se-

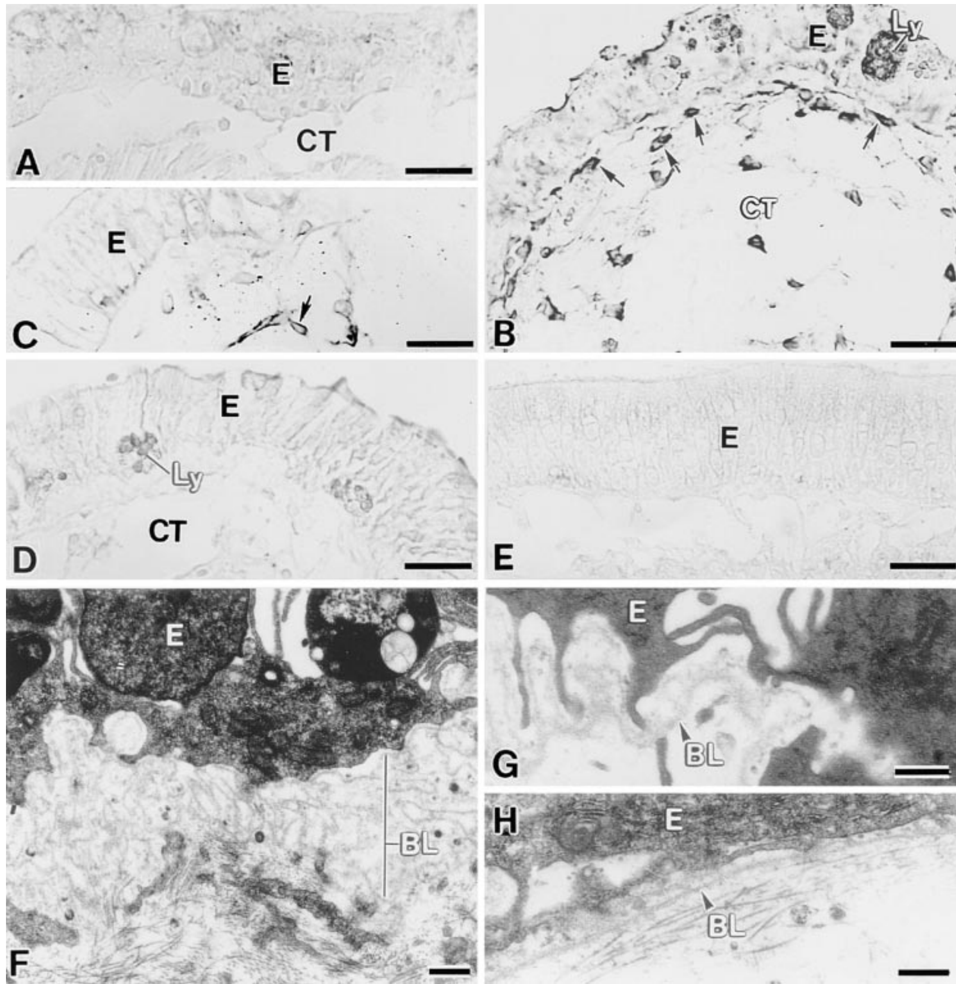


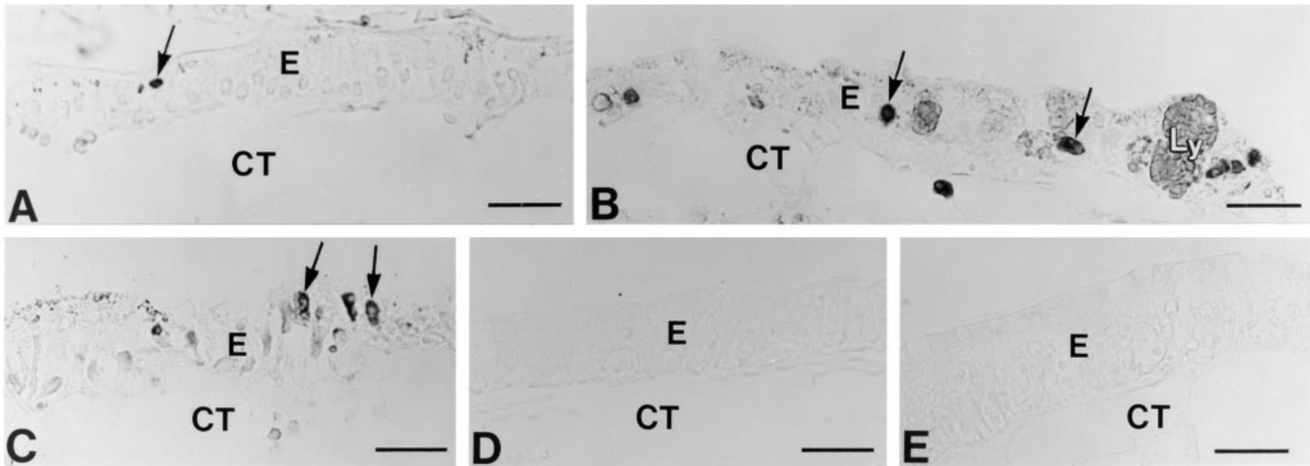
Figure 4. ST3 expression correlates with ECM remodeling during TH-induced metamorphosis in vitro. Fragments of the small intestine from stage 58 tadpoles were cultured in vitro in the presence (A–D) or absence (E) of a physiological concentration (10 nM, 3,3′,5-triiodothyronine) of TH. ST3 expression and epithelial–connective tissue interfaces were analyzed as in the legend to Fig. 2. (A) No ST3 expression was detected in the connective tissue (CT) during the first day of TH treatment. (B) ST3-expressing cells (arrows) in the connective tissue after 3 d of TH treatment. The degenerating epithelium (indicated by E) possessed many lysosome-like granules (Ly). (C) Weak staining (arrow) remained in the connective tissue after 5 d of treatment with TH. (D) No signal was detected with the preimmune serum after 3 d of TH treatment. (E) Absence of ST3 expression in the explant cultured in the absence of TH for 3 d where epithelial cells retained their simple columnar structure. (F–H) Electron micrographs of the epithelial–connective tissue interface of intestinal explants cultured for 3 d in the presence (F and H) or absence (G) of TH and in the presence (H) and absence (F and G) of 1% anti-ST3 antiserum. Note that in the presence of TH (F), the basal lamina (BL) was multiply folded. However, it remained mostly thin when the anti-ST3 antiserum was added to the TH-containing medium (H). Bars: (A–E) 20 μm; (F–H) 0.5 μm.

rum had no effect (Fig. 6 B; a slight enhancement was observed, possibly due to the stabilization of ST3 by the serum). Together, these data indicate that the anti-ST3 antibody is a function-blocking antibody.

To see if blocking ST3 function can influence intestinal remodeling, the anti-ST3 antiserum was added to the medium of intestinal organ cultures and its effect on TH-induced intestinal remodeling was analyzed. First, electron microscopic examination of the basal lamina showed that when the ST3 antiserum was included in the medium at a final concentration of 1% (the culture medium also contained 10% TH-depleted fetal bovine serum), it inhibited or delayed the remodeling of the ECM. After 3 d of TH treatment in the presence of the antibody, the basal lamina remained a thin, continuous layer (Fig. 4 H). This contrasts with the absence of the antibody but presence of TH (Fig. 4 F), although prolonged culturing eventually led to the remodeling of the basal lamina (data not shown). Thus, ST3 is directly or indirectly involved in ECM remodeling.

When cell death was analyzed with the TUNEL assay, it demonstrated that the apoptosis in the larval epithelium was also blocked by the antibody when assayed after 3 d of TH treatment (Fig. 7 A). In contrast, the preimmune serum had no effect on TH-induced apoptosis (Fig. 7 B). In addition, when an antibody generated against the His-tagged hemopexin domain of ST3, which failed to recognize the full-length ST3, was added to the culture medium, it had no effect on the TH-induced apoptosis. Finally, an antibody against another TH-induced gene, the *Xenopus* sonic hedgehog, also failed to influence TH-induced cell death (data not shown). These controls indicate that the effect of the anti-ST3 catalytic domain antibody was due to specific inhibition of ST3 function.

To further characterize this inhibitory effect, its dose dependence was determined by including increasing concentrations of the anti-ST3 serum or preimmune serum in the organ culture medium. Quantification of the apoptotic cells labeled by the TUNEL assay showed that TH treatment led to a drastic increase in apoptotic cells, whereas few cells were labeled in control intestinal fragments after 3 d of culturing (Fig. 7 C). Inclusion of preimmune serum at levels of 0.01–1% had no effect on this TH-induced cell death. In contrast, as little as 0.2% of the anti-ST3 serum caused a twofold reduction in apoptosis and the TH-induced apoptosis was nearly completely blocked by 1% of the antiserum (Fig. 7 C), which was shown to completely block



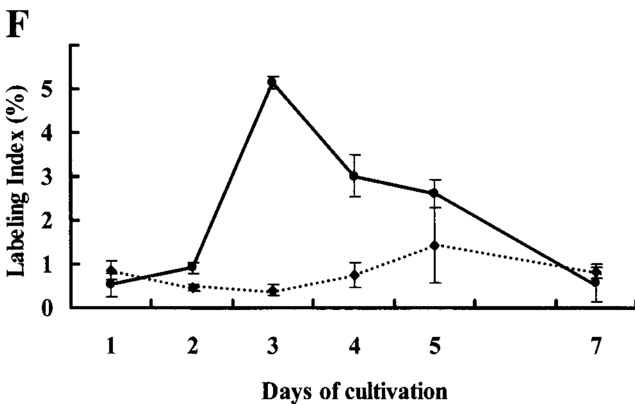


Figure 5. TH-induced epithelial apoptosis in intestinal explant in vitro correlates with ST3 expression in the underlying connective tissue. The explants were cultured in the presence (A–D) or absence (E) of TH and apoptosis was detected by using the TUNEL method. (A) Apoptotic cells (labeled nucleus, arrow) became detectable in the epithelium (indicated by E) after 2 d of treatment. CT, connective tissue. (B) Numerous labeled nuclei (arrows) were present after 3 d of treatment. The degenerating larval epithelium possesses lysosome-like granules (Ly). (C) Labeled nuclei (arrows) were still present after 5 d of TH treatment, although they were fewer than those after 3 d of treatment. (D) No apoptotic cells were detected after 7 d. The epithelium consisted of adult cells. (E) No apoptotic cells were detected in the absence of TH treatment after 3 d in culture. (F) Quantification of apoptosis by TUNEL labeling shows that cell death peaks after 3 day of TH treatment in vitro. Labeling indices of apoptotic cells of explants cultured in the presence (●) and absence (◆) of TH were measured daily. Each value represented the mean ± SD. Bars, 20 μm.

ST3 activity in vitro (Fig. 6). Interestingly, this inhibition of apoptosis appeared to be a kinetic one, as prolonged TH treatment (≥5 d) led to the degeneration of larval epithelium even in the presence of the anti-ST3 serum (data not shown; see also Fig. 8).

The ability of the ST3 antibody to inhibit epithelial transformation may be due to blocking the enzymatic function of ST3 or interfering with yet unknown pathways that involve ST3. To further demonstrate the requirement of MMP enzymatic activity for intestinal metamorphosis, a synthetic inhibitor, MMP inhibitor I, was added to the organ culture medium. After 3 d of culturing in the presence or absence of TH, the intestinal fragments were again analyzed by TUNEL assay for apoptotic cells. Like the ST3 antibody, the inhibitor inhibited TH-induced epithelial cell death (Fig. 7 D). Although this inhibitor could also inhibit other MMPs, ST3 is the only one whose expression is spatially and temporally correlated with intestinal epithelial apoptosis (Patterton et al., 1995; Ishizuya-Oka et al., 1996; Stolow et al., 1996; Damjanovski et al., 1999). Regardless, the results indicate that MMP activity is critical for intestinal metamorphosis.

Involvement of ST3 in Adult Epithelial Cell Migration

The intestinal organ culture also reproduces the second major event of the metamorphic process, i.e., the development of the adult epithelium. Upon prolonged TH treatment in vitro, adult epithelial primordia generally developed as small islets. These new epithelial cells proliferate rapidly to replace the dying larval cells. During this process the two types of epithelial cells could be distinguished by the differences in the intensity of their staining with methyl green–pyronin Y; the adult epithelial primordia consisting of undifferentiated cells were strongly stained red, whereas the apoptotic larval cells appeared pale as observed during spontaneous metamorphosis (Fig. 8). During the first 2 d, the larval epithelial cells remained healthy and stained red strongly (Fig. 8 A). After 3 d of TH treatment, the staining became weak as the larval cells underwent apoptosis (Fig. 8 B). The adult epithelial primordia were not detectable until 5 d of culturing and they were variable in size. These cells were located just beneath the larval epithelium and invaded into the underlying connective tissue as they grew in size (Fig. 8 C), again, similar to what occurs during spontaneous metamorphosis.

As was the case for larval epithelial cell death, anti-ST3 antiserum was found to alter adult primordial development when added at 0.2% or higher concentrations. In particular, the vertical invasion of the adult epithelial primodia into the underlying connective tissue was blocked (Fig. 8 D). However, cell proliferation of the adult epithelial primodia appeared to continue in the presence of the antibody. The proliferating cells expanded only horizontally

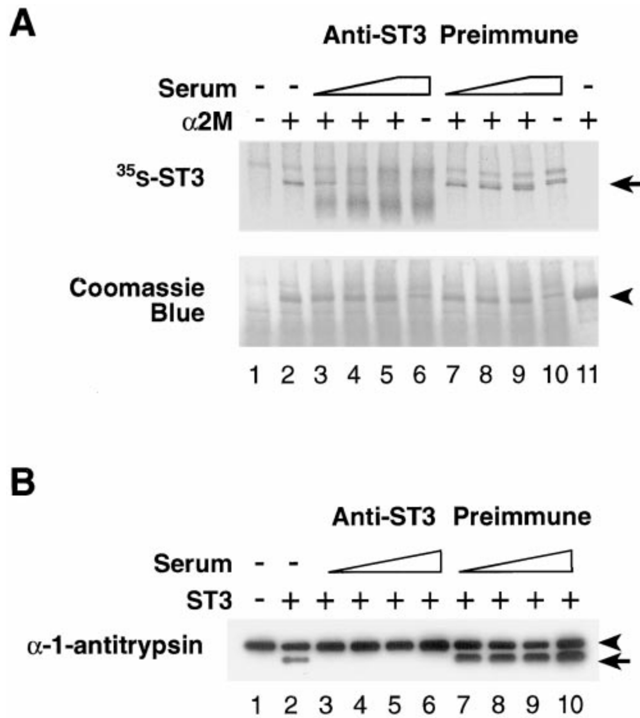


Figure 6. (A) α_2M capture assay shows that the anti-ST3 antibody blocks ST3 function. Anti-ST3 antibody inhibited the formation of ST3–α_2M complex. In vitro–synthesized, ^{35}S-labeled ST3 catalytic domain was incubated with α_2M (1 μg in 10 μl reaction) in the absence (lanes 1 and 2) or presence of anti-ST3 antiserum (lanes 3–6) or preimmune serum (lanes 7–10). The resulting mixture was electrophoresed on a native polyacrylamide gel. The gel was stained with Coomassie blue (bottom) and then dried and autoradiographed (top). The serum concentrations were 0.5% for lanes 3 and 7, 1% for lanes 4 and 8, and 2% for lanes 5, 6, 9, and 10. Lane 11 had 3.7 μg α_2M to facilitate its identification by Coomassie blue staining. The arrowhead and arrow indicate the position of α_2M and α_2M–ST3 complex, respectively. (α_2M and α_2M–ST3 complex migrated at the same position. This is because α_2M is 725 kD whereas ST3 catalytic domain is only ~20 kD. The small size difference between α_2M and α_2M–ST3 complex could not be resolved on this gel.) The faster migrating band in lanes 3–6 is the antibody–ST3 complex. The band migrating above the α_2M–ST3 complex is likely due to the nonspecific binding of ST3 by a protein in the in vitro translation extract, as it was also present in the absence of any α_2M (lane 1) and a protein at this position was also detectable by Coomassie blue staining in all lanes, except lane 11, which had only α_2M. Unlike α_2M–ST3, this band was not inhibited by the antibody. Its apparent increase in lanes 3–6 was due to the increased background smear caused by the anti-ST3 antibody. (B) Anti-ST3 antibody prevents cleavage of α1-antitrypsin by ST3. Purified ST3 catalytic domain was incubated with α1-antitrypsin in vitro in the presence of increasing concentrations (1, 2.5, 5, and 10%) of anti-ST3 or preimmune serum. The full-length and large fragment of α1-antitrypsin generated by ST3 cleavage were detected by Western blot analysis of the reaction product. Note that as little as 1% of the antiserum inhibited all of the cleavage of α1-trypsin by ST3 (lane 3) and most of the binding of ST3 to α_2M (A, lane 4). The arrow and arrowhead indicate the position of the ST3-generated large fragment and full-length α1-antitrypsin, respectively.

along the epithelial connective tissue interface but failed to protrude into the connective tissue. Again, as controls, preimmune serum had no effect on either cell proliferation or invasions (data not shown), and the explant without TH, but with the antiserum, remained unchanged throughout the cultivation (Fig. 8 E).

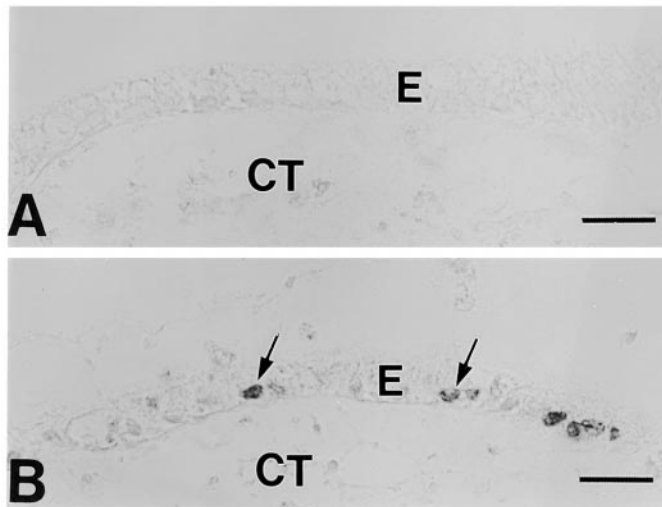
Discussion

MMPs have long been implicated to remodel ECM to facilitate organogenesis and tissue remodeling during development. Our study here has now provided strong evidence to support such a function for ST3. Our key findings are: (a) ST3 expression is correlated with ECM remodeling and epithelial transformation during intestinal metamorphosis; (b) ST3 facilitates larval epithelial cell death; and (c) ST3 is required for epithelial cell invagination into the connective tissue during intestinal morphogenesis.

ECM Remodeling Influences Intestinal Epithelial Cell Fate

Intestinal epithelial morphogenesis is one of the essential processes that occur during postembryonic development in vertebrates. In anurans, this process involves the degeneration of the larval epithelium and the development of the adult epithelium (Dauca and Hourdry, 1985; Yoshizato, 1989; Shi and Ishizuya-Oka, 1996). The epithelium lies above the basal lamina, which is a special ECM that separates the epithelium from the connective tissue. During both natural and TH-induced metamorphosis, the basal lamina changes from a continuous but thin structure to an amorphous, discontinuous, but thick structure (Figs. 2 and 3). Furthermore, the connective tissue and epithelium play a mutually interactive role to facilitate each other's development (Ishizuya-Oka and Shimozawa, 1992a,b, 1994). In addition, cells directly interact with ECM; thus, any changes in ECM can affect cell behavior. In fact, studies using several cultured cell lines have shown that proper cell–ECM interactions are important for cell survival (Ruoslahti and Reed, 1994; Boudreau et al., 1995; Frisch and Ruoslahti, 1997; Shi et al., 1998). One of the best examples is the mammary gland epithelial cells, which undergo apoptosis when cultured on plastic dishes. Such programmed cell death can be inhibited by culturing the cells on an exogenous basement membrane. In addition, blocking cell–ECM interactions with specific anti-integrin antibodies induces apoptosis of this and various other types of cells (Boudreau et al., 1995; Frisch and Ruoslahti, 1997). Thus, it is tempting to suggest that the remodeling of the basal lamina during metamorphosis plays a critical role in the removal of the larval epithelium and development of the adult epithelium.

Direct evidence to support a role of ECM in intestinal development has come from studies using primary cell cultures. By culturing epithelial or fibroblastic cells from prometamorphic tadpole intestine, Su et al. (1997) have shown that TH can induce the proliferation of both cell types and concurrently cause the epithelial, but not fibroblastic, cells to undergo apoptosis, similar to what occurs during intestinal metamorphosis. Interestingly, adult epithelial cells from metamorphosing intestine also undergo apoptosis when treated with TH in cell cultures (Su et al.,



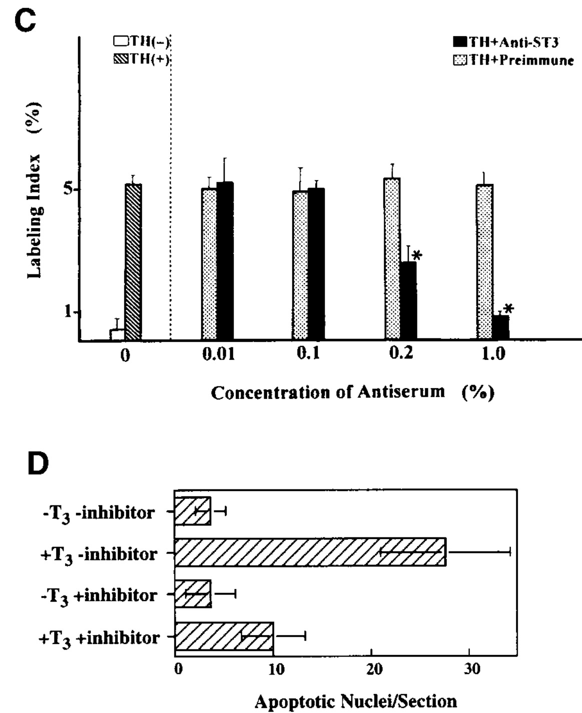


Figure 7. Blocking ST3 function inhibits TH-induced epithelial apoptosis. Intestinal explants were cultured for 3 d in the presence of TH and 1% anti-ST3 serum (A) or 1% preimmune serum (B), and apoptosis (arrows) was detected with the TUNEL method. E, epithelium; CT, connective tissue. (C) The anti-ST3 antibody inhibits TH-induced apoptosis in a dose-dependent manner. Labeling indices of apoptotic epithelial cells in the explants were measured after 3 d of treatment in the presence (hatched bar) or absence (white bar) of TH, or in the presence of TH and increasing concentrations of preimmune (dotted bars) or anti-ST3 antiserum (black bars). Each value represented the mean ± SD. High concentrations of the anti-ST3 antiserum significantly inhibits apoptosis of the epithelium ($^*P <$ 0.01) compared with those of the preimmune serum. (D) A synthetic MMP inhibitor also inhibits TH-induced epithelial apoptosis. Intestinal explants were cultured for 3 d in the presence of TH and 100 μg/ml of MMP inhibitor I. The explants were the processed as above. Bars: (A and B) 20 μm.

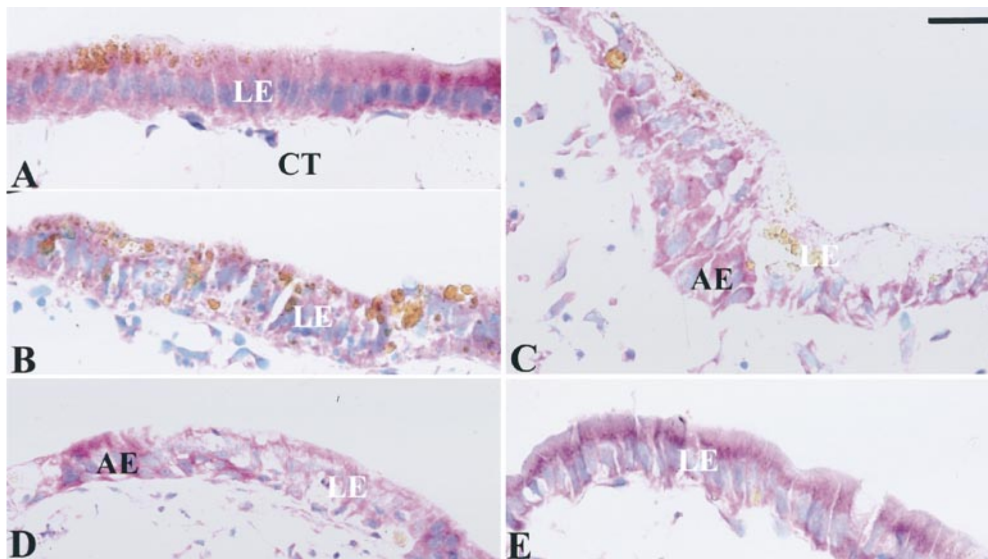


Figure 8. Anti-ST3 antibody inhibits adult epithelial primordia invasion into the connective tissue. Intestinal explants were cultured for 2–5 d in the presence (A–D) or absence (E) of TH and in the presence (D and E) or absence (A–C) of 1% anti-ST3 antiserum. A, 2 d; B, 3 d; C–E, 5 d. The larval epithelium (LE) remains stained red after 2 d (A) but its staining decreased as it underwent apoptosis after 3 d (B) in the presence of TH. The adult epithelial primordia (AE) stained red that were not detectable within the first 3–4 d, developed just beneath the degenerating larval epithelium after 5 d (C) and grew into the connective tissue (CT) in the presence of TH alone. In the presence of both TH and anti-ST3 antiserum (D), the primordia (AE) developed as well. However, they did not protrude into the connective tissue. Note that although the antiserum inhibited apoptosis as assayed after 3 d of treatment (see Fig. 7), it did not completely block the process but simply delayed it, similar to the observation that homologous deletion of gelatinase B resulted in a delay in terminal hypertrophic chondrocyte apoptosis during mouse development (Vu et al., 1998). Thus, after longer treatment, e.g., 5 d, larval epithelium was induced by TH to degenerate, as also reflected by the reduced staining in D. In the absence of TH (E), the larval epithelium remained unchanged. Preimmune serum had no effect on the adult epithelial primordia development (not shown). Bars, 20 μm.

1997), suggesting that removing the ECM support makes the cells vulnerable to TH-induced death. More importantly, larval epithelial cells cultured on various ECM-coated dishes are more resistant to TH-induced death (Su et al., 1997), supporting a key role of ECM in cell fate determination.

Dual Functions of ST3 during Intestinal Development

We have demonstrated here for the first time that the expression of ST3 proteins is spatially and temporally correlated with larval cell death and adult epithelial morphogenesis, in agreement with mRNA data reported previously (Wang and Brown, 1993; Patterton et al., 1995, Ishizuya-Oka et al., 1996, Damjanovski et al., 1999). Although it was difficult to detect ST3 protein in organ cultures after 1–2 d of TH treatment, due to low sensitivity of immunohistochemistry, ST3 has been shown to be a direct TH response, whose mRNA is induced by TH within 4–8 h (Wang and Brown, 1993; Patterton et al., 1995). Thus, ST3 protein is most likely present before larval epithelial apoptosis and could influence this process. Indeed, our results here indicate that ST3 has dual roles during intestinal metamorphosis. It is important not only for larval epithelial cell death, but also for the invasion of the adult epithelial primordia into the underlying connective tissue, a process that is critical for the morphogenesis of the adult epithelium, such as in epithelial fold formation.

It is worth noting that ST3 is the only MMP shown to be tightly correlated with larval cell death and adult epithelial morphogenesis. In situ hybridization and morphological examination indicate that ST3 is expressed in the fibroblastic cells of the connective tissue, although a definitive determination of the cell type(s) remains to be done (Patterton et al., 1995; Damjanovski et al., 1999). This suggests that as an MMP, ST3 may affect epithelial development by either directly or indirectly modifying the ECM. Indeed, ST3 expression also correlates with the extensive ECM remodeling during metamorphosis, and blocking ST3 expression inhibits ECM remodeling in vitro (Fig. 4 H; Patterton et al., 1995; Brown et al., 1996; Stolow et al., 1996; Damjanovski et al., 1999). In this paper, we present the first direct functional data to support a physiological role of ST3 during intestinal remodeling. Although ST3 is expressed in the connective tissue, it is required for epithelial transformation. A similar requirement of factors from neighboring tissues has also been observed for tail resorption in *Rana catesbeiana* (Niki et al., 1982; Niki and Yoshizato, 1986), where an epidermal factor was found to be important for mesenchymal regression, although the factor has yet to be identified.

It is worth noting that while ECM remodeling correlates with the ST3 expression, the basal lamina is actually thicker but multiply folded at the peak of ST3 expression. This suggests that ST3 causes, either directly or indirectly, cleavage of certain substrate(s). The apparent increase in the basal lamina may be due to new ECM synthesis and/or the intestinal contraction as the intestine reduces its length during metamorphosis (Shi and Ishizuya-Oka, 1996). Regardless, the result is consistent with the idea that ECM remodeling, not necessarily its degradation, plays a critical role in cell fate determination.

ST3 was initially discovered as an MMP associated with human breast cancer carcinomas and with apoptotic organogenesis (Basset et al., 1990; Lefebvre et al., 1992, 1995; Muller et al., 1993). However, direct proof for a role of ST3 in cell migration and cell fate determination had been elusive due to the difficulties of working with this enzyme. Recent gene knockout studies have demonstrated that mice lacking ST3 had reduced incidence of carcinogen-induced tumors, whereas overexpression of ST3 in cultured cells led to increased tumor incidence when the resulting cells were injected into mice (Noel et al., 1996; Masson et al., 1998). Our study provides direct evidence that ST3 is indeed important for both cell migration and cell death. For proliferating epithelial cells of the metamorphosing intestine, ST3 facilitates migration/invasion, whereas for terminally differentiated larval epithelial cells, it participates in promoting apoptosis. Such differential effects are in agreement with the observed expression profiles of ST3 in both mammals and *Xenopus* (Basset et al., 1990; Lefebvre et al., 1995; Patterton et al., 1995; Brown et al., 1996; Muller et al., 1993).

Interestingly, blocking the function of ST3 was unable to completely block the TH-induced cell death, as prolonged TH treatment eventually caused larval epithelium to degenerate (Fig. 7), suggesting that the role of ECM remodeling lies mainly at facilitating or coordinating tissue transformations but is not the determining factor for cell fate, or that the inhibition was incomplete. Such an effect also bears a similarity to the observation that homologous deletion of gelatinase B resulted in a delay in terminal hypertrophic chondrocyte apoptosis during mouse development (Vu et al., 1998).

Finally, our findings also complement recent studies with transgenic mice overexpressing another MMP, stromelysin-1, which showed that overexpression of the MMP leads to ECM remodeling and cell death (Sympson et al., 1994; Witty et al., 1995a,b).

Like these and other studies such as the recent gene knockout studies on gelatinase B (Vu et al., 1998) and MT1-MMP (Holmbeck et al., 1999), it is unknown how MMPs exert their effects. They can either directly or indirectly modify the ECM to influence cell death and/or cell migration. Our results are consistent with this possibility. As discussed above, ECM remodeling can lead to apoptosis. Likewise, MMPs have long been implicated to play a role in tumor invasion. We have shown that ST3 is also important for cell migration during development. The increase in basal lamina thickness at the peak of ST3 expression suggests that selective cleavages of the ECM proteins, not massive ECM degradation, is sufficient for such an effect. In this regard, it is worth noting that specific cleavage of the ECM protein laminin-5 by MMP-2 or gelatinase A can induce cell migration (Giannelli et al., 1997). Thus, it is likely that alterations in cell–ECM interaction, not the mere loss of cell–ECM contacts, activate cell migration processes.

Alternatively, MMPs can function through other pathways. In the case of ST3, this was made possible by the discovery of the ability of this MMP to cleave a serpin, which inhibits serine proteases (Pei et al., 1994). Several studies have shown that serine proteases can influence cell behavior, including cell differentiation, growth, and apoptosis

(Turgeon and Houenou, 1997). For example, the serine protease thrombin can alter neurite outgrowth and induce neuronal cell death (Turgeon et al., 1998), whereas the serpin protease nexin I inhibits neuronal apoptosis (Houenou et al., 1995). Although it remains to be shown that ST3 cleaves serpins in vivo, these findings raise the possibility that ST3 may facilitate cell death by enhancing the activities of serine proteases through the removal of their inhibitors. Likewise, ST3 may cleave other non-ECM substrates to regulate cell behavior. Thus, the future challenges will be (a) to determine whether ST3 functions by directly or indirectly modifying the ECM, or through other pathways; (b) to identify the in vivo substrates of ST3; and (c) to determine the mechanisms underlying the effects of ST3 on cell death and migration.

We would like to thank Drs. Henning Birkedal-Hansen and Duanqing Pei for helpful suggestions and discussions during the course of this work.

Submitted: 17 March 2000
Revised: 12 July 2000
Accepted: 13 July 2000

References

Alexander, C.M., and Z. Werb. 1991. Extracellular matrix degradation. *In* Cell Biology of Extracellular Matrix, 2nd ed. E.D. Hay, editor. Plenum Press, New York. 255–302.

Basset, P., J.P. Bellocq, C. Wolf, I. Stoll, P. Hutin, J.M. Limacher, O.L. Podhajcer, M.P. Chenard, M.C. Rio, and P. Chambon. 1990. A novel metalloproteinase gene specifically expressed in stromal cells of breast carcinomas. *Nature.* 348:699–704.

Berry, D.L., R.A. Schwartzman, and D.D. Brown. 1998a. The expression pattern of thyroid hormone response genes in the tadpole tail identifies multiple resorption programs. *Dev. Biol.* 203:12–23.

Berry, D.L., C.S. Rose, B.F. Remo, and D.D. Brown. 1998b. The expression pattern of thyroid hormone response genes in remodeling tadpole tissues defines distinct growth and resorption gene-expression programs. *Dev. Biol.* 203:24–35.

Birkedal-Hansen, H., W.G.I. Moore, M.K. Bodden, L.J. Windsor, B. Birkedal-Hansen, A. DeCarlo, and J.A. Engler. 1993. Matrix metalloproteinases: a review. *Crit. Rev. Oral Biol. Med.* 4:197–250.

Brown, D.D., Z. Wang, J.D. Furlow, A. Kanamori, R.A. Schwartzman, B.F. Remo, A. Pinder. 1996. The thyroid hormone–induced tail resorption program during *Xenopus laevis* metamorphosis. *Proc. Natl. Acad. Sci. USA.* 93: 1924–1929.

Boudreau, N., C.J. Sympson, Z. Werb, and M.J. Bissell. 1995. Suppression of ICE and apoptosis in mammary epithelial cells by extracellular matrix. *Science.* 267:891–893.

Damjanovski, S., A. Ishizuya-Oka, and Y.-B. Shi. 1999. Spatial and temporal regulation of collagenases-3, -4, and stromelysin-3 implicate distinct functions in apoptosis and tissue remodeling during frog metamorphosis. *Cell Res.* 9:91–105.

Dauca, M., and J. Hourdry. 1985. Transformations in the intestinal epithelium during anuran metamorphosis. *In* Metamorphosis. M. Balls and M. Bownes, editors. Clarendon Press, Oxford. 36–58.

Dodd, M.H.I., and J.M. Dodd. 1976. The biology of metamorphosis. *In* Physiology of the Amphibia. B. Lofts, editor. Academic Press, New York. 467–599.

Frisch, S.M., and E. Ruoslahti. 1997. Integrins and anoikis. *Curr. Opin. Cell Biol.* 9:701–706.

Gavrieli, Y., Y. Sherman, and S.A. Ben-Sasson. 1992. Identification of programmed cell death in situ via specific labeling of nuclear DNA fragmentation. *J. Cell Biol.* 119:493–501.

Giannelli, G., J. Falk-Marzillier, O. Schiraldi, W.G. Stetler-Stevenson, and V. Quaranta. 1997. Induction of cell migration by matrix metalloprotease-2 cleavage of laminin-5. *Science.* 277:225–228.

Gilbert, L.I., and E. Frieden. 1981. Metamorphosis: A Problem in Developmental Biology, 2nd ed. Plenum Press, New York. 577 pp.

Gross, J., and C.M. Lapiere. 1962. Collagenolytic activity in amphibian tissues: a tissue culture assay. *Proc. Natl. Acad. Sci. USA.* 48:1014–1022.

Hay, E.D. 1991. Cell Biology of Extracellular Matrix, 2nd ed. Plenum Press, New York. 468 pp.

Holmbeck, K., P. Bianco, J. Caterina, S. Yamada, M. Kromer, S.A. Kuznetsov, M. Mankani, P.G. Robey, A.R. Poole, I. Pidoux, J.M. Ward, and H. Birkedal-Hansen. 1999. MT1-MMP-deficient mice develop dwarfism, osteopenia, arthritis, and connective tissue disease due to inadequate collagen turnover. *Cell.* 99:81–92.

Houenou, L.J., P.L. Turner, L. Li, R.W. Oppenheim, and B.W. Festoff. 1995. A serine protease inhibitor, protease nexin I, rescues motoneurons from naturally occurring and axotomy-induced cell death. *Proc. Natl. Acad. Sci. USA.* 92:895–899.

Ishizuya-Oka, A., and A. Shimozawa. 1987. Development of the connective tissue in the digestive tract of the larval and metamorphosing *Xenopus laevis. Anat. Anz.* 164:81–93.

Ishizuya-Oka, A., and A. Shimozawa. 1991. Induction of metamorphosis by thyroid hormone in anuran small intestine cultured organotypically in vitro. *In Vitro Cell. Dev. Biol.* 27A:853–857.

Ishizuya-Oka, A., and A. Shimozawa. 1992a. Programmed cell death and heterolysis of larval epithelial cells by macrophage-like cells in the anuran small intestine in vivo and in vitro. *J. Morphol.* 213:185–195.

Ishizuya-Oka, A., and A. Shimozawa. 1992b. Connective tissue is involved in adult epithelial development of the small intestine during anuran metamorphosis in vitro. *Roux's Arch. Dev. Biol.* 201:322–329.

Ishizuya-Oka, A., and A. Shimozawa. 1994. Inductive action of epithelium on differentiation of intestinal connective tissue of *Xenopus laevis* tadpole during metamorphosis in vitro. *Cell Tissue Res.* 277:427–436.

Ishizuya-Oka, A., and S. Ueda. 1996. Apoptosis and cell proliferation in the *Xenopus* small intestine during metamorphosis. *Cell Tissue Res.* 286:467–476.

Ishizuya-Oka, A., S. Ueda, and Y.-B. Shi. 1996. Transient expression of stromelysin-3 mRNA in the amphibian small intestine during metamorphosis. *Cell Tissue Res.* 283:325–329.

Ishizuya-Oka, A., S. Ueda, S. Damjanovski, Q. Li, V.C.-T. Liang, and Y.-B. Shi. 1997. Anteroposterior gradient of epithelial transformation during amphibian intestinal remodeling: immunohistochemical detection of intestinal fatty acid-binding protein. *Dev. Biol.* 192:149–161.

Jacobson, M., M. Weil, and M.C. Raff. 1997. Programmed cell death in animal development. *Cell.* 88:347–354.

Kleiner, D.E., Jr., and W.G. Stetler-Stevenson. 1993. Structural biochemistry and activation of matrix metalloproteases. *Cell Biol.* 5:891–897.

Lefebvre, O., C. Wolf, J.-M. Limacher, P. Hutin, C. Wendling, M. LeMeur, P. Basset, and M.-C. Rio. 1992. The breast cancer–associated stromelysin-3 gene is expressed during mouse mammary gland apoptosis. *J. Cell Biol.* 119:997–1002.

Lefebvre, O., C. Regnier, M.-P. Chenard, C. Wendling, P. Chambon, P. Basset, and M.-C. Rio. 1995. Developmental expression of mouse stromelysin-3 mRNA. *Development.* 121:947–955.

Masson, R., O. Lefebvre, A. Noel, M. El Fahime, M.-P. Chenard, C. Wendling, F. Kebers, M. LeMeur, A. Dierich, J.-M. Foidart, P. Basset, and M.-C. Rio. 1998. In vivo evidence that the stromelysin-3 metalloproteinase contributes in a paracrine manner to epithelial cell malignancy. *J. Cell Biol.* 140:1535–1541.

Matrisian, L.M. 1992. The matrix-degrading metalloproteinases. *Bioessays.* 14: 455–463.

McAvoy, J.W., and K.E. Dixon. 1977. Cell proliferation and renewal in the small intestinal epithelium of metamorphosing and adult *Xenopus laevis. J. Exp. Zool.* 202:129–138.

Muller, D., C. Wolf, J. Abecassis, R. Millon, A. Engelmann, G. Bronner, N. Rouyer, M.C. Rio, M. Eber, G. Methlin, P. Chambon, and P. Basset. 1993. Increased stromelysin-3 gene expression is associated with increased local invasiveness in head and neck squamous cell carcinomas. *Cancer Res.* 53: 165–169.

Murphy, G., R. Willenbrock, T. Crabbe, M. O'Shea, R. Ward, S. Atkinson, J. O'Connell, and A. Docherty. 1994. Regulation of matrix metalloproteinase activity. *Ann. NY Acad. Sci.* 732:31–41.

Nagase, H. 1998. Cell surface activation of progelatinase A (pro MMP-2) and cell migration. *Cell Res.* 8:179–186.

Nagase, J., K. Suzuki, T. Morodomi, J.J. Enghild, and G. Salvesen. 1992. Activation mechanisms of the precursors of matrix metalloproteinases 1,2, and 3. *Matrix Suppl.* 1:237–244.

Nieuwkoop, P.P., and J. Faber. Normal table of *Xenopus laevis.* North Holland Publishing, Amsterdam. 252 pp.

Niki, K., H. Namiki, S. Kikuyama, and K. Yoshizato. 1982. Epidermal tissue requirement for tadpole tail regression induced by thyroid hormone. *Dev. Biol.* 94:116–120.

Niki, K. and K. Yoshizato. 1986. An epidermal factor which induces thyroid hormone-dependent regression of mesenchymal tissues of the tadpole tail. *Dev. Biol.* 118:306–308.

Noel, A.C., O. Lefebvre, E. Maquoi, L. VanHoorde, M.P. Chenard, M. Mareel, J.-M. Foidart, P. Basset, and M.-C. Rio. 1996. Stromelysin-3 expression promotes tumor take in nude mice. *J. Clin. Invest.* 97:1924–1930.

Odake, S., Y. Morita, T. Morikawa, N. Yoshida, H. Hori, and Y. Nagai. 1994. Inhibition of matrix metalloproteinases by peptidyl hydroxamic acids. *Biochem. Biophys. Res. Commun.* 199:1442–1446.

Parks, W.C., and R.P. Mecham. 1998. Matrix Metalloproteinases. Academic Press, New York. 362 pp.

Patterton, D., W.P. Hayes., and Y.-B. Shi. 1995. Transcriptional activation of the matrix metalloproteinase gene stromelysin-3 coincides with thyroid hormone–induced cell death during frog metamorphosis. *Dev. Biol.* 167:252–262.

Pei, D., and S.J. Weiss. 1995. Furin-dependent intracellular activation of the human stromelysin-3 zymogen. *Nature.* 375:244–247.

Pei, D., G. Majmudar, and S.J. Weiss. 1994. Hydrolytic inactivation of a breast carcinoma cell-derived serpin by human stromelysin-3. *J. Biol. Chem.* 269: 25849–25855.

Reddy, V.Y., P.E. Desrochers, S.V. Pizzo, S.L. Gonias, J.A. Sahakian, R.L. Levine, and S.J. Weiss. 1994. Oxidative dissociation of human α2-macroglobu-

lin tetramers into dysfunctional dimers. *J. Biol. Chem.* 269:4683–4691.
Ruoslahti, E., and J.C. Reed. 1994. Anchorage dependence, integrins, and apoptosis. *Cell.* 77:477–478.
Sang, Q.A., and D.A. Douglas. 1996. Computational sequence analysis of matrix metalloproteinases. *J. Protein Chem.* 15:137–160.
Shi, Y.-B., and A. Ishizuya-Oka. 1996. Biphasic intestinal development in amphibians: embryogenesis and remodeling during metamorphosis. *Curr. Top. Dev. Biol.* 32:205–235.
Shi, Y.-B., Q. Li, S. Damjanovski, T. Amano, and A. Ishizuya-Oka. 1998. Regulation of apoptosis during development: input from the extracellular matrix. *Int. J. Mol. Med.*. 2:273–282.
Stetler-Stevenson, W.G., S. Aznavoorian, and L.A. Liotta. 1993. Tumor cell interactions with the extracellular matrix during invasion and metastasis. *Annu. Rev. Cell Biol.* 9:541–573.
Stolow, M.A., D.D. Bauzon, J. Li, T. Sedgwick, V.C.-T. Liang, Q.A. Sang, and Y.-B. Shi. 1996. Identification and characterization of a novel collagenase in *Xenopus laevis*: possible roles during frog development. *Mol. Biol. Cell.* 7:1471–1483.
Su, Y., Y. Shi, M.A. Stolow, and Y.-B. Shi. 1997. Thyroid hormone induces apoptosis in primary cell cultures of tadpole intestine: cell type specificity and effects of extracellular matrix. *J. Cell Biol.* 139:1533–1543.
Sympson, C.J., R.S. Talhouk, C.M. Alexander, J.R. Chin, S.M. Clift, M.M. Bissell, and Z. Werb. 1994. Targeted expression of stromelysin-1 in mammary gland provides evidence for a role of proteinases in branching morphogenesis and the requirement for an intact basement membrane for tissue-specific gene expression. *J. Cell Biol.* 125:681–693.
Timpl, R., and J.C. Brown. 1996. Supermolecular assembly of basement membranes. *Bioessays.* 18:123–132.
Turgeon, V.L., and L.J. Houenou. 1997. The role of thrombin-like (serine) proteases in the development, plasticity and pathology of the nervous system. *Brain Res. Rev.* 25:85–95.
Turgeon, V.L., E.D. Lloyd, S. Wang, B.W. Festoff, and L.J. Houenou. 1998. Thrombin perturbs neurite outgrowth and induces apoptotic cell death in enriched chick spinal motoneuron cultures through caspase activation. *J. Neurosci.* 18:6882–6891.
Tryggvason, K., M. Hoyhtya, and T. Salo. 1987. Proteolytic degradation of extracellular matrix in tumor invasion. *Biochim. Biophys. Acta.* 907:191–217.
Uria, J.A., and Z. Werb. 1998. Matrix metalloproteinases and their expression in mammary gland. *Cell Res.* 8:187–194.
Van Wart, H.E., and H. Birkedal-Hansen. 1990. The cysteine switch: a principle of regulation of metalloproteinase activity with potential applicability to the entire matrix metalloproteinase gene family. *Proc. Natl. Acad. Sci. USA.* 87:5578–5582.
Vu, T.H., M.J. Shipley, G. Bergers, J.E. Berger, J.A. Helms, D. Hanahan, S.D. Shapiro, R.M. Senior, and Z. Werb. 1998. MMP-9/gelatinase B is a key regulatory of growth plate angiogenesis and apoptosis of hypertrophic chondrocytes. *Cell.* 93:411–422.
Vukicevic, S., H.K. Kleinman, F.P. Luyten, A.B. Roberts, N.S. Roche, and A.H. Reddi. 1992. Identification of multiple active growth factors in basement membrane matrigel suggests caution in interpretation of cellular activity related to extracellular matrix components. *Exp. Cell Res.* 202:1–8.
Wang, Z., and D.D. Brown. 1993. Thyroid hormone–induced gene expression program for amphibian tail resorption. *J. Biol. Chem.* 268:16270–16278.
Werb, Z., and J.R. Chin. 1998. Extracellular matrix remodeling during morphogenesis. *Ann. NY Acad. Sci.* 857:110–118.
Werb, Z., C.J. Sympson, C.M. Alexander, N. Thomasset, L.R. Lund, A. MacAuley, J.J. Ashenase, and M.J. Bissel. 1996. Extracellular matrix remodeling and the regulation of epithelial stromal interactions during differentiation and involution. *Kidney Int. Suppl.* 54:S68–S74.
Witty, J.P., J.H. Wright, and L.M. Matrisian. 1995a. Matrix metalloproteinases are expressed during ductal and alveolar mammary morphogenesis, and misregulation of stromelysin-1 in transgenic mice induces unscheduled alveolar development. *Mol. Biol. Cell.* 6:1287–1303.
Witty, J.P., T. Lempka, R.J. Coffey, Jr., and L.M. Matrisian. 1995b. Decreased tumor formation in 7,12-dimethylbenzanthracene-treated stromelysin-1 transgenic mice is associated with alterations in mammary epithelial cell apoptosis. *Cancer Res.* 55:1401–1406.
Woessner, J.F., Jr. 1991. Matrix metalloproteinases and their inhibitors in connective tissue remodeling. *FASEB (Fed. Am. Soc. Exp. Biol.) J.* 5:2145–2154.
Yoshizato, K. 1989. Biochemistry and cell biology of amphibian metamorphosis with a special emphasis on the mechanism of removal of larval organs. *Int. Rev. Cytol.* 119:97–149.
Yoshizato, K., S. Kikuyama, and N. Shioya. 1980. Stimulation of glucose utilization and lactate production in cultured human fibroblasts by thyroid hormone. *Biochem. Biophys. Acta.* 627:23–29.