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Tissue-dependent induction of apoptosis by matrix metalloproteinase stromelysin-3 during amphibian metamorphosis

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Abstract

Matrix metalloproteinases (MMPs) are a superfamily of Zn²⁺-dependent proteases that are capable of cleaving the proteinaceous component of the extracellular matrix (ECM). The ECM is a critical medium for cell-cell interactions and can also directly signal cells through cell surface ECM receptors, such as integrins. In addition, many growth factors and signaling molecules are stored in the ECM. Thus, ECM remodeling and/or degradation by MMPs are expected to affect cell fate and behavior during many developmental and pathological processes. Numerous studies have shown that the expression of MMP mRNAs and proteins associates tightly with diverse developmental and pathological processes such as tumor metastasis and mammary gland *In vivo* involution. evidence to support the roles of MMPs in these processes has been much harder to get. Here we will review some of our studies on the MMP11 or stromelysin-3 during the thyroid hormone-dependent amphibian metamorphosis, a process that resembles the so-called postembryonic development in mammals (from a few months before to several months after birth in humans when organ growth and maturation take place). Our investigations demonstrate that stromelysin-3 controls apoptosis in different tissues via at least two distinct mechanisms.

Keywords

Matrix metalloproteinase; stromelysin-3; metamorphosis; *Xenopus laevis*; tumor invasion; apoptosis

Introduction

In multicellular organisms, the vast majorities of the cells are in contacts with each other and/or with the extracellular matrix (ECM) (Hay, 1991). The ECM is a complex structure composed of proteins and other macromolecules (Hay, 1991; Timpl and Brown, 1996). The ECM serves as a structural support media for tissues/organs and interacts with cells through cell surface receptors such as integrins (Brown and Yamada, 1995; Damsky and Werb, 1992; Schmidt and others, 1993). The ECM can also regulate the availability and levels of many signaling molecules such as growth factors stored in the ECM (Vukicevic and others, 1992; Werb and others, 1996). Thus, the ECM and its remodeling can influences cell shape,

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behavior, and ultimately cell fate via multiple pathways (Hay, 1991; Ruoslahti and Reed, 1994).

ECM remodeling and degradation is believed to be largely through the action of matrix metalloproteinases (MMPs). MMPs are extracellular or membrane-bound proteolytic enzymes (Alexander and Werb, 1991; Barrett and others, 1998; Birkedal-Hansen and others, 1993; Fu and others, 2009; McCawley and Matrisian, 2001; Nagase, 1998; Parks and Mecham, 1998; Pei, 1999). MMPs can be divided into 5 groups based on their domain organizations (Fig. 1). The first and most common group of MMPs, e.g., collagenase 1, has five domains, the pre-and pro-peptides, a catalytic domain, a hinge region, and the hemopexin domain (Fig. 1). The second group includes gelatinase A (MMP2) and B (MMP9), which contain the fibronectin (FN)-like domain within the catalytic domain (Fig. 1). The membrane type (MT)-MMPs, e.g., MT1-MMP, belong to a group with a C-terminal transmembrane domain as well as a furin-recognition motif RXXR at the end of the pro-peptide (Fig. 1). Another group includes stromelysin-3 (ST3, MMP11), MMP23, and the amphibian-specific MMP N6 (Fu and others, 2009). MMPs in this group have the furin-recognition motif RXXR but lack the C-terminal transmembrane domain (Fig. 1) (Fu and others, 2009). Finally, MMP7, MMP26 and the amphibian-specific MMP N2 (Fu and others, 2009) lack the hemopexin domain (Fig. 1).

MMPs are synthesized as pre-enzymes and the pre-peptide is cleaved upon secretion into the ECM as pro-enzymes with exceptions of those contain a furin-recognition motif RXXR (Fig. 1). The pro-enzymes are enzymatically inactive due to the formation of the fourth coordination bond with the catalytic Zn^{2+} ion by the conserved cysteine residue in the pro-peptide (van Wart and Birkedal-Hansen, 1990). The pro-enzymes are activated through proteolytic removal of the pro-peptide (Barrett and others, 1998; Birkedal-Hansen and others, 1993; Kleiner and Stetler-Stevenson, 1993; Murphy and others, 1999; Nagase, 1998; Nagase and others, 1992). On the other hand, the MMPs with a furin-recognition motif RXXR, such as ST3 and MT-MMPs, are activated intracellularly through a furin-dependent process (McCawley and Matrisian, 2001; Pei and Weiss, 1995; Sato and Seiki, 1996). The activated MMPs have different but often overlapping substrate specificities (Barrett and others, 1998; McCawley and Matrisian, 2001; Overall, 2002; Uria and Werb, 1998). Together, they are capable of cleaving all protein components of the ECM as well as many non-ECM extracellular or membrane-bound proteins (Barrett and others, 1998; McCawley and Matrisian, 2001; Overall, 2002; Uria and Werb, 1998). Thus, MMPs are expected to affect cells through both ECM-dependent and ECM-independent pathways.

Extensive expression studies have shown that MMPs are associated with a number of pathological processes such as tumor invasion and metastasis (Fingleton and others, 1999; Hotary and others, 2003; Lochter and Bissell, 1999; Lopez-Otin and Matrisian, 2007; Lynch and others, 2007; MacDaougall and Matrisian, 1995; Martin and Matrisian, 2007; Masson and others, 1998; Nelson and others, 2000; Stetler-Stevenson and others, 1993; Tryggvason and others, 1987). In addition, the expression of the mRNAs and proteins for various MMPs also shows strong correlations with tissue remodeling and organogenesis during development in various animal species ranging from human to amphibians (Matrisian and Hogan, 1990; Salamonsen and Woolley, 1999; Sang, 1998; Sarra and others, 2002; Uria and Werb, 1998; Vu and Werb, 2000). Furthermore, a number of *in vivo* studies by using MMP inhibitors and/or MMP knockout mice support the involvement of at least some MMPs in mammalian development (Chin and Werb, 1997; Holmbeck and others, 1999; Shapiro, 1998; Stickens and others, 2004; Wiseman and others, 2003; Zhou and others, 2000). On the other hand, mice lacking any one particular MMP, including ST3, often show little or weak phenotype (Masson and others, 1998; Shapiro, 1998; Stickens and others, 2004; Wiseman and others, 2003), with the exception of mice lacking MT1-MMP, which

develop a number of severe postnatal defects including dwarfism, craniofacial dysmorphism, impaired endochondral ossification and angiogenesis and die within several weeks after birth (Holmbeck and others, 1999; Zhou and others, 2000). This is likely due to redundancy in MMP function during development. In addition, the importance of MMPs may vary in different organs and this would result in MMP knockout animals that appear normal but have defects in certain organs/tissues. For example, mice lacking MMP13 (collagenase 3) have abnormal skeletal growth plate development (Stickens and others, 2004) and mice lacking MMP2 (gelatinase A) or MMP3 (stromelysin-1) have altered mammary gland branching morphogenesis (Wiseman and others, 2003). Thus, a proper model system is critical for studying the function of MMPs in development *in vivo*.

Amphibian metamorphosis is a postembryonic developmental process initiated and controlled by thyroid hormone (T3) (Dodd and Dodd, 1976; Shi, 1999). T3 has both non-genomic and genomic effects on target tissues/cells. The non-genomic effects of T3 is mediated through its binding to cell surface proteins and cytosolic proteins to alter signaling pathways within the cell (Bassett and others, 2003; Davis and Davis, 1996; Davis and Davis, 2002; Davis and others, 2005; Guigon and Cheng, 2009; Shi and others, 1996; Storey and others, 2006). Its genomic or nuclear effects are due to the regulation of target gene expression via binding to thyroid hormone receptors (TRs). TRs are transcription factors belonging to the nuclear receptor super-family and modulate gene expression by binding to specific DNA sequences in target genes (Mangelsdorf and others, 1995; Shi, 2009; Tsai and O'Malley, 1994). Molecular and genetic studies have shown that TRs are both necessary and sufficient to mediate the metamorphic effects of T3 (Buchholz and others, 2003; Buchholz and others, 2006; Buchholz and others, 2004; Das and others, 2002; Nakajima and Yaoita, 2003; Schreiber and Brown, 2003; Schreiber and others, 2001; Schreiber and others, 2009; Shi, 2009). Thus, T3 induces a gene regulation cascade in individual tissues/organs to effect their transformations. Extensive analyses have been carried out to identify and functionally characterize T3-regulated genes during metamorphosis by using *Xenopus laevis* as a model (Brown and Cai, 2007; Buchholz and others, 2007; Das and others, 2006; Das and others, 2009; Gilbert and others, 1996; Shi, 1999). Among the genes regulated by T3 during *Xenopus laevis* metamorphosis are several MMP genes, including ST3 (Fujimoto and others, 2006; 2007; Hasebe and others, 2006; Hasebe and others, 2007; Jung and others, 2002; Patterson and others, 1995; Shi and Brown, 1993; Shi and Ishizuya-Oka, 2001; Stelow and others, 1996; Wang and Brown, 1993). Expression and functional studies suggest that different MMPs have different roles during *Xenopus laevis* metamorphosis. In this paper, we will review some of these studies by focusing on ST3, for which most of the functional studies have been carried out.

Regulation of MMP genes during *Xenopus laevis* metamorphosis

A role of MMPs in amphibian metamorphosis was first implicated in early half a century ago by the discovery of drastic increases in collagen degradation activity in the resorbing tadpole tail (Gross and Lapiere, 1962). 30 years later, the two frog MMP genes, collagenase-3 and ST 3, were identified as T3 inducible genes in *Xenopus laevis* (Brown and others, 1996; Patterson and others, 1995; Shi, 1999; Shi and Brown, 1993; Wang and Brown, 1993). Around the same time, another frog MMP, the *Rana catesbeiana* collagenase-1, was cloned by screening an expression cDNA library with an antiserum against purified *Rana* tail collagenase (Oofusa and others, 1994). These three frog MMP genes are all upregulated by T3 during metamorphosis. Subsequently, many other MMPs have been cloned in *Xenopus laevis* and all found to be regulated either directly by T3 at the transcriptional level via TR or indirectly through other mechanisms (Brown and others, 1996; Damjanovski and others, 1999; Das and others, 2009; Fu and others, 2006; Fujimoto and others, 2006; 2007; Hasebe and others, 2006; Hasebe and others, 2007; Ishizuya-Oka and others, 1996; Jung and others,

2002; Oofusa and others, 1994; Patterton and others, 1995; Sawada and others, 2001; Stolow and others, 1996; Wang and Brown, 1993). In addition, a genome wide analysis indicates that *Xenopus laevis* and the highly related species, *Xenopus tropicalis* have homologs of essentially all mammalian MMPs plus a few novel ones (Fu and others, 2009), suggesting the existence of additional MMPs participating in frog metamorphosis.

There are three major changes that take place during metamorphosis (Dodd and Dodd, 1976; Gilbert and others, 1996; Shi, 1999). The larval specific organs such as the tail and gills are resorbed completely during metamorphosis and the adult specific ones such as the limbs develop de novo. The vast majority of the organs such as the intestine and brain are present both in tadpoles and adults and are remodeled during metamorphosis to accommodate the change from being aquatic and herbivorous to being terrestrial and carnivorous. These diverse transformations involve both the removal of unwanted larval cells through programmed cell death or apoptosis and proliferation followed by differentiation of adult cell types. Extensive analyses have demonstrated the apoptotic removal of larval cells during metamorphosis in a number of organs, including the tail and intestine (Shi, 1999). During metamorphosis, the tadpole intestine is transformed from a simple tubular structure with primarily a single layer of larval epithelial cells into a complex one with a multiply folded adult epithelium surrounded by elaborate connective tissue and muscle layers (Fig. 2A) (Schreiber and others, 2005; Shi and Ishizuya-Oka, 1996). This transition involves apoptotic removal of essentially all larval epithelial cells around stages 60-62 (Fig. 2A). Similarly, tail resorption involves the removal of all cell types by the end of metamorphosis. It has been well documented that the muscle and epidermal cells die through apoptosis, mostly after stage 62 (Fig. 2A) (Dodd and Dodd, 1976; Yoshizato, 1989).

Given the importance of cell-ECM interactions for cell fate and behavior and the ability of MMPs to remodel ECM, MMPs are expected to participate in the apoptotic removal of the larval cells. Expression analyses have provided the first evidence to support this. For example, ST3 has little expression in premetamorphic *Xenopus laevis* tadpoles (Patterton and others, 1995). Its mRNA is strongly upregulated by stage 58 in the tadpole intestine, prior to any detectable epithelial apoptosis. It reaches very high levels by stage 60 when cell death is at high levels in the larval epithelium, and is absent by the end of metamorphosis (Fig. 2B). Similarly, in the tail, ST3 mRNA is highly upregulated by stage 60, later than in the intestine but again ahead of major cell death in the tail after stage 62 that leads to rapid tail shortening. Even in the developing limb, ST3 is expressed at much higher levels around stage 54 when inter-digital cell death takes place (Patterton and others, 1995). Thus, ST3 expression is temporally correlated with and more importantly precedes cell death in different organs during metamorphosis. Two other MMPs, MT1-MMP (MMP14) and MMP9-TH (one of the two duplicated MMP9 or gelatinase B in *Xenopus laevis*), have similar temporal expression profiles as ST3 (Fujimoto and others, 2006; 2007; Hasebe and others, 2007). On the other hand, several other MMPs, including gelatinase A (MMP2), collagenase-3 (MMP13), and collagenase-4 (MMP18), are upregulated later than ST3 in intestine, suggesting that they are unlikely involved in initiating cell death in this organ but rather in post-apoptotic ECM remodeling/removal (Fujimoto and others, 2006; 2007; Hasebe and others, 2006; Patterton and others, 1995; Stolow and others, 1996), although all are upregulated during tail resorption (Fujimoto and others, 2006; 2007; Hasebe and others, 2006; Jung and others, 2002; Patterton and others, 1995; Shi and Brown, 1993; Stolow and others, 1996; Wang and Brown, 1993).

In situ hybridization and immunohistochemical analyses have shown that ST3 mRNA and protein are expressed in most of the fibroblasts of the connective tissue underlying intestinal epithelium around stages 60-61 (Damjanovski and others, 1999; Ishizuya-Oka and others, 1996; Patterton and others, 1995). The mRNAs of several other MMPs are also expressed in

the connective tissue of the intestine during metamorphosis, although the expression of collagenase-3 and -4 appears to be more sporadic, probably due to lower levels of expression (Damjanovski and others, 1999; Hasebe and others, 2006; Hasebe and others, 2007). In the tail, *in situ* hybridization analyses of collagenase-3, -4, and ST3 expression during tail resorption show that only ST3 mRNA is spatially and temporally associated with apoptosis (Berry and others, 1998b; Damjanovski and others, 1999; Fujimoto and others, 2006; 2007). In particular, ST3 mRNA is highly expressed at stages 62-64 in the connective tissue underlying the degenerating epidermis and surrounding the dying muscles. In contrast, high levels of collagenase-3 and collagenase-4 mRNAs are present only in the connective tissue surrounding the notochord, where few apoptotic cells are detected. On the other hand, the spatial expression patterns of MT1-MMP and gelatinase A are similar to those of ST3 in the tail during metamorphosis (Hasebe and others, 2006). These results suggest that ST3, MT1-MMP, and gelatinase A likely play an active role in the apoptotic degeneration of the tail epidermis and muscles while collagenase-3 or -4 may have little direct effect on the apoptosis in these tissues.

Expression studies indicate that MMPs are also expressed in other tissues/organs during *Xenopus* metamorphosis, including the developing limbs, remodeling head, and resorbing gill (Berry and others, 1998a; Berry and others, 1998b; Fujimoto and others, 2007; Patterson and others, 1995; Stolor and others, 1996; Wang and Brown, 1993). *In situ* hybridization shows that they are often associated with tissue degeneration events, likely involving apoptosis (Berry and others, 1998a; Berry and others, 1998b). Such associations may not be surprising as ECM removal is required during tissue degeneration. However, more detailed studies are needed to determine if any of these MMPs actively participate in the apoptotic process in these tissues/organs.

In addition to their involvement during larval tissue degeneration, all of the MMPs are expressed at stage 62 or later in the intestine when adult tissue morphogenesis takes place (Fujimoto and others, 2006; 2007; Hasebe and others, 2006; Hasebe and others, 2007; Patterson and others, 1995; Stolor and others, 1996). Most are highly expressed at stage 62 but repressed after stage 64. During this period, adult epithelial cells and cells of the connective tissue and muscles differentiate. The morphogenesis of intestinal folds also progresses through migration of the epithelial and/or fibroblastic cells, thereby establishing a crest-trough axis of the intestinal fold that is similar to the villus-crypt axis in higher vertebrates (Shi and Ishizuya-Oka, 1996). Thus, it is likely that MMPs also participate in the establishment of new adult ECM to facilitate differentiation and/or cell migration during adult epithelial morphogenesis.

Roles of ST3 in ECM remodeling and apoptosis during metamorphosis

Compared to the extensive studies on MMP expression, there have been relative few reports of functional analyses of MMPs during metamorphosis. Organ cultures have provided the first opportunity to investigate the roles of MMPs during tissue remodeling. As MMPs are extracellular proteins, it is possible to block their function in such cultures by adding inhibitors to the culture medium. For example, when tail fins are cultured *in vitro* in the presence of T3, they undergo resorption. The addition of TIMP(s) (the naturally occurring tissue inhibitors of metalloproteinases) purified from bovine dental pulp can block this T3-induced resorption (Oofusa and Yoshizato, 1991), supporting the requirement of MMP activity in this process. Similarly, intestinal metamorphosis, including the apoptosis of the larval epithelial cells, can be reproduced *in vitro* by treating intestinal fragments from premetamorphic tadpoles with physiological concentrations of T3. The addition of a synthetic MMP inhibitor to such cultures also inhibits T3-induced apoptosis (Ishizuya-Oka and others, 2000). While these studies demonstrate the importance of MMPs during

metamorphosis, they do not reveal the role of any specific MMPs as the TIMPs and synthetic inhibitors are capable of inhibiting the activities of many MMPs.

We have focused on the role of ST3 during metamorphosis based on the strong spatiotemporal correlation of its expression with apoptosis in various organs. Furthermore, ST3 expression is similarly induced by T3 in intestinal organ cultures just like during natural metamorphosis (Ishizuya-Oka and others, 2000). Its mRNA and protein levels correlate with the thickening and folding of the ECM (basal lamina) separating the epithelium and connective tissue as well as the apoptosis of the larval epithelial cells. In order to study the role of ST3 in such organ cultures, we have generated a function-blocking polyclonal antibody against the catalytic domain of *Xenopus* ST3 (Ishizuya-Oka and others, 2000). When this antibody was added to the culture medium of tadpole intestinal fragments, it also inhibited T3-induced epithelial cell death (Ishizuya-Oka and others, 2000). Interestingly, ECM remodeling induced by T3, i.e., the thickening and folding of the basal lamina, was inhibited as well. Thus, ST3 is required for both ECM remodeling and larval epithelial cell death. After longer culturing in the presence of T3, adult epithelial cells proliferated as clusters of undifferentiated cells or islets that invaginated into the connective tissue (Ishizuya-Oka and others, 2009; Ishizuya-Oka and Shimozaawa, 1991). The antibody was able to block the expansion of the proliferating adult epithelial islets into the connective tissue. Instead, these T3-induced adult epithelial islets expanded only laterally along the epithelium-connective tissue interface. Thus, ST3 is critical for ECM remodeling, larval cell death, and adult cell migration but not for adult epithelial cell proliferation in the T3-induced intestinal remodeling *in vitro* (Ishizuya-Oka and others, 2000).

To investigate whether ST3 can affect cell fate, *in vivo* we have carried out transgenesis to overexpress MMPs during *Xenopus* development. Transgenic expression of *Xenopus laevis* ST3 and collagenase-4 as well as a mammalian membrane-type MMP under the control of the constitutive CMV promoter causes lethality during late embryonic and early tadpole stages (Damjanovski and others, 2001), consistent with the tight regulation of MMP expression during *Xenopus* embryogenesis (Damjanovski and others, 2000). Thus, we have developed a double promoter construct to express MMPs under the control of a heat shock-inducible promoter (Fu and others, 2002) and at the same time express the green fluorescent protein (GFP) in the tadpole eyes under the control of a second promoter, the γ -crystallin promoter. This allows one to rear wild type and transgenic tadpoles together to ensure no variation in animal growth and treatment conditions between the two groups and later identify the transgenic ones by simply examining their eyes under a fluorescent dissecting microscope. The expression of transgenic MMPs can be induced any time by simply heat shocking the animals for brief periods daily. Consistent with the findings from transgenic expression of ST3 under the control of the CMV promoter, heat shock induction of ST3 expression in the ST3 transgenic animals generated using this double promoter construct also leads to lethality during early embryonic stages (Fu and others, 2002). Interestingly, induction of ST3 at premetamorphic tadpole stages (between stage 45 to 54) for up to a few weeks has little effect on overall growth and development (Fu and others, 2005), indicating, perhaps not surprisingly, that proper regulation of MMP function is not as critical for tadpole organ/tissue function during animal growth period as for organ development during embryogenesis.

When premetamorphic ST3 transgenic tadpoles at stage 54 were heat shocked for 4 days, the precocious transgenic overexpression of ST3 alone were able to induce larval epithelial apoptosis (Fu and others, 2005), accompanied by the activation of fibroblasts (i.e., with enriched rough endoplasmic reticulum) just beneath the epithelium and cell-cell contacts between epithelial cells and the fibroblasts. These changes are similar to those observed during natural metamorphosis, but were absent in heat shock-treated non-transgenic animals

or transgenic animals expressing a catalytically inactive ST3 mutant (Fu and others, 2005). In addition, the ECM or basal lamina separating the epithelium and connective tissue was also altered by the transgenic expression of ST3. In some areas, including areas where epithelial cells and fibroblasts contact, the ECM was thicker and in other areas, it appeared to be absent. Thus, the ECM in the transgenic animals was different from the thick, multiply folded basal lamina in the small intestine during metamorphosis. Another difference from the metamorphosing intestine is that adult epithelial cell islets were not observed in ST3 expressing transgenic animals. Thus, ST3 expression alone is sufficient to induce some but not all T3-induced metamorphic program in the intestine (Fu and others, 2005). These *in vitro* and *in vivo* studies complement each other and support a critical role of ST3 in ECM remodeling and larval epithelial cell death during intestinal metamorphosis.

When the tail of the ST3 transgenic animals was analyzed, it was found that precocious expression of ST3 led to apoptosis predominantly in the muscles with relatively little in the epidermis (Fig. 3) (Mathew and others, 2009). On the other hand, during natural metamorphosis, comparable levels of apoptotic cells are detected in both the epidermis and muscles of the tail around stage 61, right before rapid reduction in tail length when ST3 mRNA levels are significantly up-regulated (Fig. 4A) (Mathew and others, 2009). By stage 63, with even higher levels of ST3 expression, the number of apoptotic cells in both tissues, especially the epidermis, increases dramatically (Fig. 4B) (Damjanovski and others, 1999; Mathew and others, 2009). Interestingly, when wild type premetamorphic tadpoles were treated with physiological levels of T3 for several days (before any significant reduction in tail length), cell death was much higher in the muscles than in the epidermis and the ST3 mRNA level induced by T3-treatment was also much lower than that at stage 63 (Mathew and others, 2009). These results suggest that very high levels of ST3 expression in the connective tissue underlying the epidermis are needed for epidermal apoptosis in the tail during metamorphosis. In this regard, it is of interest to note that the ST3 mRNA levels in the heat shocked transgenic tadpole tail were very high, comparable to those in the tail of stage 63 tadpoles (Mathew and others, 2009). However, due to the ubiquitous nature of the heat shock inducible promoter, the transgenic ST3 mRNA was expressed in all cell types and its level in the connective tissue underlying epidermis was thus likely lower than the endogenous ST3 mRNA level at stage 63 tadpoles during natural metamorphosis, consequently insufficient to induce significant epidermal apoptosis.

Multiple pathways for ST3 to induce apoptosis during metamorphosis

Unlike almost all other MMPs, ST3 has only weak activities toward ECM proteins but much stronger activities toward non ECM proteins α 1-proteinase inhibitor and IGFBP-1 (Amano and others, 2004; Ishizuya-Oka and others, 2000; Manes and others, 1997; Murphy and others, 1993; Pei and others, 1994). While it is possible that ST3 may be able to cleave ECM proteins under *in vivo* conditions during development as efficiently as other MMPs, *in vitro* these findings suggest that ST3 likely function by cleaving non-ECM proteins. Interestingly, a yeast-two-hybrid screen led to the identification of the 67 kD laminin receptor (LR) as a substrate of ST3 (Amano and others, 2005a; Amano and others, 2005b). LR is synthesized as a 37 kD precursor protein. The majority of the precursor proteins is localized intracellularly and may be involved in translational and others, 1998; Auth and Brawerman, 1992) (Ardini regulation. A small fraction of the precursor proteins are inserted in the plasma membrane and become the 67 kD LR through (Buto and others, 1998; possibly heterodimerization and/or posttranslational modifications Castronovo and others, 1991; Landowski and others, 1995; Malinoff and Wicha, 1983; Menard and others, 1997; Rao and others, 1983; Starkey and others, 1999). *In vitro*, purified *Xenopus laevis* ST3 catalytic domain can efficiently cleave both *Xenopus laevis* and human LR synthesized *in vitro* or *in E. coli* (Amano and others, 2005b). More importantly, the two ST3 cleavage sites are

located between the transmembrane domain and laminin binding sequence within the extracellular half of LR, while several other MMPs cleave LR outside of this region (Amano and others, 2005b). Thus, ST3 may be unique in its ability to cleave LR to separate its cell-binding portion from its laminin-binding part, thus disrupting cell-ECM interaction.

During metamorphosis, both LR mRNA and protein are expressed fairly constantly in the intestine and tail (Amano and others, 2005a; Mathew and others, 2009), in contrast to the metamorphosis-specific expression of ST3. On the other hand, LR fragments of the sizes expected from ST3 cleavage are present in the intestine and tail at the climax of metamorphosis when ST3 is highly expressed, whereas little such fragments are present in pre- or post-metamorphic intestine or premetamorphic tail (Fig. 5A, B) (Amano and others, 2005a; Mathew and others, 2009). More importantly, precocious expression of ST3 upon heat shock treatment of premetamorphic tadpoles with transgenic ST3 under the control of a heat shock-inducible promoter leads to the formation of these fragments in the absence of ST3 in the intestine, although not in the tail (Fig. 5B) (Amano and others, 2005a; Mathew and others, 2009). These results suggest that LR is an *in vivo* substrate of ST3 during intestinal remodeling. The lack of LR cleavage in the tail of transgenic animals suggest that either LR is not directly cleaved by ST3 in the tail during natural metamorphosis or the level of ST3 expressed in the tail of transgenic animals is somehow insufficient to allow significant cleavage of the LR in the tail.

If LR cleavage by ST3 plays a role in apoptosis of the cells in the tail and the intestine, one would expect LR to be expressed in the apoptotic cells. Indeed, immunohistochemical analysis has revealed that LR is highly expressed in the differentiated epithelial cells but not in the connective tissue of premetamorphic intestine (Amano and others, 2005a). As intestinal metamorphosis proceeds and ST3 expression rises, LR expression increases in the connective tissue but decreases in the dying larval epithelial cells. There is no detectable signal in the proliferating adult epithelial cells. However, as the adult epithelial cells differentiate, they begin to express LR just when ST3 expression is repressed. These results suggest an interesting model for LR function in the intestine. That is, LR expression on the cell surface may be important for the survival of differentiated intestinal epithelial cells. The upregulation of ST3 expression during metamorphosis leads to the cleavage of LR on larval epithelial cells. This results in the reduced epithelial cell-ECM interaction and thus facilitates larval epithelial cell death. On the other hand, the proliferating, undifferentiated adult epithelial cells are not associated with the basal lamina and thus do not require LR for attachment to the ECM. Consequently they are not affected by ST3. The high levels of LR expression in the fibroblasts of the connective tissue during metamorphosis may be related to the role of intracellular LR in protein synthesis as the fibroblasts are highly activated with extremely rich rough endoplasmic reticulum, where large amounts of actively translating ribosomes are present.

In the resorbing tail, LR is expressed in the epidermis and the fibroblasts underneath the epidermis and surrounding the muscles but appear to be absent in the muscle cells (Mathew and others, 2009). Interestingly, western blot analysis of the isolated epidermis and muscles of the tail at stage 63 (when the tail has resorbed to half of the original length) showed that LR cleavage products as observed in the whole tail (Fig. 5B) were present in the isolated epidermis but not the muscles (Fig. 5C). Thus, LR cleavage only occurs in the epidermal region during tail resorption. Given the LR cleavage occurs only when high levels of epidermal apoptosis and ST3 expression are present during metamorphosis, it is very likely that LR cleavage plays a role in epidermal cell death. These findings suggest that in the tail, ST3 may induce apoptosis via two distinct mechanisms. In the epidermis, LR is expressed and likely important for proper interaction between the cells and the underlying ECM. When high levels of ST3 are expressed around stage 63, ST3 cleaves LR to facilitate epidermal

cell death. In ST3 transgenic animals or premetamorphic tadpoles treated with T3, most of the apoptosis observed are in the muscles but not in the epidermis, consistent with the lack of detectable LR cleavage. It is unclear at present why the level of transgenic ST3 expression was sufficient to cleave LR on the intestinal epithelial cells but not on the tail epidermal cells. Nonetheless, the strong correlation among the expression of ST3, LR cleavage, and apoptosis in the LR-expressing epidermis or epithelium supports a role of LR in cell-ECM interaction for these cells and its cleavage in their death. On the other hand, the muscle cells do not express LR. Even low levels of ST3 expression are sufficient to cause muscle cell death. Here, clearly a different mechanism is employed. It is very likely that ST3 cleaves other cell surface or extracellular proteins to affect the muscles.

Conclusion

ST3 was first cloned from human breast cancer (Basset and others, 1990). Since then, extensive expression studies have shown that ST3 is expressed under many pathological conditions including cancer, wound healing, atherosclerotic lesions, and rheumatoid arthritis (Ahmad and others, 1998; Basset and others, 1997; Chenard and others, 1996; Kossakowska and others, 1996; Lochter and Bissell, 1999; Nawrocki and others, 1994; Schonbeck and others, 1999; Tetu and others, 1998; Wolf and others, 1992). In addition, while ST3 has little expression in adult organs under normal physiological condition, it is highly expressed in a number of developmental processes such as embryo implantation and mammary gland involution (Alexander and others, 1996; Basset and others, 1990; Chin and Werb, 1997; Lefebvre and others, 1995). In mammals, at least in the developing limb and in mammary gland during post-lactation involution, ST3 expression also correlates with apoptosis (programmed cell death) (Basset and others, 1990; Lefebvre and others, 1992; Uria and Werb, 1998), suggesting that ST3 have an evolutionally conserved role in facilitating cell death during normal physiological processes. Despite extensive biochemical studies on various MMPs have revealed their biochemical and *in vitro* molecular properties and led to the identification of many ECM and non-ECM substrates (Barrett and others, 1998; McCawley and Matrisian, 2001; Overall, 2002; Uria and Werb, 1998), it has been much more difficult to study the biological functions of MMPs *in vivo*. Specific gene knockout can provide direct evidence for the roles of an MMP in development. Unfortunately, likely due to redundancy in MMPs activity in development, mice lacking individual MMPs have very weak or little phenotypes (Shapiro, 1998; Stickens and others, 2004; Vu and others, 1998; Wiseman and others, 2003) with the exception of MT1-MMP (Holmbeck and others, 1999; Zhou and others, 2000). This is also true for ST3 knockout mice (Masson and others, 1998). Thus, the physiological functions for most MMPs, including ST3, remain unknown during mammalian development.

The frog metamorphosis model has provided the first direct evidence to support an important role of ST3 in regulating physiological cell death during vertebrate development. The intestinal organ culture studies indicate that ST3 function is required for ECM remodeling and epithelial cell death during T3-induced metamorphosis. Transgenic analysis of ST3 in premetamorphic tadpoles complements the organ culture findings by demonstrating that ST3 alone is sufficient to induce several changes, including epithelial cell death, in the tadpole intestine that normally occur during natural metamorphosis when ST3 is also highly expressed. The differential effects of transgenic ST3 overexpression on tail epidermal and muscle cells indicate the existence of tissue specificity in the induction of apoptosis by ST3 during metamorphosis. More importantly, the identification and characterization of LR as an *in vivo* substrate of ST3 during metamorphosis suggest that ST3 induces cell death via at least two pathways: one through the cleavage of LR to alter cell-ECM interaction for apoptosis of the intestinal epithelial cells and tail epidermal cells while the second one independent of LR cleavage for apoptosis in the tail muscles. A major

challenge in the near future is to identify ST3 substrate(s) involved in muscle cell death and to investigate whether and how LR and other ST3 substrates participate in the induction of apoptosis by ST3. Furthermore, it will be of importance to determine whether ST3 regulates cell death via similar pathways during mammalian development.

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Fig. 1.

Structure of MMPs. MMPs generally contain five domains. These are the pre-and pro-peptides, catalytic domain, hinge region, and hemopexin domain, respectively. Some exceptions exist. These include matrilysin, which does not have a hemopexin domain, and membrane type MMPs (MT-MMPs), which contain a transmembrane/cytoplasmic (TM) domain at the carboxyl end, and gelatinases, which contain a fibronectin (FN)-like domain. A conserved peptide sequence is present in the pro-peptide where a C residue (underlined) is involved in coordination with the catalytic Zn^{2+} atom in the inactive pro-enzyme. In the catalytic domain, a conserved region contains three H residues (underlined) that coordinate with the catalytic Zn^{2+} atom. Finally, MT-MMPs and ST3 contain the conserved RXKR sequence for furin-dependent intracellular activation of the enzymes.

B.

Fig. 2.

(A). Stage-dependent apoptosis in the intestine and tail during *Xenopus laevis* metamorphosis. Tadpole tail consists of several tissues such as epidermis (Ep), muscle (M), connective tissue (CT), and notochord (Nc), all of which are removed by stage 66. The tails at stages 62-66 are drawn to the same scale to show the resorption, while the tadpoles and intestinal cross-sections (middle) at different stages are not in the same scale in order to highlight the morphological differences. Tadpole small intestine has a single epithelial fold, where much of the connective tissue is localized, while a frog has a multiply folded intestinal epithelium, with elaborate connective tissue and muscle. Asterisks : proliferating cells of adult intestinal epithelium (AE). Open circles: apoptotic cells of larval intestinal epithelium (LE).

(B). Organ-dependent temporal regulation of ST3 during *Xenopus laevis* metamorphosis. The mRNA levels are based on (Patterson and others, 1995) with the expression level in tail at stage 64 set to 100.

Wild type (WT)

Transgenic (TG)

Fig. 3.

Transgenic overexpression of ST3 preferentially induces apoptosis in the muscles of the tadpole tail. Premetamorphic transgenic (B) and sibling wild type (A) tadpoles were subjected to daily heat shock treatment to induce transgene expression. After 4 days the tadpoles were sacrificed and the tail was isolated for TUNEL assay to detect apoptotic cells. M: muscle, Ep: epidermis, Mc: melanocytes; arrows: apoptotic cells. (C) Quantification of the apoptotic cells in the muscle or epidermal region per an arbitrarily defined unit area sections of the tail of wild and transgenic tadpoles. Note that ST3 over expression led to an increase in apoptosis in both the muscle and epidermal regions but significantly only in the muscle region. *, P value = 0.05. For more details, see (Mathew and others, 2009).

Fig. 4.

High levels of ST3 expression correlate with cell death in the metamorphosing tail. (A). ST3 mRNA attains high levels by stage 62, when tail length reduction is to begin during natural metamorphosis, and reaches even higher levels by stage 63, when the tail is resorbed to half of its original length.

(B). Apoptotic cells per defined unit area of the tail at stage 61 and stage 63. M: muscles; Ep: epidermis. For more details, see (Mathew and others, 2009).

A

B

C

Fig. 5.
(A). LR cleavage is present at stages 62 and 63 when ST3 expression is very high in the tail. Total protein was isolated from tail of tadpoles at various stages. The proteins were subjected to western blotting with anti-*Xenopus* LR antibody. For more details, see (Mathew and others, 2009).
(B). LR is cleaved in the tail and intestine at the climax of metamorphosis as well as in the intestine but not tail of premetamorphic transgenic tadpoles overexpressing ST3. Total protein was isolated from tail or intestine of heat shock (HS)-treated transgenic (TG) or wild type premetamorphic animals or naturally metamorphosing tadpoles at stage 62 or 63. 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 tail. (C). LR is cleaved in the epidermis but not the muscles of the tail at the climax of metamorphosis. 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. Note that the LR in the muscle lane was from the connective tissue surrounding the muscle cells. The black arrowhead indicates full-length LR. The gray arrowheads indicate likely non-specific cleavage products of LR. The open and closed circles indicate the expected ST3 cleavage products. For more details, see (Mathew and others, 2009).