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Matrix metalloproteinase function in non-mammalian model organisms

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1. ABSTRACT

Matrix metalloproteinases (MMPs), adamalysins, astacins, and serralysins are members of the metzincin superfamily of proteases. MMPs constitute a large protein family of both secreted and membrane-tethered enzymes that are synthesized as zymogens (proMMP) and activated by a cysteine-switch mechanism. First described over 50 years ago by Gross and Lapiere as a collagenolytic activity in amphibian tissues, the human MMP family now encompasses 23 different genes whose encoded proteins are capable of cleaving a variety of extracellular matrix protein substrates. Since their expression is upregulated in many cancer cell types, MMPs have received much attention particularly in the areas of tumor progression and metastasis. However, in terms of normal developmental processes, much less is known regarding MMP function and substrate identity. Data from knockout mouse studies support the notion that MMPs are not essential regulators of embryonic development, suggesting redundancy between MMPs or the presence of subtle phenotypes. However, studies on MMP function in other model systems indicate a larger role for MMP-dependent proteolysis during embryonic processes. Here, we review the current knowledge of MMPs from diverse model systems ranging from flowering plants and invertebrates to non-mammalian vertebrates.

2. INTRODUCTION

First described in amphibian tissues (1), matrix metalloproteinases (MMPs) are proteolytic enzymes and members of the metzincin superfamily of zinc-dependent endopeptidases (2). MMPs are capable of cleaving extracellular matrix (ECM) and non-ECM proteins including adhesion molecules, ligands, and receptors. The human genome contains 23 MMP genes and 4 genes encoding tissue inhibitors of metalloproteinases (TIMPs). Most human MMP proteins have a similar domain organization that includes a signal peptide, propeptide domain, catalytic domain, hinge region, and a hemopexin domain. While many MMPs are secreted as inactive zymogens requiring proprotein convertase activity to remove the N-terminal propeptide domain for activation (3), six MMPs are membrane-tethered and are activated in the trans-Golgi network by convertases (4) or by alternative mechanisms such as auto-proteolysis (5). These so-called MT-MMPs possess either a glycosylphosphatidylinositol (GPI) anchor or a type I transmembrane domain with a short cytoplasmic tail and are regulated by vesicular trafficking pathways (6).

It has long-been recognized that MMP-mediated collagenolytic activity correlates with tumor histology/grade (7) (reviewed in (8)). Thus, the general consensus is that increased MMP-dependent ECM degradation promotes tumor progression and metastasis. This thinking has prompted much interest in the field of MMP research. Knockout mice for several different MMPs have been created and while some show no developmental defects (9-13), some exhibit severe skeletal and connective tissue abnormalities (14,15). In cases where MMP loss-of-function phenotypes are apparent, these

Table 1. Numbers of MMPs and TIMPs in non-mammalian model systems

Organism	Secreted MMPs	MT-MMPs	TIMPs
Flowering Plants (*Arabidopsis thaliana*)	2	3	?
Hydra	1	?	?
Amphioxus	1	?	?
Sea Urchin (*Strongylocentrotus purpuratus*)	17	9	10
Worm (*Caenorhabditis elegans*)	5	1	?
Fly (*Drosophila melanogaster*)	1	1	1
Frog (*Xenopus laevis*)	23	≥3	2
Zebrafish	~16	~11	6
Chicken	≥3	≥3	≥2

are not observed until postnatal stages after completion of embryogenesis. These findings are unexpected given the large-scale cell migration and morphogenetic events that occur during mouse embryogenesis (16). The MMP14/MT1-MMP knockout mice have the strongest post-embryonic phenotypes (14,15) and this membrane-tethered MMP is a key activator of the proMMP2 zymogen (17). It is therefore notable that mice deficient for both MMP14 and MMP2 show a stronger phenotype than either single mutant, dying shortly after birth with a variety of defects (18). At minimum, the mouse MMP knockout data indicate that specific MMPs are not essential regulators of embryonic development. Is this a simple matter of redundancy between MMP family members or are the MMP knockout phenotypes extremely subtle or both?

Further insight into MMP function during normal development is being provided by studies utilizing classical non-mammalian model systems such as worms, flies, frogs, and fish. These systems have wide-ranging numbers of *MMP* genes (Table 1) and exhibit various *MMP* loss-of-function phenotypes. The goal of this review is to describe and highlight the function of MMPs expressed by diverse developmental model systems including plants, invertebrates, and non-mammalian vertebrates.

3. FLOWERING PLANTS

Arabidopsis thaliana is a flowering plant native to Africa, Asia, and Europe and is used as a model organism because of its many attributes including short life cycle, small genome, and genetics. The *A. thaliana* genome contains five genes encoding MMP-like proteins and these have been named *At1-* to *At5-MMP* (19). Each of these genes encodes a metalloproteinase with a signal peptide, propeptide domain, and catalytic domain. Therefore, structurally these putative MMPs are most similar to human MMP7 and MMP26 because they lack a C-terminal hemopexin-like domain characteristic of most MMP family members (3). Notably, At2-, At4-, and At5-MMP contain predicted C-terminal GPI anchor sites to link these proteins to the plasma membrane (19-22). Proteolytic assays performed utilizing purified/recombinant catalytic domains demonstrated enzymatic activity and distinct substrate specificity for all five At-MMPs (23). Here, similarities were observed between the cleavage site preferences for At2/3-MMP and At1/4-MMP suggesting the potential for common *in vivo* substrates, though this would require experimental confirmation. In the developing plant, differential *MMP* gene expression levels were noted for *At1-*, *At2-*, *At3-*, *At4-*, and *At5-MMP* in the flower, leaf, root, and stem at 14 days. An *A. thaliana At2-MMP* mutant exhibited growth abnormalities at 6 weeks associated with defects in root, leaf, and shoot length (24). At 10 weeks of development, *At2-MMP* mutants also had increased chlorophyll degradation, accelerated senescence, and reduced cell size compared to wild-type plants (24). Defects in germination were not observed.

4. HYDRA

Hydra is a member of the phylum Cnidaria and thus arose early during metazoan evolution, before the separation of protostome and deuterostome branches. Due to the ability to regenerate their entire body, hydra is a popular model organism for studying the cellular basis of tissue remodeling (see (25,26) for reviews). Notably, a hydra matrix metalloproteinase (HMMP) and its associated ECM degradation were shown to be required for regeneration of both the head and foot process (27,28). Analysis of the HMMP protein sequence (484 amino acids) identified several features in common with vertebrate MMPs including a signal peptide, propeptide domain, catalytic domain, hinge region, and a C-terminal hemopexin domain (27). It lacks both a transmembrane domain and GPI anchor site and is thus likely to be a secreted protein. Compared to both secreted and membrane-tethered human MMPs, HMMP has an overall amino acid sequence identity of 31-35% and as high as 47% when only the catalytic domains are compared. While both the cysteine-switch and Zn^{2+}-binding regions are highly conserved between HMMP and vertebrate MMPs, HMMP has unique properties such as the lack of key cysteine residues in the hemopexin domain (27). While HMMP is able to cleave a variety of hydra ECM substrates (laminin and collagen I), it is unable to degrade certain human ECM proteins including laminin, fibronectin, and both types I- and IV-collagen (27). The inability of hydra MMPs to cleave human ECM substrates might be due to differences in hemopexin domain structure that could prevent hydra

MMPs from physically interacting with human ECM proteins. HMMP enzymatic activity is inhibited by the synthetic MMP inhibitors GM6001 (galardin/ilomastat) and Matlistatin and by recombinant TIMP1 (27).

The hydra body plan is organized as an epithelial bilayer consisting of ectoderm and endoderm with a layer of ECM in between. Experiments utilizing whole-mount *in situ* hybridization have shown that *HMMP* mRNA is expressed within the endoderm of the adult polyp along the entire body length (27). However, *HMMP* is enriched in both the tentacles and the foot process just above the cells of the basal disc (27). It is notable that this mRNA expression pattern mirrors that of the HMMP cleavage substrate laminin β1. In terms of HMMP function, long-term exposure of adult hydra polyps to GM6001 caused the reversible de-differentiation of epithelial basal disc cells or alternatively the inhibition of transdifferentiation of body column cells to basal disc cells (27). Therefore, HMMP functions to regulate cell differentiation likely through effects on ECM cleavage and remodeling. In terms of foot process tissue regeneration, the expression of *HMMP* mRNA is strongly upregulated at sites of amputation and to continue for at least 48 hours during foot regeneration (27). During head regeneration, both *HMMP* and genes encoding the ECM proteins laminin β1 and collagen I are upregulated three hours after decapitation with increases in protein levels being observed several hours later (28). Typical of other metazoans, hydra ECM consists of basement membrane and fibrillar interstitial ECM (29) and both are lost and then reformed during tissue regeneration following amputation. Notably, the biogenesis of ECM during tissue regeneration is associated with the expression of *HMMP* suggesting a regulatory role for proteolysis during cell-ECM interactions underlying ECM assembly and cell differentiation (28).

5. AMPHIOXUS

The first reported cloning of an *MMP* family member in amphioxus occurred in 2012 (30). This gene was named *BbMMPL2* and encodes a 494 amino acid protein with a predicted mass of 54.7. kDa. This MMP contains a propeptide domain, catalytic domain, hinge region, and C-terminal hemopexin domain. Sequence analysis indicated that amphioxus BbMMPL2 has an overall identity of 33-36% with human MMPs (higher identity in the catalytic domain) (30). BbMMPL2 contains the highly conserved serine at position 214 indicative of vertebrate MMP family members. In terms of proteolysis, recombinant active BbMMPL2 was shown to cleave gelatin, type IV collagen, fibronectin, and laminin, an enzymatic activity that was inhibited by EGTA, GM6001, and human TIMP1/TIMP2 (30). While *BbMMPL2* mRNA is expressed in various adult organs including hepatic diverticulum, gut, ovary, and testes, expression in the developing embryo remains to be determined (30).

6. SEA URCHIN

In contrast to the organisms discussed thus far, the sea urchin (*Strongylocentrotus purpuratus*) genome contains at least 26 potential *MMP* genes (*Sp-MMP*) and 10 *TIMP* genes (31,32). Interestingly, there are at least nine membrane-tethered MMPs in the sea urchin genome (*Sp-MT-MMP*). Because most sea urchin *MMP* genes are the result of duplications of ancestral genes, it is not possible to determine orthologous relationships between sea urchin and vertebrate *MMPs*. Several sea urchin MMP proteins possess the typical architecture found in vertebrate MMPs such as catalytic and hemopexin domains, while several have unique structures (31). Utilizing a whole-genome tiling array approach, it was found that 19/26 sea urchin *MMP* genes and 9/10 *TIMP* genes are transcribed during embryogenesis (blastula and gastrula stages) (33). These data suggest that sea urchin MMPs might regulate specific morphogenetic processes associated with early development. Indeed, it is well recognized that a collagen-containing ECM is required for sea urchin gastrulation (34). Further work is needed to determine whether sea urchin ECM proteins are actual proteolytic substrates for MMP family members. One study on adult sea urchins from the order Camarodonta (*Paracentrotus lividus*) provided evidence that MMP activity might regulate the tensile strength of specific ligaments (35).

7. WORM

The genome of the nematode *Caenorhabditis elegans* contains at least 6 genes encoding potential MMPs (named zinc metalloproteinase; *ZMP-1* through *ZMP-6*) (36,37). With some variation, worm MMP proteins have typical vertebrate MMP domains including a signal peptide, hinge region, catalytic domain, and hemopexin domain (36). ZMP-5 does not contain an inhibitory cysteine-switch sequence and ZMP-1 has a predicted GPI membrane anchor. Amino acid identity between ZMP-2 and ZMP-3 and human MMP1 is 34% and 45%, respectively (38). ZMP-3 demonstrated proteolytic activity towards gelatin but not type I collagen or carboxymethylated transferrin (38). Using a fluorescence-quenching substrate, it was shown that the enzymatic activities of both ZMP-2 and ZMP-3 are inhibited by recombinant human TIMP1 and TIMP2 and by synthetic chemical MMP inhibitors (BB94/batimatstat and CT543) (38). Functional studies utilizing RNAi to knockdown *zmp-2* expression demonstrated a requirement for ZMP-2 during development from larval to adult stages (36). In addition, the molting process was retarded in *zmp-2* knockdown animals suggesting a potential role for this protease in the process of ecdysis.

One of the most studied *C. elegans* MMPs is ZMP-1 and its role during the process of anchor cell (a specialized uterine cell) invasion of the basement

membrane (reviewed in (39)). The larval basement membrane is similar to that from vertebrates containing the ECM proteins laminin, type IV collagen, perlecan, and nidogen (40). During the 90-minute transmigration process, an anchor cell first develops an invasive front that interfaces with the basement membrane followed by the formation of protrusions that will breach the basement membrane. Loss of function mutations in the transcription factor *fos-1a* produce anchor cells that can form polarized protrusions and target them to the basement membrane but that are unable to breach this ECM barrier (37). Although *zmp-1* is a transcriptional target of FOS-1a, null mutations in *zmp-1* only mildly affect anchor cell invasion indicating that this MMP functions redundantly with other proteases (37). Such a redundant and/or subtle role for ZMP-1 during anchor cell invasion suggests that the process of breaching the basement membrane is complex and involves more than proteolytic degradation of ECM proteins. Indeed, elegant work by Ihara *et al.* demonstrated that at later stages of uterine/vulval development, large ECM gaps form through a process of basement membrane sliding and targeted cellular adhesion (41).

8. FLY

Since the identification of two fruit fly *MMPs* over a decade ago, *Drosophila melanogaster* has continued to be an informative model for the study of MMP function (reviewed in (42)). The fly *Mmp* genes were originally named *Dm1-MMP* and *Dm2-MMP* (43,44) and are now more commonly referred to as *Mmp1* and *Mmp2* to better align with human *MMP* nomenclature. However, fly *Mmp1* and *Mmp2* are not orthologous to human *MMP1* and *MMP2* (45). Fly *Mmp1* encodes a secreted protein while the Mmp2 protein contains a GPI anchor site and localizes to cell membranes (43,45). Each of these MMPs contains typical domains found in vertebrate proteins including a signal peptide, propeptide domain, catalytic domain, hinge region, and hemopexin domain (43,44). Both exhibit proteolytic activity towards peptide substrates and Mmp1 was additionally shown to cleave fibronectin and type IV collagen. The *D. melanogaster* genome encodes just one TIMP (called Timp) and it is most similar to human TIMP3 (46). Notably, Timp can inhibit both Mmp1 and Mmp2 in addition to several human MMPs; Mmp1 is also inhibited by human TIMPs (45,46). These data once again confirm the conservation between invertebrate and vertebrate MMPs.

Analyses of *Mmp1* gene expression by northern blot first identified transcripts at late stages of embryogenesis (15 hours of development), after gastrulation (45). By contrast, *Mmp2* was detected between 8-12 hours (45). Each MMP is expressed in diverse embryonic and larval tissues including the site of dorsal closure, mesoderm, ectoderm, the developing gut, and cells of both the central and peripheral nervous systems (45). Despite expression in embryonic tissues, *Mmp1Mmp2* double mutant animals complete embryogenesis and hatch (45). While these data indicate that fly metalloproteinases are not essential for embryogenesis, they do not show whether Mmp1 or Mmp2 play subtle roles in specific cellular processes underlying embryonic development. At post-embryonic stages, Mmp1 and Mmp2 are required for tissue remodeling related to tracheal growth, metamorphosis, and wound healing (45,47,48). Fly tumor cells also utilize Mmp1 activity to metastasize to the ovary (49). Taking advantage of the genetic tools of the fly system, a structure-function study was performed to interrogate the role of the Mmp1 catalytic and hemopexin domains (47). Interestingly, while the catalytic domain is required for all Mmp1-dependent functions, the hemopexin domain is not required for tracheal tissue remodeling (47). The hemopexin domain however, is required for tissue invasion during metamorphosis. These findings underscore the specificity of interactions between Mmp1 and different substrates and implicate non-hemopexin domains in substrate recognition events required for certain developmental processes.

9. FROG

There have been 26 *MMPs* identified in *Xenopus laevis* (50). Twenty of these have vertebrate homologs and fifteen have human identified homologs. The other six MMPs represent *MMPs* that are novel to *X. laevis*. Since *X. laevis* has a pseudotetraploid genome several *MMPs* have duplicate copies including *XMMP1*, *XMMP7*, *XMMP13*, *XMMP14*, *XMMP24*, and *XMMP28*. Of the six novel *MMPs*, XMMP-N1 and *XMMP-N3* are found in both *X. laevis* and *X. tropicalis*. The other four novel MMPs are found only in *X. tropicalis*. Based on the similarities of these novel *MMPs* to other known *MMPs*, these *MMPs* probably arose by gene duplication (50). The *X. laevis* genome also encodes at least two TIMP homologs (TIMP2 and TIMP3) (51).

During development macrophages must migrate from the blood islands, where they form, throughout the body. Three MMPs have been shown to play a role in this migration: XMMP7, XMMP9 and XMMP18. XMMP7 lacks a hemopexin domain and has a variety of ECM substrates (50). XMMP9 is a gelatinase. XMMP18 is known to cleave gelatin and collagen (52). Early in embryogenesis *XMMP7* is expressed in the area where the blood islands eventually form. As development progresses it begins to be expressed throughout the embryo between the epidermis and mesoderm in a spotted pattern indicating its localization within single cells (53). Supporting this notion, *XMMP7* colocalizes with the macrophage marker POX2 (52). Both *XMMP9* and *XMMP18* have similar expression patterns to *XMMP7* (52) and appear to be expressed specifically and exclusively within macrophages during development.

Through loss of function and rescue experiments it was shown that XMMP7, XMMP9 and XMMP18 are each individually necessary for macrophage migration. Loss of function of any one of these MMPs inhibits macrophage migration and there are several possible mechanisms whereby these MMPs function (52,53). XMMP7 cleaves and activates XMMP9 and potentially XMMP18 (52). β4-integrin and syndecan-1 are present in the area of the migrating macrophages and these are known substrates of XMMP7. It is therefore possible that these proteins play a role in the migration process through interactions with XMMP7 (53). Another possible mechanism is through the MMP-dependent cleavage of chemokines. Chemokines may be an unidentified substrate of either XMMP7, XMMP9 or XMMP18 (52). Current data indicate that these three MMPs promote macrophage migration by cleavage of ECM, activation of chemokines, or a combination of both.

XMMP14 is a membrane bound MMP that cleaves many ECM substrates including fibronectin, laminin and collagen (50). It begins to be expressed at gastrulation and continues through hatching. Its expression is contained in the hindbrain at rhombomeres three and five and in migrating neural crest cells (53). *XMMP2*, a gelatinase, is also expressed in migrating neural crest cells starting at tailbud stage. In later stages of embryogenesis, *XMMP14* and *XMMP2* are co-expressed (54) and XMMP14 has been shown to activate XMMP2 (53). Their co-expression in neural crest indicates an important role during the migration of these cells but the exact mechanism is unknown (54).

There remain several frog *MMPs* whose expression patterns have been characterized yet their biological function remains unknown. *XMMP28* is expressed at low levels within the fertilized egg (55). Its expression increases starting during mid-neurulation where it is localized in the developing neural tissue. Later in development it colocalizes with the axon marker acetylated-tubulin. It continues to be expressed throughout development in the peripheral and central nervous systems. Due to the expression of *XMMP28* later in neurulation, it is predicted that XMMP28 is involved in the maturation but not migration of the neurons (55).

XMMP16/MT3-MMP is a membrane-tethered MMP that also has a soluble form produced by the cleavage of its extracellular domain (56). Its mRNA begins to be expressed during the tailbud stage in the anterior neural tissue. Through the use of mRNA injection experiments it was shown that the membrane bound and the soluble forms have different roles during development. Those embryos injected with mRNA encoding the soluble form of XMMP16 showed neural tube closure defects and defects in the somites. Injection of the soluble form also showed an increase in gelatinase activity. This could be through the activation of the gelatinase XMMP2 because XMMP16 can activate XMMP2. The defects and increased gelatinase activity were not present in embryos injected with full length *XMMP16* mRNA. Although the exact mechanism and role of XMMP16 in development is still unknown, these data indicate that the membrane bound and soluble forms have distinct roles (56). *XMMP15*, another membrane-tethered MMP, is expressed during all stages of embryogenesis (53). Early in development it is expressed throughout the ectoderm while at later stages *XMMP15* localizes to the ventral ectoderm (53) suggesting a possible function in the epidermis.

XMMP11 is classified as a stromelysin (57). *XMMP11* mRNA is expressed in the branchial arches and the dorsal endoderm in the tailbud stage embryo. *XMMP11* overexpression causes death during late embryogenesis (57). XMMP24 is a transmembrane MMP that has been shown to cleave fibronectin and proteoglycans (57). Overexpression of *XMMP24* also caused late stage lethality and showed a dose dependent effect on the length of the body axis. Notably, XMMP11 was shown to cleave the non-integrin laminin receptor during intestinal remodeling events associated with metamorphosis (58,59).

10. ZEBRAFISH

Compared with humans, the zebrafish genome has a similar number of *mmp* and *timp* genes (~27 *MMPs* and ~6 *TIMPs*), yet the complexity of zebrafish MMP and TIMP family members is lower. For a detailed sequence and phylogenetic analysis of zebrafish *MMPs* and *TIMPs*, see (60). While zebrafish appear to lack orthologs of human *MMP1*, *3*, *8*, *10*, *12*, *26*, *27*, and *TIMP1* (60), the zebrafish genome contains homologous genes for each of the six human membrane-tethered *MMPs* including additional isoforms that might have arisen after teleost genome duplication (11 zebrafish versus 6 human genes) (61). It is unclear whether each zebrafish membrane-tethered MMP isoform has a specific biological function. Zebrafish MMPs contain typical domains found in the orthologous vertebrate proteins including propeptide, catalytic, and hemopexin domains. The differences in secreted and membrane-tethered MMPs present in humans and zebrafish might reflect differences in the type and/or spatial distribution of specific ECM substrates.

Gene expression patterns have been described for several zebrafish *MMPs* and *MMP* isoforms including *mmp2*, *mmp9*, *mmp13*, *mmp14*, *mmp15*, *mmp16*, *mmp17*, *mmp23*, *mmp24*, and *mmp25* (62-71). Zebrafish *mmp2* exhibits low expression at early embryonic stages prior to somitogenesis and a dynamic pattern at later stages localizing to the somites, pectoral fins, epidermis, otic vesicle, pharyngeal arches, and jaw cartilage (68). By contrast, embryonic *mmp9* expression is restricted

to the notochord, myeloid cells (similar to *mmp13a*), and potentially a site of hematopoiesis termed the intermediate cell mass (69). Unlike human *mmp23* that is predominantly expressed in reproductive tissues (72), zebrafish *mmp23bb* expression is largely restricted to the liver (65). Work from our lab has shown that zebrafish *mmp14a* and *mmp14b* are broadly expressed during gastrulation and at later stages localize to the pectoral fins, jaw cartilage, neurocranium, and neuromasts of the head (62). Expression of *mmp14a* has also been reported in the optic tectum and nerve and in retinal ganglion cells (73). Recently, *mmp25b* was shown to exhibit expression in several sensory neurons (63).

Utilizing semi-quantitative RT-PCR our lab has also shown that other membrane-tethered *MMPs* display varying expression levels from the onset of gastrulation (shield stage) through 3 days post-fertilization (66). For example, while *mmp15a*, *mmp15b*, and *mmp16a* exhibit stronger expression during gastrulation, *mmp16b*, *mmp17a*, and *mmp24* do not. All of the membrane-tethered *MMPs* are strongly expressed at 24 and 48 hours post-fertilization (62,66). Further analyses identified weak and ubiquitous *mmp15a* expression in the early embryo but tissue-specific expression at later stages in an area resembling the cardinal vein tail plexus and in the brain, mandibular arch, pectoral fins, spinal cord neurons, epidermis, and caudal hematopoietic tissue (66) (Figure 1). By contrast, *mmp15b* is expressed in the early embryo being detected in prechordal plate mesoderm, anterior neural plate, the polster, hindbrain, and pronephric duct (66). At later developmental stages (after 24 hours post-fertilization), *mmp15b* exhibits highly specific expression in the proctodeum, pharyngeal arches, pharyngeal pouches, caudal notochord, posterior lateral line primordium, and otic vesicle (66) (Figure 2).

During embryonic development, the composition, density, and rigidity of the ECM each has the potential to regulate multiple cell behaviors including motility, where matrix proteins act as both a physical barrier to directed cell migration and a scaffold that cells move on and through. Studies of tumor cell migration have demonstrated a critical role for membrane-tethered MMPs during both invasion of collagen and transmigration through type IV collagen and laminin containing basement membranes (74,75). Notably, membrane-tethered MMPs also cleave fibronectin (76), an ECM protein important for cell behaviors associated with gastrulation in multiple organisms such as mouse (77,78), frog (79,80), and zebrafish (81,82). The abundant expression of fibronectin and laminin in zebrafish gastrula-stage embryos (81) suggests that membrane-tethered MMPs might play a significant role during this developmental process. Work from our lab has shown that Mmp14a and Mmp14b regulate planar cell polarity underlying convergence and extension gastrulation cell movements (62). The gastrulation phenotypes produced by morpholino-mediated *mmp14* knockdown in wild-type embryos are subtle. However, in the compromised genetic background of *knypek*^{m119} where a null mutation in the Wnt co-receptor Glypican4 (83) disrupts planar cell polarity, *mmp14* knockdown greatly exacerbates the convergence and extension defect (62) (Figure 3). These findings suggest that there is a relationship between planar cell polarity and zebrafish Mmp14 function in migrating gastrula cells. Indeed, utilizing a human cancer cell model system, we found that the core planar cell polarity protein Vang-like 2 influences cell surface levels and proteolytic activity of human MMP14 (84-86). Our ongoing studies aim to specifically define the role of zebrafish Mmp14 during gastrulation.

Unlike fibronectin, collagens are broadly expressed at later developmental stages (after 24 hours post-fertilization) and also have the potential to influence numerous cell migration events (67,68,87). Zebrafish lack the collagenases Mmp1 and Mmp8 but have two Mmp13 homologs (*mmp13a* and *mmp13b*), a collagenase whose human ortholog has overlapping and distinct cleavage substrates compared with MMP1 and MMP8 (60,76). Interestingly, *mmp13a* exhibits a highly restricted embryonic expression pattern localizing to macrophages (67) where it was shown to regulate their migration after acute injury (88). In a study by Leigh *et al.* (64), *mmp17b* expression in migrating neural crest cells was investigated utilizing both morpholino-mediated *mmp17b* knockdown and chemical inhibition of MMP. In each case, neural crest cell migration became altered with cells localizing to the posterior trunk as opposed to being dispersed along the anterior-posterior axis. The authors provide evidence that RECK (REversion-inducing-Cysteine-rich protein with Kazal motifs) inhibits human recombinant MMP17 and that zebrafish Mmp17b physically interacts with RECK raising the possibility that these proteins functionally interact to regulate neural crest cell migration (64).

11. CHICKEN

Not many MMPs from the chicken have been identified and classified. Those that have include homologs of three of the transmembrane MMPs, MMP14, MMP15, and MMP16 (89,90). Although these and other *MMP* genes have been cloned from chicken, few of these were investigated for expression patterns and roles in development. The chicken genome also includes at least two *TIMP* genes (91) and a novel simple hemopexin domain containing MMP, MMP22 (50).

One of the most described chicken MMPs during development is MMP2, shown to play a role in neural streak ingression, cornea formation, cell migration during embryogenesis and skeletal and heart development (90,92-94). MMP2 is a gelatinase that in

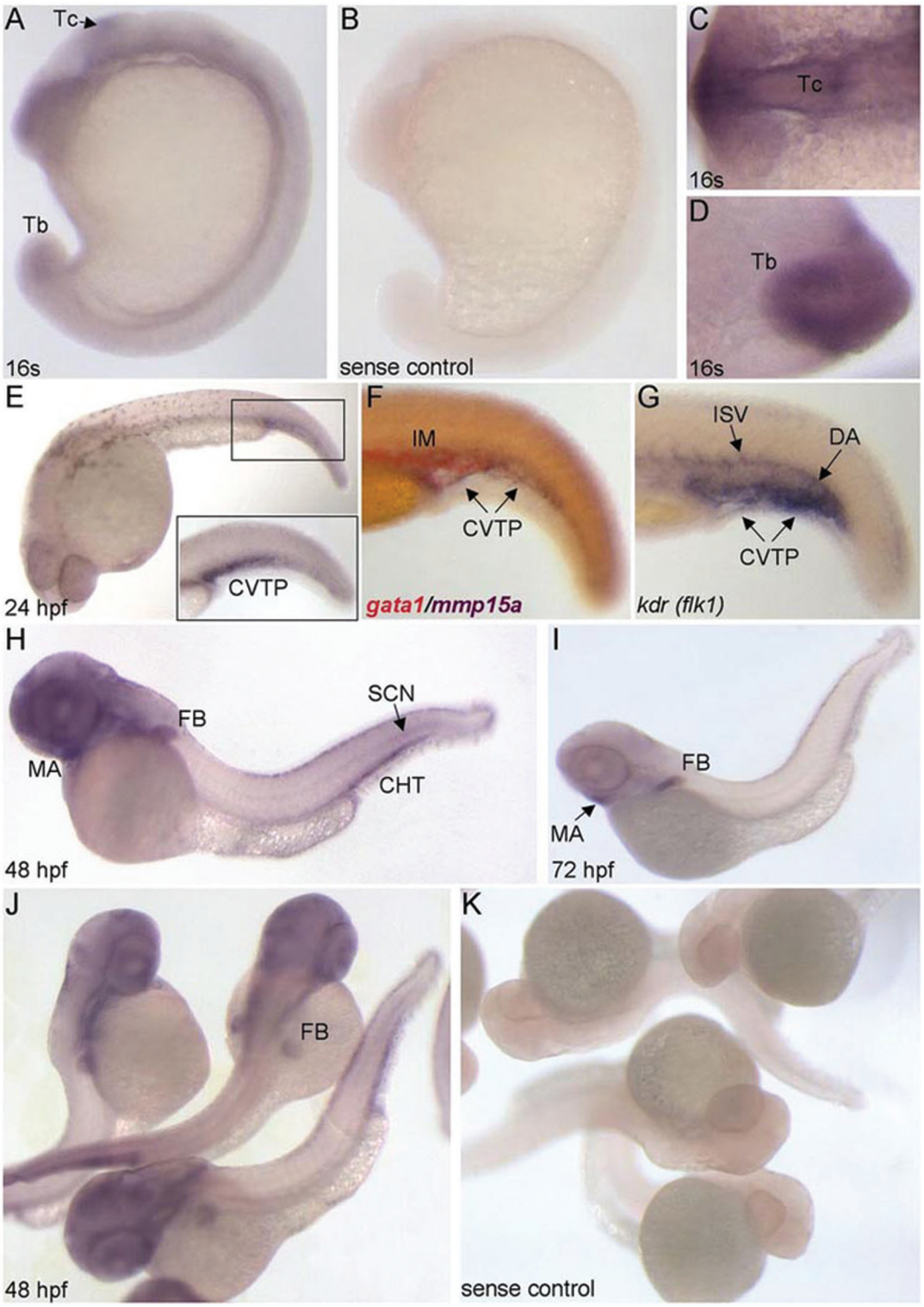

Figure 1. Expression pattern of zebrafish mmp15a examined using whole-mount RNA in situ hybridization. (A and B) Lateral views of 16-somite stage (16s) embryos with ventral to the left and anterior to the top. Close-up dorsal view of the tectum, Tc (C) and ventral view of the tailbud, Tb (D). (E-G) Lateral views of 24 hpf embryos labeled for mmp15a, gata1/mmp15a, and kdrl (flk1). Inset in panel (E) shows close-up of the cardinal vein tail plexus, CVTP. (H, J, and K) Lateral/dorsal views of 48 hpf embryos with anterior to the left (H). (I) Lateral view of 72 hpf embryo with anterior to the left. CHT, caudal hematopoietic tissue; DA, dorsal aorta; FB, fin bud; IM, intermediate cell mass; ISV, intersegmental vessels; MA, mandibular arch; SCN, spinal cord neurons. Adapted from (66) and used with permission from Elsevier.

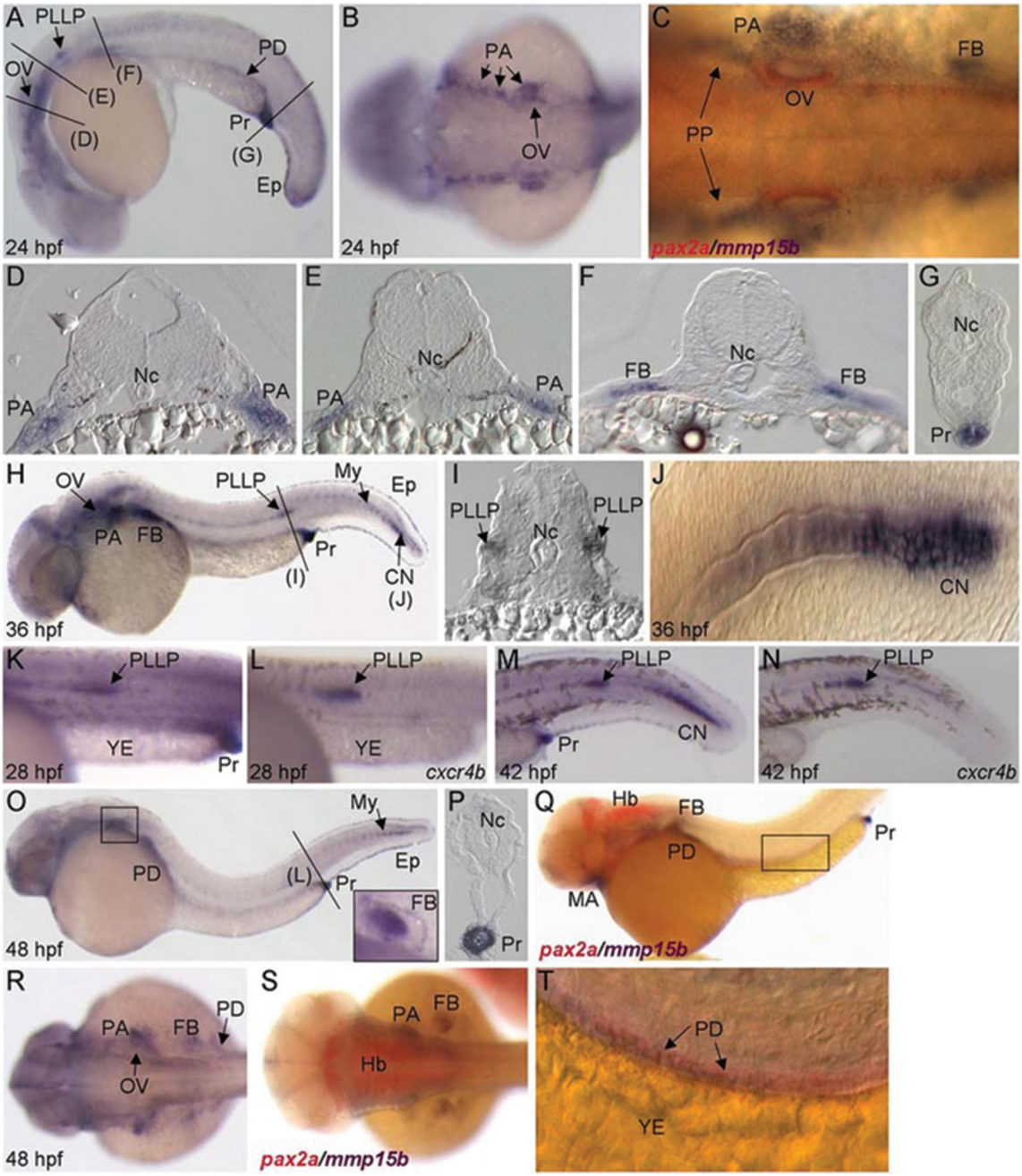

Figure 2. Expression pattern of zebrafish mmp15b during pharyngula and hatching periods. (A) Lateral view of 24 hpf embryo with anterior to the left. Black lines denote cross-section slices shown in panels (D-G). (B and C) Dorsal views of 24 hpf embryos with anterior to the left. Embryo in panel (C) is double-labeled with mmp15b and pax2a (red). (D-G) Cross-sections of 24 hpf embryos at positions marked in panel (A). (H and J) Lateral views of 36 hpf embryos with anterior to the left. (I) Cross-section of 36 hpf embryo trunk (position shown in panel H) showing expression in posterior lateral line primordia, PLLP. Mmp15b and cxcr4b expression at 28 hpf (K and L) and 42 hpf (M and N), lateral views with anterior to the left and dorsal to the top. YE, yolk extension. (O, Q, and T) Lateral views of 48 hpf embryos with anterior to the left. Inset in panel (O) shows close-up of mmp15b expression in the fin bud, FB. (P) Cross-section through the proctodeum (Pr) of a 48 hpf embryo (position shown in panel O). (R and S) Dorsal views of 48 hpf embryos with anterior to the left. (Q, S, and T) Double labeling with pax2a (red) highlights mmp15b expression in pharyngeal arches (PA) and pronephric duct, PD. CN, caudal notochord; Ep, epidermis; Hb, hindbrain; MA, mandibular arch; My, myoseptum; Nc, notochord; OV, otic vesicle; PP, pharyngeal pouch. Adapted from (66) and used with permission from Elsevier.

mammals is proteolytically activated by MMP14 (17). However, chicken MMP14 lacks the domain that cleaves and activates MMP2 in other species (90). It was speculated that this missing domain in MMP14 means that another MMP is responsible for activating MMP2 in the chicken. The very similar expression of *MMP2* and

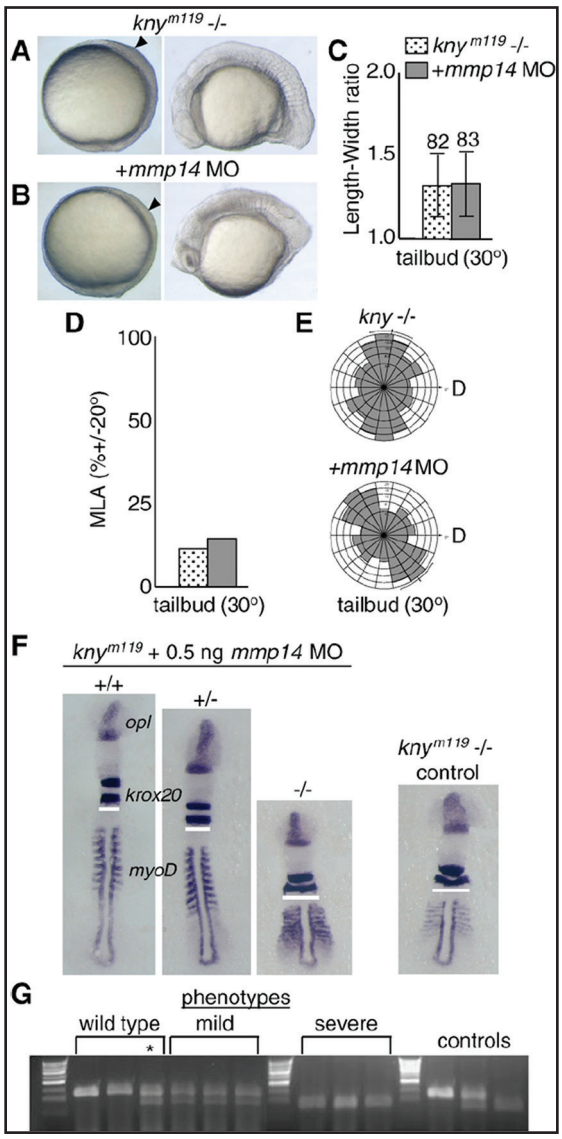

Figure 3. Genetic interaction between zebrafish mmp14 and non-canonical Wnt (planar cell polarity) signaling. (A and B) Injection of mmp14 MO into knym119 homozygous mutant embryos greatly intensified the C and E phenotypes as indicated by shorter and broader embryonic axes. Note the posteriorly shifted head region of embryos at the tailbud-stage. The embryos depicted in the right panels of A and B are approximately 1 day old. (C-E) LWR and MLA of mmp14 MO injected knym119 homozygous mutant embryos were quantified at the end of gastrulation (tailbud-stage) and the results displayed graphically and in Rose diagrams. Total cell numbers from at least three embryos are indicated. D, dorsal. (F) Clutch of knym119 embryos injected with a low dose of mmp14a and mmp14b MOs (0.5. ng each MO/embryo). RNA probes that mark mesodermal (myoD) and ectodermal (odd paired-like and krox20) tissues as indicated. Note shorter and broader (horizontal lines) embryos. Compare +/+ embryo to +/− and compare −/− embryo injected with mmp14 MO to −/− control embryo. (G) Representative single-embryo PCR genotyping results. Note that the 80 bp band is not visible on these gels. Occasionally, heterozygous knym119 siblings injected with low doses of mmp14 MO failed to develop a detectable C and E phenotype (asterisk). This result is consistent with data obtained from mmp14 MO injected wild-type embryos (Figure 3D). Adapted from (62) and used with permission from Elsevier.

MMP15 throughout development suggests that MMP15 may fulfill this role in the chicken (89).

Chicken MMP2 plays a crucial role in the epithelial-mesenchymal transition (EMT) of neural crest cells. During EMT cells transition from static polarized epithelial cells to motile mesenchymal cells (95). This process involves many molecular and morphological changes within the epithelial cells such as loss of polarity and cell-cell adhesion, loss of E-cadherin, and reorganization of the cytoskeleton. Cells must then invade and migrate through the basement membrane, a process that requires proteases that cleave ECM components of the basement membrane. After migrating through the basement membrane, mesenchymal-like cells form new adhesions and interactions with the ECM in their new microenvironment. EMT is an important process for embryonic morphogenesis, wound healing, and cancer metastasis. During chicken development, *MMP2* expression increases at the onset of EMT then diminishes after the EMT process is complete. Inhibition studies in chicken embryos show that without MMP2 the cells of the neural crest fail to undergo EMT. Lack of MMP2 does not affect the migration of neural crest cells that have already undergone EMT. These findings were similar although opposite in the somites and dermal tissue. The expression of *MMP2* appears as the somites and the dermal tissue begin EMT and also dissipates after EMT. With the inhibition of MMP2 in these tissues, EMT still takes place but cell migration fails after EMT. This indicates that in the neural crest MMP2 is necessary for EMT while in the somites and dermal tissue MMP2 is necessary for cell migration after EMT (92). *MMP2* expression begins in the epiblast cells in the area where the primitive streak forms starting at day 2 of development. *MMP11* and *MMP16* are also found in the primitive streak and injection of MMP inhibitors produced embryos where primitive streak cells failed to ingress (94). MMP2, MMP11 and MMP16 have a variety of ECM substrates and it is thought that these MMPs break down the ECM within the primitive streak to allow cells to ingress (94).

Another tissue in the chicken embryo that expresses *MMP2* is the developing cornea (93). Expression here begins at day 7 and continues throughout the development of the cornea. *MMP16* begins to be expressed at day 5 of development and also continues throughout the formation of the cornea. *MMP13* is expressed early in cornea development but its expression is gone by day 7. Interestingly, the MMP13 substrate type IX collagen also disappears from the cornea by day 7 (93). Formation of the cornea starts when the primary stoma swells. Type IX collagen binds hyaluronic acid in the primary stoma. When type IX collagen is cleaved, it releases hyaluronic acid, which retains water, and causes the swelling of the primary stoma. It was shown that MMP2, MMP13, and MMP16 play a role in the cleavage of type IX collagen and consequently the swelling of the

primary stoma (93). Once the primary stoma has swollen, neural crest cells migrate into the primary stoma. The neural crest cells express high levels of CD44v6 on their surface. CD44v6 binds to many ECM proteins regulating cell migration and is cleaved by MMP16. Therefore, MMP16 further contributes to the formation of the chicken cornea by regulating cell migration into the cornea (93).

Chicken *MMP2* is also expressed in the forming heart (94), in the limb buds where the tendons form (92), in branchial arches (92), and in the developing long bones (90); however, the role of MMP2 in these tissues is unknown. *MMP14*, *MMP13*, and *MMP16* are also expressed in the forming skeletal tissue indicating they may regulate development of the skeleton (90).

12. CONCLUSIONS

MMPs are important regulators of cell behavior because they directly influence how cells interact with their microenvironment. Cell-ECM interactions underlie key developmental processes at both the cellular and tissue levels including migration and morphogenesis. The use of non-mammalian developmental model systems has and will continue to provide needed insight for our understanding of MMP function. Work utilizing fly, with its two *Mmp* genes and many genetic tools, has addressed the question of redundancy and the role of these proteases during fly development (45). Work utilizing zebrafish, with its amenability to microscopic imaging and genetic manipulation, has identified a role for Mmp14 during gastrulation and an interaction with components of the planar cell polarity pathway (62,85). Important areas for future research with these and other non-mammalian model systems include *in vivo* identification of MMP substrates and dissection of pathways regulating MMP enzymatic activity and vesicular trafficking.

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