Expression and Activity of MMPs during Zebrafish (*Danio rerio)* Development.

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Abstract

Embryonic morphogenesis results from the growth and structural remodeling of tissues at the micro and mesoscopic scales. The degradation of the extracellular matrix (ECM) by matrix metalloproteinases (MMPs) is fundamental to this process, yet in vivo models for the study of MMP activity and regulation are limited. Recent advances have made the zebrafish an attractive system for studying this process, however, commercially available antibodies against MMPs have invariably been raised against mammalian homologues, and few are useful for studies using the zebrafish. Similarly, substrates developed for the detection of specific subsets of MMP activity have not been tested on zebrafish. The objectives of this study were to develop effective immunological reagents for the study of MMPs in zebrafish and to test commercially available narrow-spectrum fluorogenic MMPs substrates in *in vivo* zymography. We tested a suite of thirty antibodies directed against fifteen peptide epitopes predicted to occur in zebrafish homologues of these ECM remodeling enzymes, using westerns and whole mount immunostaining of formaldehyde and TCA-fixed embryos. 11 of these antibodies were found to be effective in one or more of these assays, and we report here on the ontogeny of their antigens during the first 72 hours of embryonic development. We also report the effective use of synthetic peptide substrates susceptible digestion by narrow spectra of MMPs as *in vivo* zymography substrates. These data will serve as a foundation for the use of zebrafish as a model system for investigating the regulation of ECM remodeling during development, regeneration, aging and pathological processes.

Conclusions

1) Antibodies raised against peptides predicted to occur in zebrafish MMPs can be used to detect these antigens in western blots of embryonic homogenates and immunostains of whole embryos.
2) Several MMPs may be present in complexes that are not easily denatured, and/or as glycosylated proteins with significantly higher than expected masses.
3) MMPs 2, 11, 17, 24 and 28 are not detectable prior to the onset of somite formation during zebrafish development.
4) Several MMPs have become very abundant by 48 hours of development
5) MMPs are abundant in ECM rich tissue boundaries undergoing morphogenesis (e.g. maturing somite boundaries, the elongating notochord, etc.) and migrating cells and elongating neurons
6) Synthetic peptide substrates connecting fluorphores to quenchers can be used in differential *in vivo* zymography to detect spatially heterogeneous patterns of proteolytic activity that likely reflects MMP activity.

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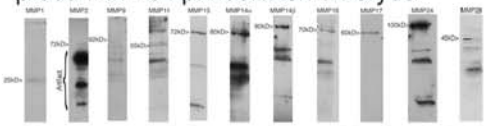
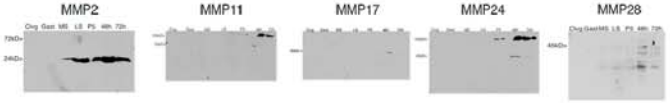
Anti-MMP-peptide antibodies recognize proteins present in 48 hpf zebrafish embryos



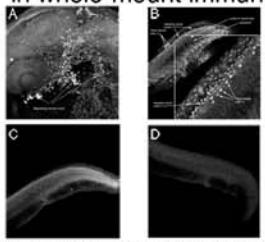
Epitope-affinity purified antibodies were tested on western blots of 48 hpf zebrafish embryo homogenates (350 μg total homogenate). Several antibody reacted with antigens on these blots, reproducibly labeling bands of consistent molecular weights. Some antibodies (anti-MMP2, 9,11, 16, 17 and 28) labeled bands of the expected molecular weight for these proteins, as well as lower MW bands that are likely proteolytic fragments of these antigens. Other antibodies (anti-MMP13, 14α, 14β, and 24) labeled bands of considerably higher MW than expected, likely due to extensive glycosylation of the proteins.

Dynamic expression of MMP-antigens during development



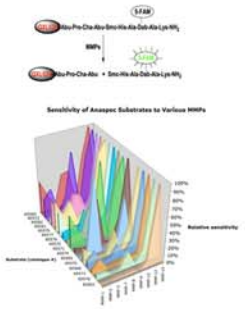
Western blots of staged embryo homogenates (Clvg: cleavage (1-3 hpf), Gast: gastrulation (6-10 hpf), MS: mid-somitogenesis (13-15 hpf), LS: late somitogenesis (18-20 hpf), P5 (24 hpf), 48h (48 hpf), 72h (72 hpf))(350 μg/lane) probed with anti-MMP antibodies show dynamic patterns of expression. MMP2 is first detected after the onset of somite formation, and accumulates rapidly. MMP11 is not detected before or after 48 hpf. MMP17 expression also appears to peak at 48 hpf, but it remains detectable at 72 hpf. MMP24 is detected at 24 hpf (prim-5), and accumulates afterwards, however lower MW products are less abundant at 72 hpf than earlier. MMP28 is detected as early as mid-somitogeneis, but becomes most abundant at 48 hpf.

Some MMPs can be detected in whole-mount immunostains

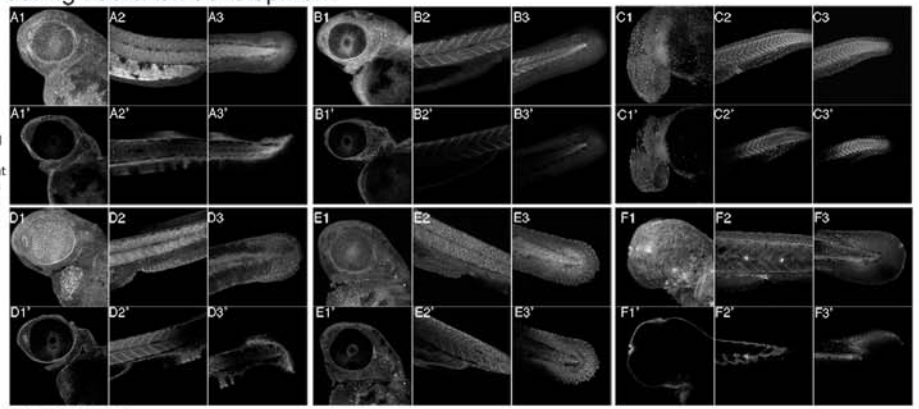


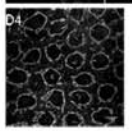
Confocal micrographs of 24 hpf (prim-5) embryos stained with anti-MMP2 (A & B) and anti-MMP9 (C & D). In panel A, migrating cranial neural crest cells are labeled with anti-MMP2 antibody. In panel B, MMP2+ neural crest can be seen in the trunk, as well as large cells in the dorsal neural tube that may be Rohon-Beard cells. MMP9 immunostaining is more diffuse, but stains the posterior mesoderm (including the notochord) in the elongating tail (panel C). This signal can be abolished by pre-adsorbing the antibody with the peptide antigen against which it was raised (panel D).

Fluorogenic peptide substrates can be used to detect MMP activity



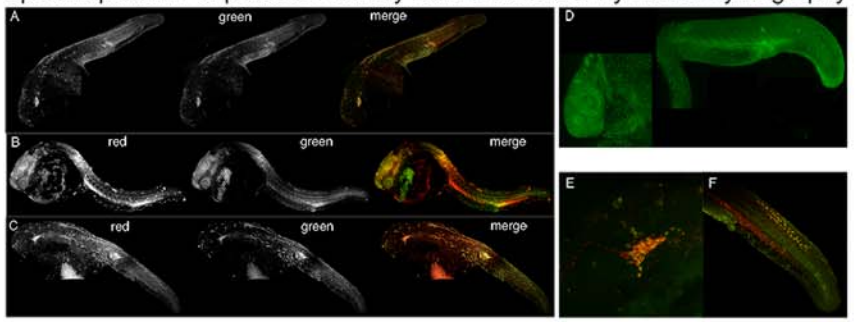
MMPs and TIMP2 show specific patterns of localization at both the cell and tissue level during zebrafish development





Confocal micrographs of 24 hpf (panel C) and 48 hpf (all other panels) stained with anti-MMP2 (A), anti-MMP11 (B), anti-TIMP2 (C & F), anti-MMP13 (D), and anti-MMP24 (E). Except for panel D4, all sets of images represent summed projections of confocal stacks of the head (1), trunk (2) and tip of the tail (3). Prime designations are individual optical sections from the projected stack shown above. Panel D4 is a high magnification image of the corneal epithelium from the embryo shown in D1.

Specific patterns of protease activity can be detected by *in vivo* zymography



In vivo zymography using multiple substrates reveals patterns of specific proteolytic activity in the living zebrafish embryo. Co-injection of substrate I tagged with both green and red fluorphores (A) reveals the same pattern of degradation in both chanels as expected. Injection of an unquenched peptide (D) shows ubiquitous fluorescence as expected. Co-injection of substrates with different peptides linking red and green fluorphores to their respective quenchers reveals spatially distinct patterns of substrate hydrolysis (B & C), consistent with spatially heterogeneous patterns of MMP activity. Panels E and F show high magnification details of C, focusing on the trigeminal ganglion and elongating tail, respectively.