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# Characterization of the zebrafish *matrix metalloproteinase 9* gene and its developmental expression pattern ☆

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**Abstract**

Members of the matrix metalloproteinase (MMP) family are important for the remodeling of the extracellular matrix in a number of biological processes including a variety of immune responses. Two members of the family, MMP2 and MMP9, are highly expressed in specific myeloid cell populations in which they play a role in the innate immune response. To further expand the repertoire of molecular reagents available to study zebrafish myeloid cell development, the *matrix metalloproteinase 9* (*mmp9*) gene from this organism has been identified and characterized. The encoded protein is 680 amino acids with high homology to known MMP9 proteins, particularly those of other teleost fish. Maternal transcripts of *mmp9* are deposited in the oocyte and dispersed throughout the early embryo. These are replaced by specific zygotic transcripts in the notochord from 12 h post fertilization (hpf) and also transiently in the anterior mesoderm from 14 to 16 h post fertilization. From 24 h post fertilization, *mmp9* expression was detected in a population of circulating white blood cells that are distinct from macrophages, and which migrate to the site of trauma, and so likely represent zebrafish heterophils. In the adult, *mmp9* expression was most prominent in the splenic cords, a site occupied by mature myeloid cells in other species. These results suggest that *mmp9* will serve as a useful marker of mature myeloid cells in the zebrafish.


*Keywords:* Matrix metalloproteinase 9; Myelopoiesis; Blood cells; Zebrafish

## 1. Results and discussion

Both primitive and definitive hematopoiesis in zebrafish is comparable anatomically, morphologically, and genetically to that in mammals (Amatruda and Zon, 1999). There is also strong conservation of hematopoietic gene expression and function at the molecular level (Thompson et al., 1998; Liao et al., 2000). Indeed most of the transcription factors identified as critical for hematopoietic development in mammals have orthologues in zebrafish (Weinstein et al., 1996; Brownlie et al., 2003; Davidson and Zon, 2004). Erythroid development has been extensively studied in this organism, aided by the ease of identifying red hemoglobin pigment in the transparent zebrafish embryo that has facilitated direct visual screens for mutants affecting this process. These studies of erythropoiesis have provided considerable insight into the process, with several mutants found to carry lesions in zebrafish orthologues of genes associated with human disease (Ransom et al., 1996; Weinstein et al., 1996; Brownlie and Zon, 1999; Davidson and Zon, 2004).

Several laboratories, including ours, are now using zebrafish to study myelopoiesis. The zebrafish possesses macrophages, which are highly motile, large, round cells with cytoplasmic phagosomes that act as a bacterial defense mechanism (Herbomel et al., 1999; Crowhurst et al., 2002). Two types of granulocytic cells are also recognized in this organism. The most abundant are the neutrophil equivalents or "heterophils", which possess a segmented nucleus of typically 2–3 lobes and are thought to be

☆ GenBank Accession Number of the zebrafish *mmp9* gene is NP_998288

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involved in host defense including acute inflammatory responses (Lieschke et al., 2001). In contrast, eosinophil/basophil granulocytes are less prevalent and have several uniquely piscine morphological features. These cells are produced by distinct anterior and posterior precursor cell populations in the embryo, the latter of which eventually moves to the adult kidney (Bennett et al., 2001; Herbomel et al., 1999; Lieschke et al., 2001). Thus far, the analysis of myeloid development has relied on the use of only a limited number of gene markers including *runx1* (Kalev-Zylinska et al., 2002), *spi1* (Bennett et al., 2001; Lieschke et al., 2001), *c/ebp* (Lyons et al., 2001), *L-plastin* (Herbomel et al., 1999), and *lysozyme* (Liu and Wen, 2002). To fully exploit zebrafish as a model system for the study of myelopoiesis, more specific molecular markers for the various stages of maturation are required.

Matrix metalloproteinases (MMPs) are a family of zinc-containing multi-domain enzymes that are involved in the regulation of the extracellular matrix by degrading and remodeling its components. Two such members of this family, MMP2 and MMP9 (also known as gelatinase A and B, respectively), have specificity for denatured collagens (gelatins) and intact collagen type IV and appear to play a key role in metastasis (Stahle-Backdahl et al., 1992; Nagase and Woessner, 1999). In mammals, MMP9 is mainly expressed in neutrophils and eosinophils, where it serves as a key marker for their differentiation (Dahlen et al., 1999). Therefore, to add to the repertoire of reagents for analyzing zebrafish myelopoiesis, we have identified and characterized the zebrafish *mmp9* gene.

### 1.1. Cloning and sequence analysis of zebrafish mmp9

BLASTX searching of zebrafish EST databases with human MMP9 identified 13 ESTs with high homology (GenBank Accession Nos. AI330810, AI331880, AI384291, AI385100, AW174507, AW421610, BG729541, BG737785, BG883189, BG883887, BI325744, BI326480, and BI473301). One clone, BG883189, derived from a zebrafish kidney library, was obtained and sequenced in full. Using sequence information derived from both this cDNA clone and the other ESTs, the complete coding sequence of the zebrafish *mmp9* gene was assembled (Fig. 1A). The *mmp9* cDNA is 2800 bp long, featuring a 2040 bp open reading frame that encodes a protein of 680 amino acids. Analysis of the conceptual translation product identified a zinc-containing catalytic domain, a hemopexin-like domain and three fibronectin domains, which is typical of matrix metalloproteinases.

Alignment of the encoded protein with eighteen other matrix metalloproteinases enabled a phylogenetic tree to be constructed from this alignment to examine potential evolutionary relatedness (Fig. 1B). All MMP9 proteins including the putative zebrafish *mmp9* formed a clade that was clearly distinct from the related MMP2 sequences, with high supporting bootstrap values. Greatest homology was to those of other teleost fish, including carp (82% identity), rainbow trout (71%), and medaka (69%). These results are consistent with the identified zebrafish sequence representing the orthologous *mmp9* gene.

### 1.2. Developmental expression pattern of zebrafish mmp9

To investigate the timing of *mmp9* gene expression during zebrafish development, RT-PCR analysis was performed on total RNA derived from staged embryos. The primer pair was designed to amplify over an intron/exon boundary to overcome problems associated with the amplification of genomic DNA, while reverse transcriptase (RT) was omitted in duplicate amplifications as a negative control (Fig. 2A). Transcripts corresponding to *mmp9* (~200 bp) were first detected in unfertilized eggs, indicating maternal origin, and continuing throughout development (at least to 5 dpf), presumably representing zygotic expression (Fig. 2Ai).

WISH analysis was used to determine the temporal/spatial expression pattern of this gene. Strong expression of *mmp9* was first detected in embryos that had reached the 6-somite stage (12 hpf) suggesting low levels of maternal transcript abundance prior to this stage. Before 12 hpf, *mmp9* expression was observed as faint diffuse expression in the posterior region of the embryo (data not shown). By the 6-somite stage (12 hpf) the expression domain of *mmp9* became more localized to the posterior region of the developing notochord (Figs. 2B and C), confirmed by a cross-section of embryos at this stage (Fig. 2D). Interestingly, by 10-somites (14 hpf), in addition to the continual expression in the posterior notochord, a second domain of *mmp9* expression was also observed in the midline region of the anterior mesoderm of the embryo, with both midline regions

Fig. 1. Characterization of the zebrafish *mmp9* gene. (A) Sequence Analysis. Full-length nucleotide sequence and deduced protein sequence (bold) of zebrafish *mmp9* with residue numbers indicated. The conserved domains of the encoded Mmp9 protein were analysed with the Conserved Domain program at the GenBank website (www.ncbi.nlm.nih.gov). These include a matrix metalloprotease catalytic domain (red), a zinc catalytic domain (blue), three fibronectin domains (green), and a hemopexin-like domain (pink). (B) Phylogenetic tree of matrix metalloproteinases. Radial phylogenetic tree constructed using TreeView (Page, 1996) based on a sequence alignment produced with ClustalX (Heringa, 1999). Bootstrap values shown were calculated from 100 predicted trees. The following sequences from GenBank were used: Dr Mmp9, (this study NP_998288); Bt Mmp2, NP_777170; Bt Mmp9, CAA55127; Cc Mmp9, BAB39390; Dr Mmp2, AAP74482; Gg Mmp2, NP_989751; Gg Mmp9, AAG47650; Hs MMP2, AAH02576; Hs MMP9, CAC10459; Hs MMP-12, P39900; Mm Mmp2, NP_032636; Mm Mmp9, BAB23442; Ol Mmp2 BAA85769; Ol Mmp9, BAA85770; Om Mmp2 BAA78479; Om Mmp9, CAC85923; Po Mmp9, BAB68366; Rn Mmp9, AAA90911; Xl Mmp9, AAD41624. Abbreviations: Bt, *Bos taurus* (cow); Cc, *Cyprinus carpio* (carp); Dr, *Danio rerio* (zebrafish); Gg, *Gallus gallus* (chicken); Hs, *Homo sapiens* (human); Mm, *Mus musculus* (mouse); Ol, *Oryzias latipes* (medaka fish); Om, *Oncorhynchus mykiss* (rainbow trout); Po, *Paralichthys olivaceus* (Japanese flounder); Rn, *Rattus norvegicus* (Norway rat); Xl, *Xenopus laevis* (African clawed frog). Scale bar indicates 0.1 nucleotide substitutions per site.