THE ROLE OF MATRIX METALLOPROTEINASES IN ZEBRAFISH (*DANIO RERIO*) EMBRYOGENESIS AND THEIR REGULATION BY GLUCOCORTICOIDS

by

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A Dissertation submitted to the

Graduate School-New Brunswick

Rutgers, The State University of New Jersey

and

University of Medicine and Dentistry of New Jersey

in partial fulfillment of the requirements

for the degree of

Doctor of Philosophy

Joint Graduate Program in Toxicology

written under the direction of

Dr. Keith Cooper

and approved by

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New Brunswick, New Jersey

January, 2008

ABSTRACT OF THE DISSERTATION

The Role of Matrix Metalloproteinases in Zebrafish (*Danio rerio*) Embryogenesis and

Their Regulation by Glucocorticoids

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Matrix metalloproteinases (MMPs) play a pivotal role during development due to

their ability to remodel the extracellular matrix, a function necessary for proper cellular

migration and tissue morphogenesis. Studies have demonstrated that MMP gene

expression can be either inhibited or induced by glucocorticoids in a variety of model

systems and that exposure to glucocorticoids causes developmental abnormalities in

several species. We hypothesize that glucocorticoid-induced teratogenesis is mediated

through the glucocorticoid receptor (GR) and results from altering the expression and

activity of the MMPs. Using the zebrafish (*Danio rerio*) as a model of development, the

data presented here demonstrate that embryonic exposure to the glucocorticoids

dexamethasone or hydrocortisone (100 mg/L) increased expression of the gelatinases

MMP-2 (~1.5 fold) and MMP-9 (7.6 to 9.0-fold), and the collagenase MMP-13 (2.5 to

4.9-fold) at 72 hours post fertilization (hpf). *In situ* hybridization experiments confirmed

these increases in MMP expression and demonstrated that the majority of transcript was

localized rostrally. Enzyme activity was also increased at 72 hpf for both MMP-2 and

MMP-9 (~3-fold), and MMP-13 (~13-fold) following glucocorticoid treatment, and

substrate specificity was confirmed via several MMP inhibitors. Acute exposure to

glucocorticoids resulted in numerous developmental abnormalities, most commonly altered craniofacial morphogenesis. Morpholino knockdown studies demonstrated that appropriate expression of MMP-2, MMP-9, and MMP-13 are necessary for proper zebrafish embryogenesis since morphants exhibited deleterious alterations in phenotype, and revealed that MMP-2 may compensate for loss of MMP-9 function. Co-treatment of zebrafish embryos with each glucocorticoid and the GR antagonist RU486 resulted in attenuation of glucocorticoid-induced increases in MMP expression (52-84% decrease) and activity (41-94% decrease), as well as a partial rescue in the abnormal craniofacial phenotypes. Taken collectively, these data show that dysregulation of MMP-2, MMP-9, and MMP-13 during embryogenesis, whether increased (as with glucocorticoids) or decreased (as with morpholinos), can lead to irregular developmental phenotypes. The teratogenic effects resulting from prolonged treatment with glucocorticoids may stem from this dysregulation of the MMPs. Finally, these results suggest that in the embryonic zebrafish, dexamethasone and hydrocortisone function through the GR, and that activation of this receptor can modulate MMP expression.

DEDICATION

To my family – my graduate career has been a very long and trying road and is by far the most challenging endeavor I have ever pursued. I want you to know that you have always served as my source of motivation. I would never have been able to achieve this goal were it not for the values and work ethic you instilled in me, and for the continued support you've provided me over these many years. Know that the work described on these pages can be attributed as much to your encouragement and tolerance as it can be to the innumerable hours I've spent in laboratory. I love you all and will never forget what you've done for me.

To my wife Wendy – words simply cannot describe the debt of gratitude I owe you. You are my best friend, you are my foundation. I'm sure I've been intolerable at times, but I never would have guessed that based on the consistent compassion and love you've provided me. You are my whole world and I hope that I can give back to you what you've so easily given to me. I love you with my whole heart.

ACKNOWLEDGEMENTS

On a personal note, I would like to thank Caren Villano for the numerous hours we've spent troubleshooting and validating the methods used during this research. A tremendous point of pride with this work comes with the fact that essentially all of the methods describe herein were novel for our laboratory, and without Caren's knowledge and patience, this process would have been much more arduous. I would also like to acknowledge all of the other graduate students and undergraduates in the Cooper and White labs who have been integral in providing the support needed to maintain the zebrafish colony over all of these years. I've greatly enjoyed the time we've spent together both inside and outside the laboratory. Finally, I would like to express thanks to my committee members for their valuable insights and continued direction and support.

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LIST OF ABBREVIATIONS

ANOVA	analysis of variance
AP-1	activator protein-1
BLAST	Basic Local Alignment Search Tool
bp	base pair(s)
Ca^{2+}	calcium ion
CCL	ceratohyal cartilage length
cDNA	complementary deoxyribonucleic acid
CSF	colony-stimulating factor
DIG	digoxigenin
DMF	dimethylformamide
DNA	deoxyribonucleic acid
dpf	days post fertilization
DTT	dithiothreitol
DX	dexamethasone
ECM	extracellular matrix
ECOSAR	Ecological Structure Activity Relationships
EDTA	ethylenediaminetetraacetic acid
EL	embryo length
EPIWIN	Estimation Programs Interface for Windows
fg	femtograms(s)
FKBP5	FK506 binding protein 5
Gb	gigabase(s)

GILZ	glucocorticoid induced leucine zipper
GITR	glucocorticoid-induced tumor necrosis factor receptor
GPI	glycosylphosphatidylinositol
GR	glucocorticoid receptor
GRE	glucocorticoid response element
h	hour(s)
HC	hydrocortisone
HEPES	4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid
HPA	hypothalamic-pituitary-adrenal
hpf	hours post fertilization
HSP	heat shock protein
ID	intercranial distance
IFN-γ	interferon-γ
IL-1	interleukin-1
IL-2	interleukin-2
IL-4	interleukin-4
kDa	kilodaltons
LJL	lower jaw length
M	molar
μg	microgram(s)
μL	microliter(s)
μm	micrometer(s)
μM	micromolar

mg	milligram(s)
min	minute(s)
mL	milliliter(s)
mm	millimeter(s)
mM	millimolar
MMP	matrix metalloproteinase
MMPI	matrix metalloproteinase inhibitor
MO	morpholino oligonucleotide
MR	mineralcorticoid receptor
MS-222	tricaine methane sulfonate
MT-MMP	membrane-type matrix metalloproteinase
NaCl	sodium chloride
NCBI	National Center for Biotechnology Information
NF-AT	nuclear factor of activated T cells
NF-κB	nuclear factor-κB
ng	nanogram(s)
nGRE	negative acting glucocorticoid response element
nm	nanometer(s)
nM	nanomolar
p	probability level for statistical significance
PEA-3	polyomavirus enhancer activator-3
PIE	pharmaceuticals in the environment
qRT-PCR	quantitative reverse transcription polymerase chain reaction

QSAR	quantitative structure activity relationship
RECK	reversion-inducing-cysteine-rich protein with kazal motifs
RNA	ribonucleic acid
rRNA	ribosomal ribonucleic acid
RT-PCR	reverse transcription polymerase chain reaction
SC	solvent control
sGITR	soluble glucocorticoid-induced tumor necrosis factor receptor
SMILES	Simplified Molecular Input Line Entry System
SOX9b	SRY-box containing gene 9b
Sp-1	selective promoter factor-1
Tcf-4	T-cell factor-4
TGF-β	transforming growth factor-β
TIMP	tissue inhibitor of metalloproteinase
TNF-α	tumor necrosis factor-α
TNF-NGF	tumor necrosis factor-nerve growth factor
USEPA	United States Environmental Protection Agency
VCAM-1	vascular cell adhesion molecule-1
VLA-4	very late antigen-4
YSL	yolk syncytial layer
ZFIN	Zebrafish Information Network
ZIRC	Zebrafish International Resource Center
Zn^{2+}	zinc ion

1.0. GENERAL INTRODUCTION

1.1. Zebrafish as a Model System

1.1.1. General Description and Advantages

The zebrafish (*Danio rerio*) is a cyprinoid teleost native to streams and rivers in India (Briggs 2002). Recently, zebrafish have emerged as the pre-eminent vertebrate model system for clarification of the roles of specific genes and signaling pathways in development (Spitsbergen and Kent 2003). However, their value as a model extends far beyond their involvement in developmental biology. Advances in mutation screening, genomic resources, and transgenesis procedures have made the zebrafish amendable to studying immunology (Yoder *et al.* 2002), human congenital and genetic disease (Dooley and Zon 2000; Penberthy *et al.* 2002; Ward and Lieschke 2002), regulatory physiology (Briggs 2002), reproductive function (Laan *et al.* 2002) and endocrine disruption (Kazeto *et al.* 2004), among others.

From a biotechnological perspective, advantages of using the zebrafish as a model system include the relatively low space requirements and maintenance costs of husbandry facilities following initial setup investments. Zebrafish housed in compact recirculation or flow-through systems can be stimulated to breed year-round under appropriate photoperiod conditions, and females can produce hundreds of eggs per day with high fertilization success. This allows many replicates and/or test concentrations to be carried out on a single batch, thereby strengthening statistical evaluation. Fertilization and development occur *ex utero*, and embryogenesis takes only 2 or 3 days depending on the environmental conditions. Both the chorion and the embryo itself are translucent prior to hatching and are well suited for visualization of tissues and organs, *in situ* hybridization, cell lineage/transplantation studies, and microinjection of DNA or RNA constructs

(Holder and Xu 1999; Lele and Krone 1996). Organogenesis occurs rapidly, and by 5 to 6 days post-fertilization (dpf), zebrafish develop distinct organs and tissues including brain, heart, liver, intestines, bones, muscles, sensory organs and a nervous system (Rubinstein 2003). Since zebrafish are vertebrate organisms, the organs and tissues have proven to be similar to those of mammals at the anatomical, physiological, and molecular levels (Parng 2005).

The zebrafish genome has been fully sequenced and found to be approximately 1.6 Gb distributed across 25 pairs of chromosomes maintained in the diploid state (2n = 50) (Freeman *et al.* 2007; Wixon 2000). Despite the fact that zebrafish proteins exhibit <70% identity with their human equivalents, they display considerably higher conservation in their functional domains, including substrate binding regions, ligand binding regions, enzyme catalytic domains, DNA binding motifs and nucleus translocation signal sequences (Parng 2005). This further supports the use of zebrafish as a model of human disease since many of the toxicants or drugs known to interact with these functional domains elicit comparable effects across both model systems (Langheinrich 2003; Shin and Fishman 2002). In addition, large scale mutagenesis screens have identified a considerable number of zebrafish mutants exhibiting phenotypes similar to those observed in human disease states (Driever *et al.* 1996; Haffter *et al.* 1996). These mutant phenotypes have subsequently been linked to genes involved in human disease, thereby emphasizing the utility of zebrafish as a suitable model for studying these disorders as well as possible treatments. This process has been aided by the fact that at least 28 groups of zebrafish and human genes are syntenic, thus assisting

in the identification and positional cloning of candidate disease genes (Barbazuk *et al.* 2000; Talbot and Hopkins 2000).

1.1.2. Developmental Staging of Zebrafish

It is well established that the development of the zebrafish is similar to the embryogenesis which occurs in higher ordered vertebrates, including humans and other mammals. This is supported by a study demonstrating that of known mammalian teratogens, 36 of 41 have caused teratogenicity in the zebrafish (Nagel 2002). The various stages of embryonic development are well characterized (**Figure 1**) (Kimmel *et al.* 1995) and can be visualized in real-time without the need for invasive procedures or sophisticated imaging techniques. Knowledge of the staging series is paramount, not only because single clutches of eggs have been shown to develop asynchronously, but also because coupling this information with the optical clarity of the embryo as previously discussed allows for elegant embryonic manipulations to distinguish the relative influences of cell autonomous effects versus non-cell-autonomous effects (Spitsbergen and Kent 2003).

Zebrafish development can be divided into several key periods, each consisting of various stages (**Table 1**) (Kimmel *et al.* 1995). The main periods of development are as follows in chronological order: zygote period, cleavage period, blastula period, gastrula period, segmentation period, pharyngula period, and hatching period. The zygote period occurs from 0 - 0.75 hours post fertilization (hpf) until the first cleavage occurs and constitutes a number of cellular changes, one of which is cytoplasmic streaming to form the blastodisc. The cleavage period (0.75 - 2.25 hpf) consists of the 2-cell stage to the 64-cell stage during which time the blastomeres divide in 15 minute intervals at regular

orientations. The blastula period (2.25 - 5.25 hpf) is comprised of the 128-cell stage to 30%-epiboly. The term blastula is used to refer to the period when the blastodisc appears ball-like, and notable processes occurring during this time include midblastula transition, formation of the yolk syncytial layer (YSL), and the onset of epiboly (spreading of the YSL and blastodisc over the yolk cell, defined by percent coverage of the yolk cell). Gastrulation (i.e., gastrula period; 5.25 - 10.33 hpf) encompasses 50%-epiboly through the bud stage, at which time the primary germ layers, antero-posterior axis and dorso-ventral axes are formed through morphogenetic cell movements of involution, convergence, and extension. During the segmentation period (10.33 - 24 hpf), somites form in an anterior to posterior wave, organogenesis results in the formation of rudimentary primary organs, and the body plan becomes recognizable with the development of the optic lobes and tail bud. In the pharyngula period (24 - 48 hpf), the embryo continues to elongate and cell differentiation persists, pigmentation is observed in both the retina and embryo body, organs become functional, rudiments of the pectoral fins are present, and the primordia of the pharyngeal arches begin to migrate. In the final period, or hatching period (48 - 72 hpf), the pectoral fins, jaw, and gills all continue to develop, the first cartilage and bone appear, and the embryos hatch from their chorions.

1.1.3. Craniofacial Development in Zebrafish

1.1.3.1. General Description

Craniofacial patterning in zebrafish is a tightly controlled process that requires precise cellular migration, morphogenesis, growth and remodeling. The types of cells involved in craniofacial morphogenesis and the migratory paths adopted by these cells are well understood. However, the actual manner in which these cells travel through the

mesodermal mesenchyme and the molecular signaling and genes that control these movements are still under investigation given the complexity of the cellular and molecular interactions involved. Craniofacial development in vertebrate embryos involves coordinated cell movement and communication between the neural crest, mesodermal mesenchyme, and surrounding epithelia (reviewed in (Le Douarin *et al.* 1994)). These processes have previously been characterized in mouse (Chai and Maxson 2006; Hall 1980; Trainor and Tam 1995) and avian models (Couly and Le Douarin 1990; Noden 1983, 1988), while craniofacial development studies in zebrafish have emerged more recently (Kimmel *et al.* 1998; Kimmel *et al.* 2001; Neuhauss *et al.* 1996; Piotrowski *et al.* 1996; Schilling *et al.* 1996; Yelick and Schilling 2002). During zebrafish embryogenesis, cephalic neural crest cells originating from the embryonic midbrain and hindbrain migrate ventrally to eventually form the neurocranium, dermatocranium, and pharyngeal skeleton (**Figure 2**).

The neurocranium is actually derived from both mesoderm and neural crest cells, and serves to protect the brain and adjacent sensory organs of the head. Following chondrification of these cells, they comprise the ethmoid plate, trabeculae, parachordals, and occipital arches. The dermatocranium is composed of a number of bony elements such as the parasphenoid bone, cleithrum, and opercle that eventually integrate other skull components as larvae. The pharyngeal skeleton is derived solely from neural crest cells and can be divided into the mandibular, hyoid, and branchial arches. The mandibular arch (also known as the first pharyngeal arch) can further be divided in to the upper (palatoquadrate) and lower (Meckel's cartilage) jaw. Located ventrally and caudally are the elements of the hyoid arch (second pharyngeal arch), the hyosymplectic

and ceratohyal cartilages. Posterior to the first and second pharyngeal arches are five sets of bilaterally arranged ceratobranchials. The most rostrally located branchial arch structure is the basihyal cartilage, which lies dorsal to the ceratohyal cartilage of the second pharyngeal arch. In adult zebrafish, each set of branchial arches will eventually support a set of filamentous gills (Schilling *et al.* 1996).

1.1.3.2. Role of Extracellular Matrix

The extracellular matrix (ECM) is composed of a number of structural and functional elements that are secreted by resident cells including collagens, glycoproteins, glycosaminoglycans, and proteoglycans. The basic structure of the ECM consists of a gel-like ground substance comprised of glycosaminoglycans and proteoglycans within which is embedded fibrous glycoproteins such as collagen (Zagris 2001). The two main domains of the ECM are the interstitial matrix as described above and the basement membrane, a condensed, sheet-like layer formed adjacent to epithelial cells (Bosman and Stamenkovic 2003). Both domains are defined by a collagen scaffold, although the collagen constituents and their three-dimensional architecture are different (Bosman and Stamenkovic 2003). Other non-proteoglycan matrix components include hyaluronic acid, fibronectin, elastin, and laminin. This network provides structural support, anchorage for cells, and an integral signaling network through the action of cytokines and growth factors embedded in the matrix (Hillegass *et al.* 2006).

The ECM plays a central role in a number of events necessary for proper embryological development during which cell-cell and cell-matrix interactions act to regulate cell migration, adhesion, morphogenesis, proliferation, differentiation, and apoptosis. The ECM has also been implicated in guidance of neural crest cells during

embryogenesis. Control of neural crest cell migration has been a subject of much conjecture and experimentation. A number of theories to explain the manner in which neural crest cells migrate have been put forth including electrical fields (Erickson and Olivier 1983), steric hindrance by barrier tissues (Newgreen 1989), diffusible chemotactic factors (Wehrle-Haller and Weston 1995), and interaction with Eph receptor tyrosine kinases and ephrins (Krull 2001). Another alternative proposed is haptotaxis, which suggests that the migration of neural crest cells is guided by a gradient of adhesion sites within the migration substrate; in this case, the ECM (reviewed by (Henderson and Copp 1997; Perris and Perissinotto 2000). Essentially, the existence of permissive and non-permissive ECM molecules along certain migratory pathways and at specific ratios either prevents or permits migration of neural crest cells. Permissive is meant to imply that the ECM component supports neural crest cell adhesion and extensive migration, while non-permissive represents those ECM components that promote weak cell adhesion and little to no migration. Permissive ECM molecules include laminin, fibronectin, and certain collagen isoforms (Perris and Perissinotto 2000; Sternberg and Kimber 1986; Tuckett and Morriss-Kay 1986). Non-permissive ECM molecules include aggrecan, versican, and hyaluronan (Landolt *et al.* 1995; Perris and Perissinotto 2000). The viability of this mechanism is supported by the fact that neural crest cells have been shown to express integrin receptors on their cell surface which are capable of binding fibronectin, laminin, and other ECM molecules (Bronner-Fraser 1994; Lallier and Bronner-Fraser 1991, 1993; Lallier *et al.* 1992). Further support is offered by studies showing that mouse mutants with alterations in ECM parameters often possess abnormalities of neural crest cell migration as part of their phenotype (Henderson *et al.*

1997; Lofberg *et al.* 1989; Morrison-Graham *et al.* 1992; Perris 1997). Finally, microinjection of antibodies near the cranial neural tube has been implemented to functionally knock out certain cell-matrix interactions along neural crest cell migratory pathways, resulting in accumulation of neural crest cells adjacent to the site of injection and various neural tube abnormalities (Bronner-Fraser 1994; Erickson and Perris 1993). Using this approach, it has been shown that the β_1 subunit of integrin, fibronectin, laminin-heparan sulfate proteoglycan complex, tenascin, and galactosyltransferase are necessary for the proper emigration of neural crest cells (Erickson and Perris 1993).

Migration of neural crest cells that will eventually become pharyngeal cartilage occurs during the first embryonic day (Kimmel *et al.* 1998), indicated by the fact that these cells have been identified in 12 hpf embryos (Yelick and Schilling 2002). Following migration, cells form condensations of precartilage mesenchyme whose chondrification begins during the second embryonic day (Hall and Miyake 1992; Schilling and Kimmel 1997). This chondrocyte differentiation is accompanied by a period of rapid cartilage morphogenesis (Hall and Miyake 1992; Schilling and Kimmel 1997) followed by an extended period of cartilage growth (Kimmel *et al.* 1998). At approximately 5 to 6 dpf, cartilage undergoes one of two types of ossification to form bone: endochondral (ossification within cartilage) or perichondral (ossification around cartilage) (Yelick and Schilling 2002).

1.2. Matrix Metalloproteinases

1.2.1. Function and Structure of Matrix Metalloproteinases

The ECM is remodeled constantly in response to a host of cellular stimuli, and the degree to which these changes occur ranges from dynamic homeostasis (as in resting

state adult organs) to persistent remodeling that takes place during development, inflammation, wound healing and cancer (Stamenkovic 2003). Numerous proteases have been implicated in the proteolytic degradation of the ECM, most notably the matrix metalloproteinases (MMPs). MMPs are produced and excreted by a number of different cell types including keratinocytes, fibroblasts, phagocytes, and lymphocytes (Brinckerhoff and Matrisian 2002). MMPs require Zn^{2+} for proper three-dimensional structure and Ca^{2+} for stability and expression of catalytic activity (Bode *et al.* 1999; Nagase and Woessner 1999). Thus far, 24 distinct MMPs have been classified based on their substrate specificities, although in general, MMPs cleave peptide bonds before residues with hydrophobic side chains such as leucine, isoleucine, methionine, phenylalanine, or tyrosine (Visse and Nagase 2003). The main subdivisions of MMPs include the collagenases, gelatinases, stromelysins, matrilysins, membrane-type, and a sixth group distinct from the other four in both sequence and substrate specificity (**Table 2).** The collagenases degrade interstitial triple-helical fibrillar collagens, such as collagens I, II, and III, at a specific site three-fourths from the N-terminus (Visse and Nagase 2003). The gelatinases (also referred to as type IV collagenases) generally degrade basement membrane collagens and denatured collagens, also referred to as gelatins. The stromelysins, matrilysins and membrane-type MMPs (MT-MMPs) each possess a broad substrate specificity which includes proteoglycans, laminins, fibronectins, and gelatins. The main difference between these groups is inherent in their domain structure, which is discussed below. It should be noted, however, that these divisions are somewhat artificial given both the high degree of overlap among MMP substrate specificities, and the fact that MMPs are capable of degrading a number of

substrates that are not part of the ECM (Egeblad and Werb 2002; McCawley and Matrisian 2001).

All MMPs are composed of three fundamental domains, and can be further separated based on the presence or absence of other more specialized domain structures (**Figure 3**). These three fundamental domains are the 'pre' domain, the 'pro' domain, and the catalytic domain. MMPs are produced as preproenzymes containing a 'pre' domain which signals for cellular export and a 'pro' or 'propeptide' domain that must be proteolytically cleaved in order to activate the enzyme (Hulboy *et al.* 1997; Vincenti *et al.* 1996). The catalytic domain consists of two Zn^{2+} ions (structural and catalytic) and two or three Ca^{2+} ions, which as described earlier, are required for stability and expression of enzyme activity. This domain also contains both a zinc-binding motif HEXXHXXGXXH, where three histidine residues coordinate a Zn^{2+} ion, and a conserved methionine residue which forms a "Met-turn" structure responsible for supporting the structure around the catalytic zinc (Bode *et al.* 1993). MMP-2 and MMP-9 are unique in that they contain three repeats of fibronectin-type II domain inserted within the catalytic domain which enhance substrate binding (Ganea *et al.* 2007).

All MMPs except MMP-7, MMP-23 and MMP-26 contain a C-terminal hemopexin-like domain which determines substrate specificity and presents the substrate to the catalytic site (Ganea *et al.* 2007; Hillegass *et al.* 2006; Hulboy *et al.* 1997). MMP-7 and MMP-26 are sometimes referred to as 'minimal domain' MMPs since their active form corresponds to the catalytic domain alone. MMP-23, also referred to as cysteine array MMP, lacks both a hemopexin domain and the cysteine switch motif in the prodomain (to be discussed subsequently). Rather, it possesses a cysteine-rich domain

followed by an immunoglobulin-like domain (Visse and Nagase 2003). The hemopexin domain is attached to the catalytic domain by a variable hinge region which has been demonstrated to contribute to MMP specificity either by direct substrate binding, or by influencing the orientation of the hemopexin and catalytic domains (Roeb *et al.* 2002). Finally, of the six total MT-MMPs, four possess type I transmembrane domains (MMP-14, MMP-15, MMP-16, and MMP-24) and two possess glycosylphosphatidylinositol (GPI) anchors (MMP-17 and MMP-25). In both cases, these domains serve to anchor the enzyme to the cell surface. In addition, all six MT-MMPs have a furin recognition sequence RX[R/K]R at the C-terminus of the propetide domain; therefore, they are activated intracellularly and active enzymes are likely to be expressed on the cell surface (Nagase *et al.* 2006).

1.2.2. Regulation of Matrix Metalloproteinase Expression and Activity

MMP expression and activity is primarily controlled at three levels: (1) transcription; (2) proteolytic activation of the zymogen form; and (3) inhibition of the active enzyme via natural inhibitors (reviewed in (Chakraborti *et al.* 2003)). In resting-state adult tissues, the bulk of the MMPs are expressed at low levels or not at all (Stamenkovic 2003). However, there exist numerous cytokines and growth factors capable of affecting MMP expression. For example, the inflammatory cytokines interleukin-1 (IL-1) and tumor necrosis factor-α (TNF-α) stimulate MMP gene expression at the site of inflammation through the ceramide signaling pathway (Spiegel *et al.* 1996), whereas transforming growth factor-β (TGF-β) and IL-4 inhibit MMP synthesis (Schroen and Brinckerhoff 1996). MMP gene expression may also be affected by non-soluble factors such as changes in cell-cell and cell-matrix interactions. An example of such

interaction is induction of MMP-2 in T-cells through very late antigen-4 (VLA-4)-vascular cell adhesion molecule-1 (VCAM-1)-mediated adhesion to endothelial cells (Romanic and Madri 1994). Finally, MMP promoters contain several *cis*- and *trans*-acting transcription factors allowing for regulation of MMP gene expression, including activator protein-1 (AP-1), polyomavirus enhancer activator-3 (PEA-3), selective promoter factor-1 (Sp-1), β-catenin/T-cell factor-4 (Tcf-4), and nuclear factor-κB (NF-κB) (Benbow and Brinckerhoff 1997; White and Brinckerhoff 1995; Yan and Boyd 2007). These represent only a small selection of a growing number of promoter elements and illustrate the complexity with which these factors cooperate to regulate MMP gene expression.

Following translation of the mRNA, the majority of MMPs are secreted in an inactive zymogen form (subsequently referred to as pro-MMPs). In order to maintain the inactive zymogen form, a conserved cysteine-sulphydryl group within the distinctive concensus PRCGXPDV motif (the "cysteine switch") in the propeptide domain ligates the active site zinc atom, thereby maintaining latency (Nagase and Woessner 1999; Stamenkovic 2003). Removal of the prodomain via proteolytic cleavage of the COOH-terminal side of the PRCGXPDV site subsequently disrupts the cysteine-zinc interaction, allowing access to the active site (Nagase 1997). This disruption constitutes the first step in MMP activation and typically occurs in the extracellular space, at least for secreted MMPs. However, as discussed previously, several MMPs including MMP-11, MMP-28, and the MT-MMPs contain furin cleavage sites and may be activated intracellulary in the Golgi by furin-like serine protease prior to reaching the cell surface (Pei and Weiss 1995; Sternlicht and Werb 2001). Other relevant pro-MMP activators *in vivo* include the serine

proteases plasmin (Carmeliet *et al.* 1997) and kallikrein (Vincenti *et al.* 1996), nitric oxide (Gu *et al.* 2002), and a variety of activated MMPs (Cowell *et al.* 1998). The exact mechanisms of physiological extracellular activation of MMPs have yet to be elucidated, with the exception of activation of MMP-2 by MMP-14 (MT1-MMP) and tissue inhibitor of metalloproteinase-2 (TIMP-2), which has been well characterized (Strongin *et al.* 1995).

Given the diversity of MMP enzymes, there are several endogenous inhibitors which control MMP activity; however, α2-macroglobulin and TIMPs constitute the major physiological inhibitors. The primary inhibitor of MMP activity in plasma is α2-macroglobulin, which binds directly to MMPs, trapping them within the macroglobulin following proteolysis of the inhibitor bait region (Barrett 1981). TIMPs are the primary inhibitors of MMP activity in tissue and are often synthesized by the same cells that produce the MMPs. X-ray crystallographic studies have shown that TIMP molecules are shaped like wedges which slot into the active site cleft of MMPs in a similar manner to that of the endogenous substrate (Fernandez-Catalan *et al.* 1998; Gomis-Ruth *et al.* 1997). TIMPs also serve other crucial biological functions such as influencing cell proliferation, apoptosis, angiogenesis, and activation of pro-MMPs (Brew *et al.* 2000). A number of other proteins have been reported to inhibit MMPs, although their precise mechanism of action is unknown. These include the serine protease inhibitor tissue factor pathway inhibitor-2 (Herman *et al.* 2001), a C-terminal fragment of the procollagen C-terminal proteinase enhancer protein (Mott *et al.* 2000), and the GPI-anchored glycoprotein reversion-inducing-cysteine-rich protein with kazal motifs (RECK) (Oh *et al.* 2001).

1.2.3. Matrix Metalloproteinases in Development and Disease

MMPs play a vital role in a number of physiological and pathological processes. The physiological processes include development, uterine resorption following pregnancy, ovulation, and wound healing (Brinckerhoff and Matrisian 2002). Central among these is development, where MMPs participate in events related to angiogenesis, cellular migration and aggregation, and apoptosis (Lemaitre and D'Armiento 2006; Stamenkovic 2003). Dysregulation and inappropriate expression and activity of MMPs have been tied to a number of pathological disorders. Examples include rheumatoid arthritis, where MMPs are responsible for joint destruction, and tumor invasion and metastasis, where these enzymes degrade basement membranes and ECM (Folgueras *et al.* 2004; Hendrix *et al.* 2003; Rundhaug 2005). In addition to enhancing tumor invasion and metastasis through degradation of protein barriers, MMPs are capable of altering cell signaling by providing substrate to responsive cell surface receptors, releasing membrane-bound growth factors such as TGF-β, and enhancing cell migration by exposing cryptic sites that promote cellular migration (Giannelli *et al.* 1997; Mott and Werb 2004; Xu *et al.* 2001; Yu and Stamenkovic 2000). MMPs can also contribute to genomic instability and tumor progression by altering cell-cell and cell-matrix interactions, thus contributing to epithelial-mesenchymal transition and compromised genomic integrity (Radisky and Bissell 2006). Further, expression and activity of MMPs have been linked to cardiovascular and pulmonary disease, as well as degenerative neurological disorders such as multiple sclerosis (Leppert *et al.* 2001; Tayebjee *et al.* 2005).

The importance of MMPs in development and disease has been demonstrated via the creation of knockout mice. Although a number of MMP knockout mice have been

created, only MMP-2, MMP-9, and MMP-13 null mice will be mentioned here since these particular metalloproteinases are the focus of the studies presented in this thesis. The initial selection of these MMPs came as a result of both the availability of their gene sequence and the fact that they have been shown to be vital for normal development in both zebrafish and mice (Inada *et al.* 2004; Itoh *et al.* 1997; Mosig *et al.* 2007; Stickens *et al.* 2004; Vu *et al.* 1998; Zhang *et al.* 2003b). In addition, these enzymes have been directly linked to a number of disease processes and have been widely studied in this regard; therefore, there exists a large amount of background information upon which to rely for designing and interpreting the current studies.

MMP-2 null mice exhibit abnormal long bone and craniofacial development, progressive loss of bone mineral density (with associated decreases in osteoblast and osteoclast numbers), and articular cartilage destruction (Itoh *et al.* 1997; Mosig *et al.* 2007). MMP-2 deficient mice are protected against cardiac rupture and experimentally-induced abdominal aortic aneurysm, and show a delayed macrophage-driven phagocytic removal of infarcted myocardium (Longo *et al.* 2002; Matsumura *et al.* 2005). MMP-9 knockout mice display an abnormal pattern of skeletal growth plate vascularization and ossification resulting in progressive lengthening of the growth plate (Vu *et al.* 1998). These mice possess decreased lymphocytic inflammation and peribronchial mononuclear cell infiltration in a murine model of asthma (Cataldo *et al.* 2002). In an *in vitro* model using smooth muscle cells isolated from MMP-9 deficient mice, cells exhibit impaired migration, a vital step in the formation of atherosclerosis (Cho and Reidy 2002). MMP-13 null mice show evidence of severe alterations in skeletogenesis including increases in tibial and femoral trabecular bone density, defects in growth plate cartilage and primary

ossification centers, and delayed endochondral ossification (Inada *et al.* 2004; Stickens *et al.* 2004). Mice deficient in this collagenase possess atherosclerotic plaques with thin and misaligned collagen fibers (Deguchi *et al.* 2005). A recently discovered autosomal dominant disorder characterized by a mutation in the MMP-13 gene which causes it to autodegrade (referred to as spondyloepimetaphyseal dysplasia) leads to defective bone and vertebrae growth and modeling (Kennedy *et al.* 2005).

1.2.4. Matrix Metalloproteinases in Zebrafish

Recent work aimed at elucidating the evolution of vertebrate zinc proteases implemented genes from both *Ciona intestinalis* (sea squirt) and zebrafish. Overall, 83 zinc protease genes have been identified in the zebrafish genome, 26 of which are MMP orthologs and four of which are TIMP orthologs (Huxley-Jones *et al.* 2007). However, only three zebrafish MMPs have been characterized thus far in terms of expression and function, including MMP-2, MMP-9, and two isoforms of a novel membrane-type MMP (MT-MMP α and β) (Yoong *et al.* 2007; Zhang *et al.* 2003b, c). Also, a tissue inhibitor of metalloproteinase-2 (TIMP-2) has been investigated (Zhang *et al.* 2003a). Targeted gene knockdown using antisense morpholino oligonucleotides (MOs) demonstrates that MMP-2, MT-MMP α and β, and TIMP-2 are all required for normal development since blocking translation of these genes results in numerous aberrant phenotypes. MMP-2 morphants exhibit poorly defined somites, craniofacial defects, pericardial edema, and a truncated anterior-posterior axis (Zhang *et al.* 2003b). MT-MMP morphants possess a truncated anterior-posterior axis, poorly defined somites, and a complete lack of craniofacial cartilage at 72 hpf (Zhang *et al.* 2003c). TIMP-2 morphants exhibit a distorted anterior-posterior axis (body axis curvature), craniofacial defects, and

pericardial edema (Zhang *et al.* 2003a). These studies demonstrate the fundamental role played by MMPs in zebrafish embryogenesis and serve as a model that can be used to identify additional metalloproteinases that function in this process. Morpholino knockdown experiments on MMP-9 have not been conducted prior to the studies presented here (see Chapter 4), although it has been cloned, sequenced, and characterized in terms of its expression pattern in order to study zebrafish myelopoiesis (Yoong *et al.* 2007). Specifically, the authors found differential MMP-9 expression throughout embryogenesis and high levels of expression in the splenic cords that constitute the red pulp in adult zebrafish (a site typically occupied by mature myeloid cell in other organisms) (Yoong *et al.* 2007).

1.3. Glucocorticoids

1.3.1. Mode of Action and Physiological Effects of Glucocorticoids

Glucocorticoids are a class of 21-carbon steroid hormones synthesized in the adrenal cortex under control of the hypothalamic-pituitary-adrenal (HPA) axis (Duma *et al.* 2006; Jacobson 2005) (**Figure 4**). The majority of secreted glucocorticoids are bound to cortisol-binding globulin (transcortin) and albumin in the blood with various affinities (Duma *et al.* 2006; Thomson *et al.* 2007). As a result of their high lipophilicity, glucocorticoid molecules are capable of reaching virtually all tissues, including the brain, and readily diffuse across the cell membrane. Most glucocorticoid-associated effects are mediated through the glucocorticoid receptor (GR) (**Figure 5**). The GR belongs to the nuclear receptor superfamily, whose members act as ligand-dependent transcription factors. Other members of this family include steroid hormones, thyroid hormones, retinoic acid and vitamin D_3 (Stolte *et al.* 2006). The GR resides in the cytoplasm in

complex with heat shock proteins (HSPs) and several immunophilins. Glucocorticoids entering the cytoplasm bind this complex to form a non-DNA-binding oligomer which, following cytoplasmic activation and subsequent conformational changes, dissociates into the accessory proteins and the DNA-binding GR monomer. Following dimerization with a second activated GR, the GR homodimer complex enters the nucleus and binds to conserved DNA motifs known as glucocorticoid response elements (GREs) or negative acting GREs (nGREs) in the promoter regions of target genes in order to activate or repress transcription, respectively (Bruner *et al.* 1997; Karin 1998; Schaaf and Cidlowski 2002). Glucocorticoids are also capable of modulating transcription through antagonism of transcription factors such as AP-1, NF-κB, and nuclear factor of activated T cells (NF-AT) (Almawi and Melemedjian 2002; Herrlich 2001).

In vivo, glucocorticoids play a central role in glucose metabolism by stimulating gluconeogenesis. In order to provide ample substrate for this process, glucocorticoids can also stimulate lipolysis and mobilize amino acids from extrahepatic tissues (AHFS 2000). Glucocorticoids are potent immunosuppressive and antiproliferative agents capable of blocking cytokine expression. Glucocorticoids inhibit the expression of a wide variety of interleukins such as IL-1 and IL-2 (Amano *et al.* 1993; Paliogianni and Boumpas 1995), as well as interferon-γ (IFN-γ) (Kunicka *et al.* 1993), TNF-α (Crinelli *et al.* 2000), and colony-stimulating factors (CSF) (Campbell *et al.* 1993). As a result, they are widely utilized to treat autoimmune and inflammatory disorders, transplant rejection, and lymphoproliferative diseases (Almawi *et al.* 2002). Short-term antenatal glucocorticoid therapy is also utilized in pregnant women to reduce the incidence of fetal mortality, respiratory distress syndrome, and intraventricular hemorrhage in preterm

infants (Sloboda *et al.* 2005). However, these compounds are also potent teratogens, and data are conflicting concerning the efficacy and safety of antenatal therapy before 24 weeks or after 34 weeks of gestation (AHFS 2000).

1.3.2. Regulation of Matrix Metalloproteinases by Glucocorticoids

Recent studies have shown that regulation of MMP expression by glucocorticoids is more complex than originally thought. *In vitro* mammalian cell models originally demonstrated that MMP-1 expression is inhibited by exposure to glucocorticoids (Vincenti *et al.* 1996). Hydrocortisone has also been shown to decrease the amount of MMP-2 and MMP-9 in plasma isolated from human subjects following intravenous administration (Aljada *et al.* 2001). It is believed that this inhibition may contribute to the anti-inflammatory effect of these steroid hormones since glucocorticoid administration could potentially reduce the extracellular spread of inflammation. Proposed inhibitory mechanisms include sequestration of the AP-1 proteins Fos/Jun, direct binding of GRs to DNA via Fos/Jun (thus inhibiting the binding of Jun homodimers to the AP-1 site), ligand dependency and independency, and induction of TIMP transcription (reviewed in (Schroen and Brinckerhoff 1996)). However, glucocorticoids have been shown to *stimulate* MMP-9 expression through induction of soluble glucocorticoid-induced tumor necrosis factor receptor (sGITR) in murine macrophages (Lee *et al.* 2004; Lee *et al.* 2003). GITR is a member of the tumor necrosis factor-nerve growth factor (TNF-NGF) receptor family and it has been proposed that GITR could serve as a physiological counter-regulatory mediator involved in counteracting the immunosuppressive effects of glucocorticoids (Lee *et al.* 2003; Nocentini *et al.* 1997). In another study still, glucocorticoid injection repressed MMP-13

expression in uninjured rabbit knee cartilage, but enhanced MMP-13 expression in an injured state (Kydd *et al.* 2005). Taken collectively, it appears that regulation of MMPs by glucocorticoids may vary depending on cell type, MMP type, the presence of additional receptor molecules, or tissue remodeling status. In addition, this regulation has yet to be studied in a whole animal model of development as described in the subsequent studies.

1.3.3. Glucocorticoids and Glucocorticoid Receptor Signaling in Fish

As stated previously, most glucocorticoid-associated effects in mammals are mediated through the GR, and this holds true for fish as well. GR structure and function is well established in mammals and conserved among all vertebrate species analyzed thus far, including fish (Stolte *et al.* 2006) (**Figure 6A**). Similar to other members of the nuclear receptor superfamily, the fish GR consists of four domains: the (1) N-terminal domain (A/B domain), (2) DNA binding domain (C domain), (3) hinge region (D domain), and (4) C-terminal domain (E domain). The A/B domain is highly variable among different members of the superfamily in both size and composition and is involved in modulating transcriptional activity. The C domain, on the other hand, is highly conserved among virtually all vertebrate species and is responsible for DNA binding and receptor dimerization. Specifically, this domain binds to GREs in the promoter regions of target genes to initiate transcription via the action of two zinc fingers (Stolte *et al.* 2006). The D domain is involved in conformational changes and the E domain is the hormone binding domain. The fish GR is unique from that expressed in mammals in that the DNA-binding domain (C domain) contains nine additional residues between the two zinc fingers. These nine amino acid inserts are remarkably conserved

among the teleostean fish species studied thus far, and appear to be the result of alternative splicing (Stolte *et al.* 2006; Terova *et al.* 2005). It has been suggested that since these residues promote greater DNA affinity in the GR, they could have been selected to serve the large spectrum of cortisol functions in fish (Lethimonier *et al.* 2002). Such GR splice variants have been characterized in the rainbow trout (Bury *et al.* 2003) and Burtons' mouthbrooder (Greenwood *et al.* 2003; Takeo *et al.* 1996) thus far, and it is believed this could result in separate biological functions for each receptor variant (Prunet *et al.* 2006). This situation is unique to teleost fish and is consistent with the large number of duplicated genes, such as aryl hydrocarbon receptor (AhR) and aryl hydrocarbon receptor nuclear translocator (ARNT), reported in teleost fish (Andreasen *et al.* 2002; Robinson-Rechavi *et al.* 2001). To date, only a single GR has been identified in zebrafish, although given the high GR sequence homology that exists between multiple teleostean species, it is likely additional isoforms will be discovered following further examination. GR isoforms have been identified in numerous fish tissue types including brain, heart, gill, kidney, liver, and spleen (Greenwood *et al.* 2003).

Glucocorticoids are secreted from interrenal tissue situated in the head kidney region, which is analogous to the adrenal cortex in mammals (Prunet *et al.* 2006). The major circulating glucocorticoid in teleosts is cortisol (i.e. hydrocortisone) (Prunet *et al.* 2006; Vijayan *et al.* 2003) and for this reason, was selected as one of the glucocorticoids to be used for these studies. Hydrocortisone is chemically identical to cortisol, but this name is used in order to distinguish that which is produced synthetically (hydrocortisone) from endogenously produced hormone (cortisol). Biosynthesis of cortisol occurs from cholesterol and is briefly described in (**Figure 6B**). The second glucocorticoid examined

in these studies, dexamethasone, is a more potent GR agonist and was selected in order to examine differential response in the zebrafish embryos. This has been shown via *in vitro* experiments in rainbow trout, during which dexamethasone activated both variants of the GR at lower concentrations than cortisol (Bury *et al.* 2003). In separate studies, dexamethasone was able to elicit a stronger reporter gene activation than similar concentrations of cortisol (Greenwood *et al.* 2003; Takeo *et al.* 1996).

Glucocorticoids serve as key endocrine factors in teleosts, where they are involved in regulation of numerous physiological functions related to metabolism, growth, wound healing, reproduction, osmoregulation, circadian cell cycle rhythms, respiration, and the immune and stress responses (Dickmeis *et al.* 2007; Prunet *et al.* 2006). For example, exposure of rainbow trout hepatocytes to cortisol attenuated heat shock-induced heat shock protein 90 (HSP90) mRNA expression (Sathiyaa *et al.* 2001). Another recent study showed that the exogenously administered glucocorticoid beclomethasone dipropionate was able to block tailfin regeneration in zebrafish larvae, and that this effect could be abrogated by morpholino knockdown of the GR (Mathew *et al.* 2007). Beclomethasone dipropionate exposure also induced several GR target genes, including the glucocorticoid induced leucine zipper (GILZ), the FK506 binding protein 5 (FKBP5) and the SRY-box containing gene 9b (SOX9b), supporting the concept that in zebrafish, glucocorticoids act through the GR pathway (Mathew *et al.* 2007).

1.4. Objective and Hypotheses

The objective of these studies is to determine the role of MMP-2, MMP-9, and MMP-13 in normal zebrafish embryogenesis and to establish a causal link between activation of the GR via dexamethasone and hydrocortisone and changes in MMP

expression and activity. In so doing, it may be possible to elucidate the potential mechanism(s) by which glucocorticoids elicit teratogenicity. The primary phenotypic endpoint used by this laboratory to characterize these glucocorticoid-induced changes in the zebrafish model was craniofacial development. Craniofacial development in vertebrate embryos involves coordinated cell movement and communication between various cell types, and serves as a sensitive endpoint for evaluating dysmorphogenesis relating to MMP misexpression. The following hypotheses have been formulated to direct this research: (1) MMP-2, MMP-9, and MMP-13 are required for normal zebrafish embryogenesis and knocking down these genes via antisense MOs will result in abnormal phenotypes; (2) exposure to the glucocorticoids dexamethasone or hydrocortisone will alter MMP-2, MMP-9, and MMP-13 expression and activity in developing zebrafish embryos, thereby impeding normal development; and (3) glucocorticoid-induced teratogenesis is mediated through the GR and co-treatment with the GR antagonist RU486 will attenuate changes in MMP expression and activity.

The data presented here demonstrate that MMP-2, MMP-9, and MMP-13 are differentially regulated during embryogenesis and are required for proper embryonic development, including craniofacial patterning. Use of morpholinos to knockdown MMP gene function results in several gross morphological changes in the developing embryo and suggests a potential compensatory mechanism between MMP-2 and MMP-9. Further, knockdown of these MMPs causes changes in craniofacial cartilage formation and migration. Embryonic exposure to either dexamethasone or hydrocortisone also results in dysmorphogenesis (including craniofacial) and causes increases in MMP expression and activity at 72 hpf. *In situ* hybridization confirmed these increases in

MMP expression and demonstrated that the majority of transcript was localized rostrally. Use of the GR antagonist RU486 results in attenuation of increases in MMP expression and activity, confirming that dexamethasone and hydrocortisone are working through the GR, and that activation of this receptor can modulate MMP expression. These studies demonstrate the importance of MMPs in zebrafish embryogenesis and suggest that glucocorticoids may elicit teratogenicity through altered expression of these enzymes.

1.5. Figures
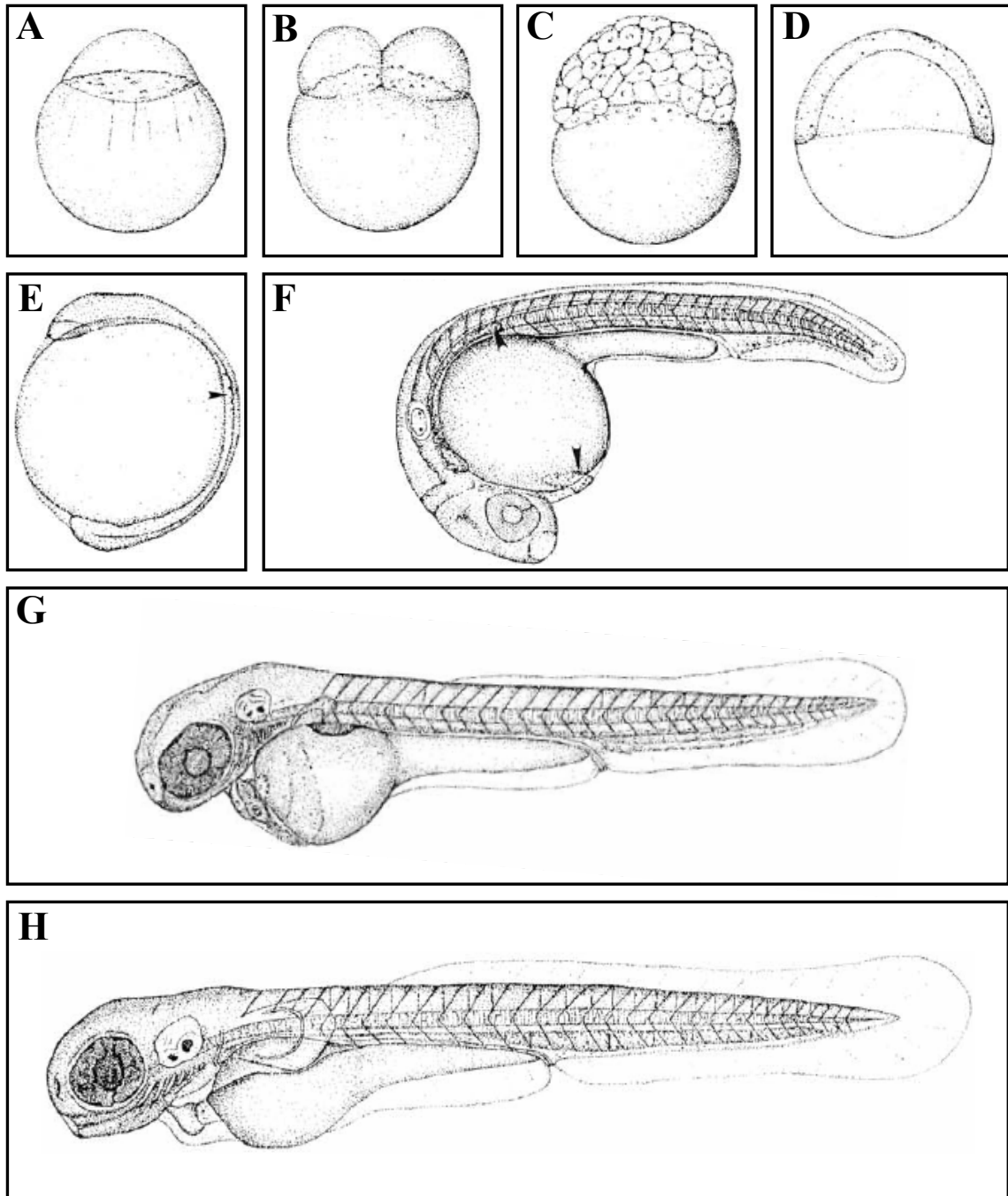
Figure 1.1. Select stages of zebrafish embryonic development. Camera lucida sketches correspond to the onset of the **(A)** zygote period, **(B)** cleavage period, **(C)** blastula period, **(D)** gastrula period, **(E)** segmentation period (arrowhead indicates the third somite), **(F)** pharyngula period (arrowheads indicate the posterior lateral line primordium on the dorsal side and the hatching gland on the yolk ball), **(G)** hatching period, and **(H)** larval period. Specific developmental processes occurring during each of these periods can be found in Table 1. Modified from Kimmel *et al.* 1995.
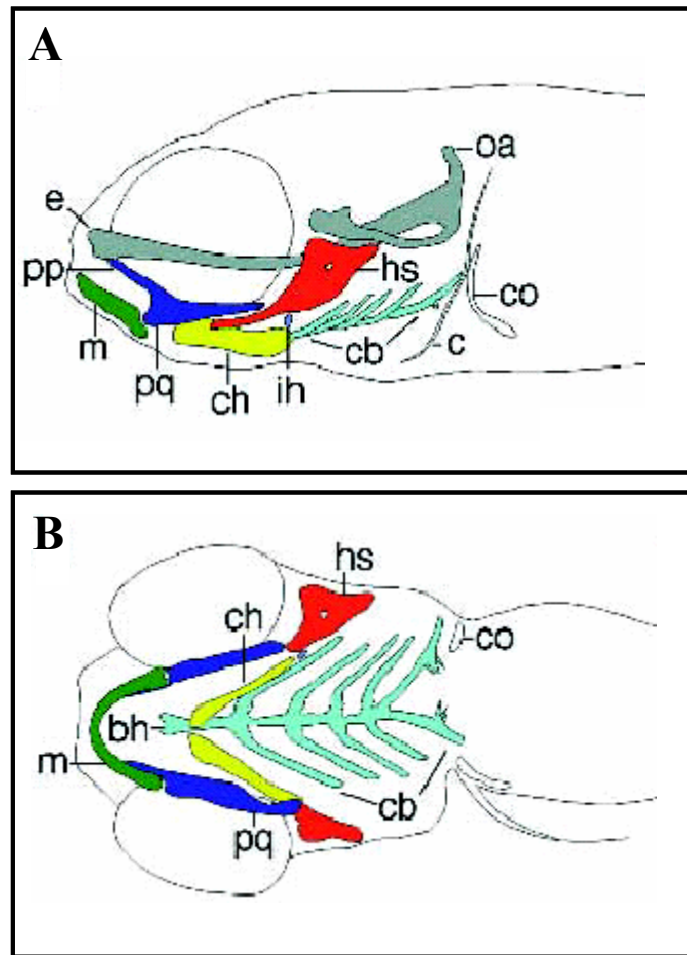
Figure 1.2. (A) Lateral and (B) ventral views of the cartilaginous elements of the zebrafish embryo head and pectoral girdle. Abbreviations: **bh**, basihyal; **c**, cleithrum; **cb**, ceratobranchial; **ch**, ceratohyal; **co**, coracoid of pectoral girdle; **e**, ethmoid plate; **hs**, hyosymplectic; **ih**, interhyal; **m**, Meckel's cartilage; **oa**, occipital arch; **pp**, pterygoid process of the palatoquadrate; **pq**, palatoquadrate. Modified from Piotrowski *et al.* 1996.
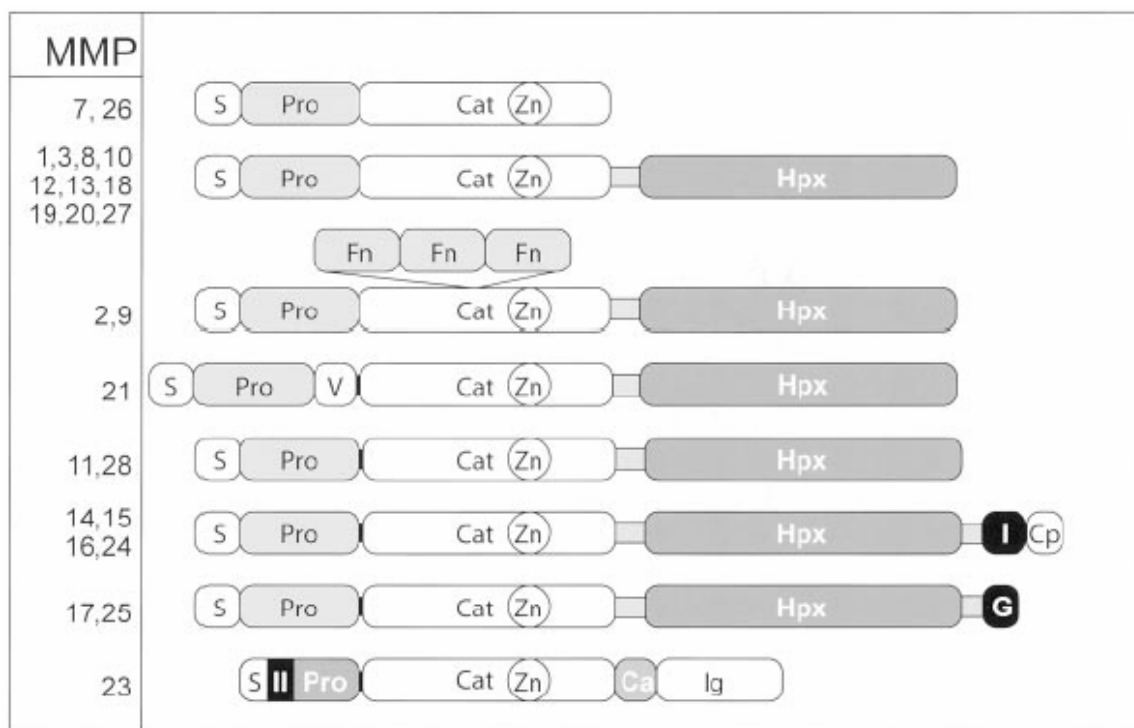
Figure 1.3. Domain structure of MMPs. Abbreviations: **S,** signal peptide ('pre' domain); **Pro**, propeptide ('pro' domain); **Cat**, catalytic domain; **Zn**, active site zinc; **Hpx**, hemopexin domain; **Fn**, fibronectin domain; **V**, vitronectin insert; **I**, type I transmembrane domain; **II**, type II transmembrane domain; **G**, GPI anchor; **Cp**, cytoplasmic domain; **Ca**, cysteine array region; **Ig**, IgG-like domain. Black bands between the propeptide and catalytic domains signify a furin cleavage site. Modified from Visse and Nagase 2003.
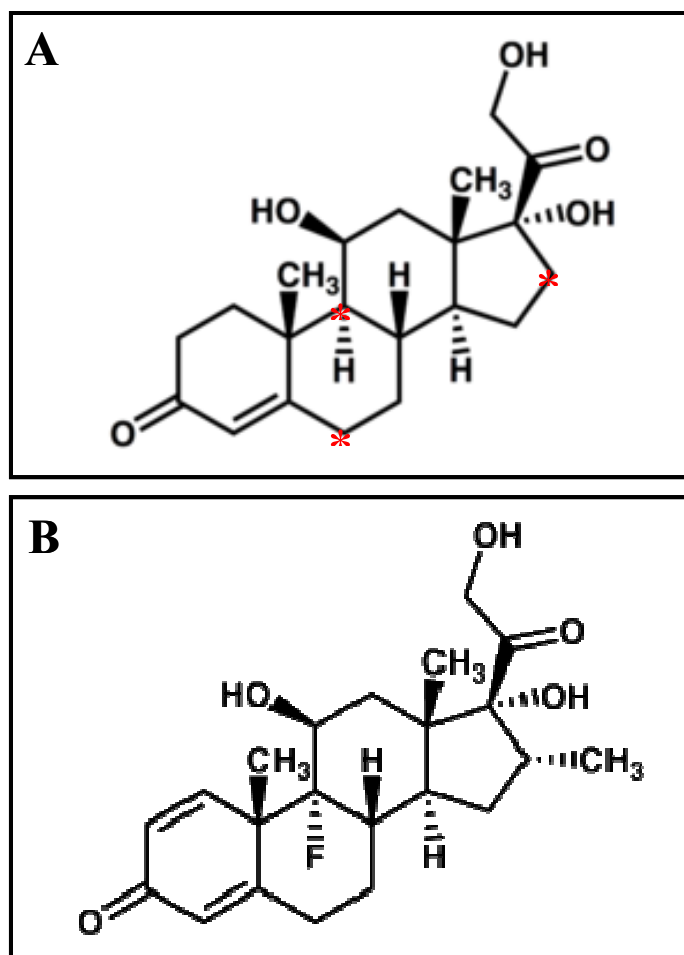
Figure 1.4. Chemical structures of (A) hydrocortisone and (B) dexamethasone. The glucocorticoid backbone consists of 21 carbons, and modifications of the glucocorticoid nucleus typically occur at positions 6, 9, and 16 (marked by "*" in the hydrocortisone structure above). These modifications typically affect pharmacologic activity, and multiple modifications may produce more pronounced effects than would be predicted on the basis of individual changes. For dexamethasone, substitution of a fluorine atom at position 9 profoundly increases glucocorticoid activity (AHFS 2000).
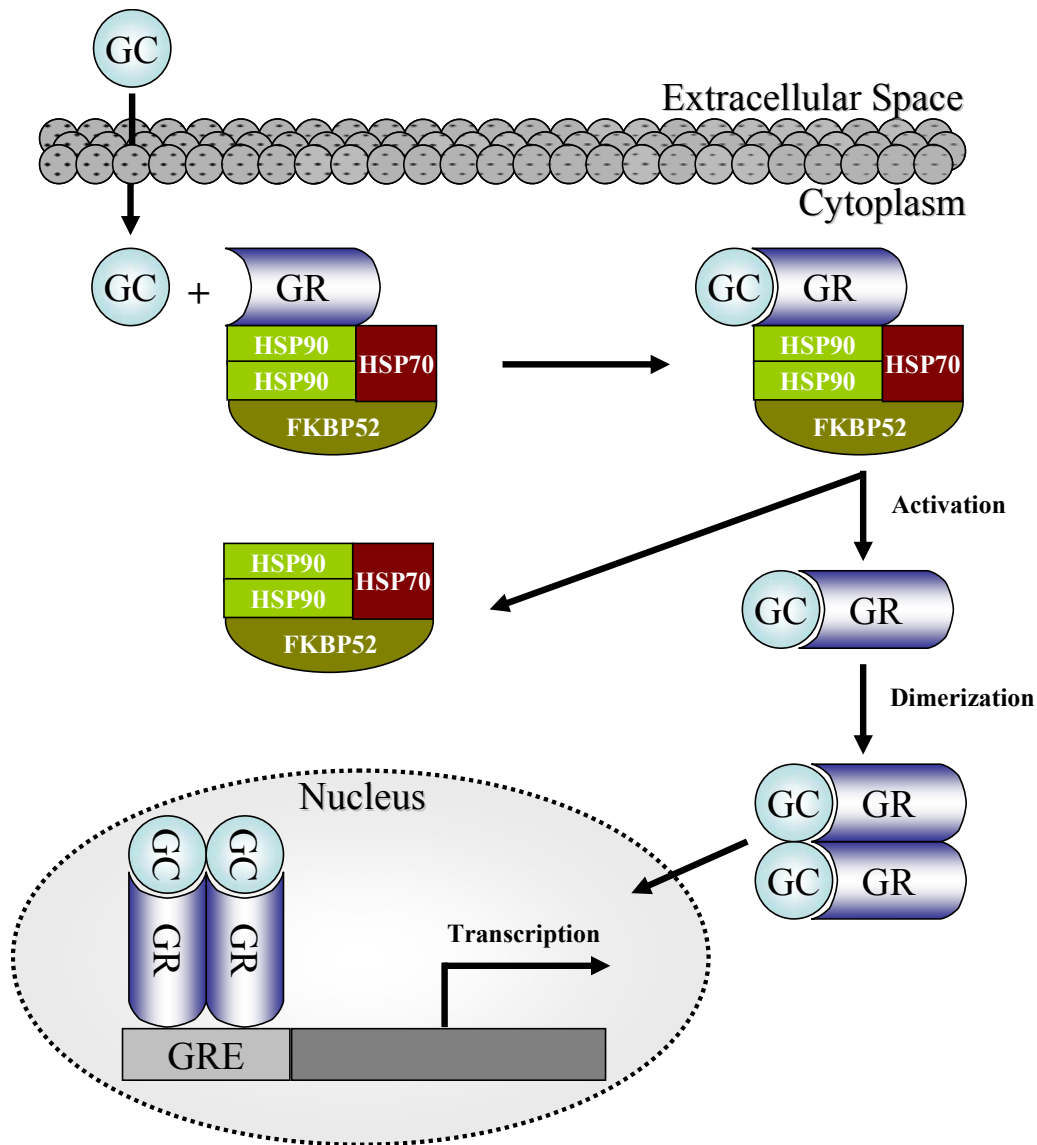
Figure 1.5. Conventional GR signaling pathway. The GR resides in the cytoplasm in complex with HSPs and the immunophilin FKBP52. Glucocorticoids entering the cytoplasm bind this complex to form a non-DNA-binding oligomer which, following cytoplasmic activation and subsequent conformational changes, dissociates into the accessory proteins and the DNA-binding GR monomer. Following dimerization with a second activated GR, the GR homodimer complex enters the nucleus and binds to the GRE in the promoter regions of target genes in order to activate or repress transcription. Abbreviations: **FKBP52**, FK506 binding protein 5; **GC**, glucocorticoid; **GR**, glucocorticoid receptor; **GRE**, glucocorticoid response element; **HSP70**, heat shock protein 70; **HSP90**, heat shock protein 90.

A   **Species (Receptor Type)**   **Domain Structure**

Rainbow Trout (MR)

| A/B | C | D | E |

Human (MR)

31%   93% 31%   72%

| A/B | C | D | E |

Rainbow Trout (GR1)

10%   92% 22%   50%

| A/B | C | D | E |

Rainbow Trout (GR2)

7%   90% 25%   56%

| A/B | C | D | E |

B

Cholesterol → 17-hydroxyprogesterone

Cholesterol ↓ Pregnenalone ↓ 17-hydroxypregnenalone

17-hydroxyprogesterone ↓ 11-deoxycortisol ↓ Cortisol ⟵┈┈┈⟶ Cortisone
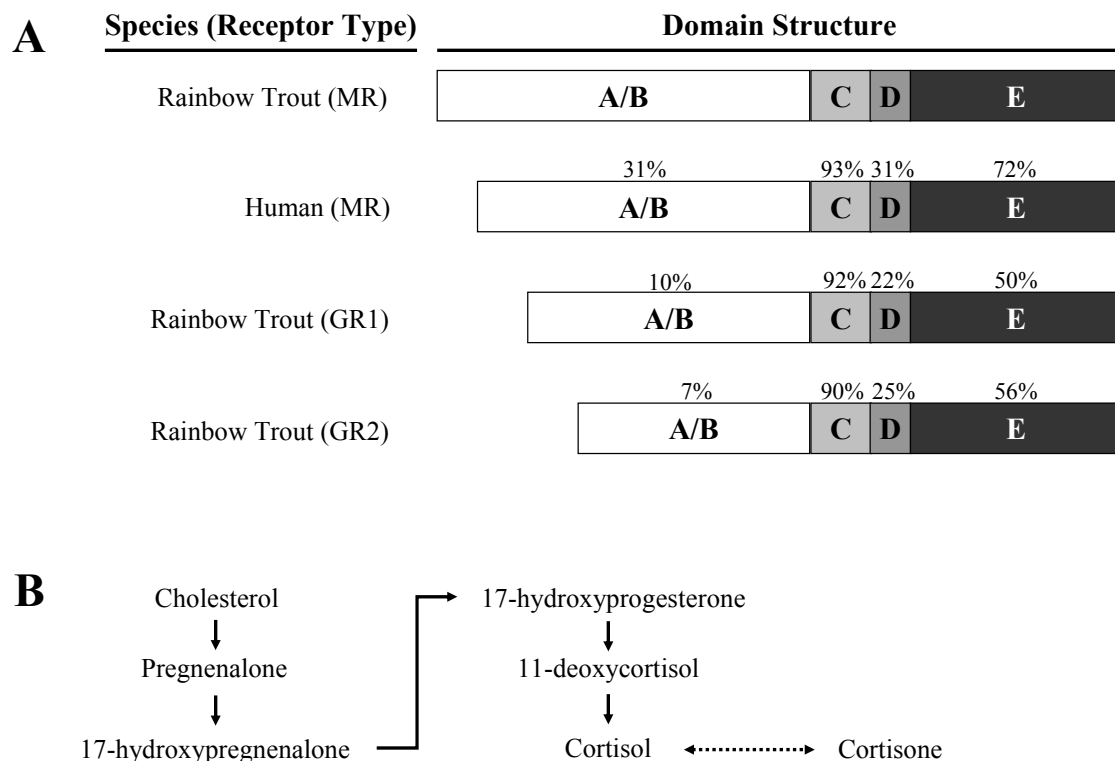
Figure 1.6. (A) Domain amino acid sequence identity between the human mineralcorticoid receptor (MR) or the rainbow trout glucocorticoid receptor (isoforms GR1 and GR2) and the rainbow trout MR. Percentages listed above each domain correspond to the sequence identity of that particular domain to the corresponding rainbow trout MR domain. Domain structures are as follows: **A/B**, N-terminal domain; **C**, DNA-binding domain; **D**, hinge region; **E**, C-terminal domain. Note the high identity between the various DNA-binding domains (≥ 90%). Modified from Prunet *et al.* 2006. **(B)** Biosynthesis of cortisol in fish. There is ready interconversion of cortisol and cortisone.

1.6. Tables

Table 1.1. Zebrafish developmental staging (modified from Kimmel *et al.* 1995).

Period	Stage	Begins (hpf)	Developmental Marker
Zygote	1-cell	0.00	Cytoplasmic streaming toward animal pole to form blastodisc
Cleavage	2-cell	0.75	Partial cleavage
	4-cell	1.00	2x2 blastomere array
	8-cell	1.25	2x4 blastomere array
	16-cell	1.50	4x4 blastomere array
	32-cell	1.75	4x8 blastomere array
	64-cell	2.00	3 blastomere tiers
Blastula	128-cell	2.25	5 blastomere tiers; irregular cleavage planes
	256-cell	2.50	7 blastomere tiers
	512-cell	2.75	9 blastomere tiers; formation of YSL
	1000-cell	3.00	11 blastomere tiers; single row of YSL nuclei; asynchronous cell cycle
	High	3.33	>11 blastomere tiers; two rows of YSL nuclei; blastodisc flattening
	Oblong	3.66	Multiple rows of YSL nuclei; blastodisc flattening
	Sphere	4.00	Blastula obtains spherical shape; flat border between blastodisc and yolk
	Dome	4.33	Yolk cell bulging toward animal pole
	30% epiboly	4.66	Blastoderm of uniform thickness forms; 30% of yolk cell covered by blastoderm
Gastrula	50% epiboly	5.25	50% of yolk cell covered by blastoderm
	Germ-ring	5.66	Germ ring visible from animal pole; 50% epiboly
	Shield	6.00	Embryonic shield visible from animal pole
	75% epiboly	8.00	Dorsal side distinctly thicker; epiblast, hypoblast, evacuation zone visible
	90% epiboly	9.00	Axis and neural plate formation; presence of brain, notochord, and muscle rudiments
	Bud	10.00	Prominent tail bud; 100% epiboly
Segmentation	1-4 somites	10.33	First somite furrow
	5-9 somites	11.66	Kupffer's vesicle, optic vesicle and neural keel form
	10-13 somites	14.00	Pronephros forms
	14-19 somites	16.00	EL=0.9 mm; otic placode, brain neuromeres
	20-25 somites	19.00	EL=1.4 mm; lens, otic vesicle, hindbrain neuromeres
	26+ somites	22.00	EL=1.6 mm; blood islands, otoliths, midbrain-hindbrain boundary
Pharyngula	Prim-5	24.00	EL=1.9 mm; early pigmentation, heartbeat
	Prim-15	30.00	EL=2.5 mm; early touch reflex, retina pigmented
	Prim-25	36.00	EL=2.7 mm; early motility, tail pigmentation
	High-pec	42.00	EL=2.9 mm; pectoral fin rudiments
Hatching	Long-pec	48.00	EL=3.1 mm; elongated pectoral fin buds; first cartilage and bone appear
Larval	Protruding-mouth	72.00	EL=3.5 mm; mouth opens and protrudes anteriorly just beyond the eye

Table 1.2. Matrix metalloproteinases and ECM substrate specificity.

Subfamily	Name(s)	ECM Substrate(s)
Collagenase	MMP-1/Collagenase-1	Collagen I, II, III, VII, VIII, X, XI; gelatin, entactin/nidogen, fibronectin, laminin, vitronectin, aggrecan
	MMP-8/Collagenase-2	Collagen I, II, III; gelatin, entactin, aggrecan, tenascin
	MMP-13/Collagenase-3	Collagen I, II, III, VI, VIII, IX, X, XIV; gelatin, fibronectin, vitronectin, aggrecan, osteonectin
	MMP-18/Collagenase-4	Collagen IV
Gelatinase	MMP-2/Gelatinase-A	Collagen I, III, IV, V, VII, X, XI; gelatin, fibronectin, fibrillin, elastin, laminin-5, vitronectin, aggrecan, osteonectin, tenascin
	MMP-9/Gelatinase-B	Collagen IV, V, XI, XIV; gelatin, decorin, fibrillin, elastin, laminins, vitronectin, aggrecan
Stromelysin	MMP-3/Stromelysin-1	Collagen III, IV, V, VII, IX, X, XI; gelatin, decorin, elastin, entactin/nidogen, fibronectin, fibrillin, laminin, vitronectin, aggrecan, osteonectin
	MMP-10/Stromelysin-2	Collagen III, IV, V; gelatin, elastin, fibronectin, aggrecan
	MMP-11/Stromelysin-3	Fibronectin, laminin, aggrecan
Matrilysin	MMP-7/Matrilysin-1	Collagen I, IV; decorin, elastin, fibrillin, fibronectin, laminin, vitronectin, aggrecan, osteonectin
	MMP-26/Matrilysin-2	Collagen IV, gelatin, fibronectin, fibrin/fibrinogen
Membrane-type	MMP-14/MT1-MMP	Collagen I, II, III; gelatin, fibronectin, vitronectin, aggrecan
	MMP-15/MT2-MMP	Fibronectin, laminin, entactin, aggrecan, proteoglycans
	MMP-16/MT3-MMP	Collagen III, fibronectin
	MMP-17/MT4-MMP	Gelatin, fibrin/fibrinogen
	MMP-24/MT5-MMP	Fibronectin, gelatin, proteoglycans
	MMP-25/MT6-MMP	Collagen IV, gelatin, laminin-1, fibronectin, proteoglycans, fibrin/fibrinogen
Other MMPs	MMP-12/Macrophage elastase	Fibronectin, elastin, laminin, proteoglycans, fibrin/fibrinogen
	MMP-19/RASI-1	Collagen IV, gelatin, laminin, fibronectin, fibrin/fibrinogen
	MMP-20/Enamelysin	Amelogenin, aggrecan
	MMP-21/XMMP	Gelatin
	MMP-23/CA-MMP	Gelatin
	MMP-27/CMMP	Gelatin
	MMP-28/Epilysin	Not determined

2.0. MATERIALS AND METHODS

2.1. Protein Sequence Analysis

MMP-13 protein sequences for zebrafish (AAQ07962), mouse (NP_032633), and human (NP_002418) were obtained from the National Center for Biotechnology Information (NCBI). Multiple sequence alignment was performed using MultAlin (Corpet 1988) and alignment output was generated using Boxshade v.3.21. Domain structure was determined using the NCBI Protein-Protein BLAST network service.

2.2. Zebrafish Strains and Husbandry

The AB strain of zebrafish (*Danio rerio*), obtained from the Zebrafish International Resource Center (ZIRC), was used for all of the experiments described. Zebrafish were maintained and bred in an Aquatic Habitats recirculation system according to a husbandry protocol approved by the Rutgers University Animal Care and Facilities Committee. Staging of embryonic zebrafish was conducted according to (Kimmel *et al.* 1995).

2.3. Real-Time (Quantitative) RT-PCR

Embryos collected for RNA isolation were snap frozen in 1.5 mL microcentrifuge tubes using liquid nitrogen and stored at -80°C. Total RNA was isolated from embryos using TRIzol (Invitrogen) and DNase-treated (DNA-free kit, Ambion) to remove genomic DNA contamination. Reverse transcription was performed on 1 μg aliquots of total RNA to produce cDNA for Real-Time RT-PCR (quantitative RT-PCR; qRT-PCR) using an iScript cDNA Synthesis Kit (BioRad). Quantitative RT-PCR reactions were

performed in triplicate using BioRad iQ SYBR Green Supermix, and cDNA amplification was performed for 40 cycles on a BioRad iCycler equipped with an iCycler iQ Detection System. Primers to zebrafish β-Actin, 28S ribosomal RNA (rRNA) MMP-2, MMP-9, and MMP-13 were used in amplification reactions (**Table 2.1**). Quantitative RT-PCR threshold cycle data were normalized to β-Actin or 28S rRNA, which served as a loading controls, and standard curves generated for MMP-2, MMP-9, and MMP-13 were used to quantify mRNA expression.

2.4. *In Situ* Hybridization

The MMP-2 RNA probe was generated using a construct generously donated by Dr. Robert Tanguay (Oregon State University) consisting of full length MMP-2 cDNA expressed in pCR-Blunt II-TOPO. The plasmid was linearized using PstI, and SP6 and T7 RNA polymerase were used to generate antisense and sense, respectively, digoxigenin (DIG)-labeled RNA probes (DIG RNA Labeling Kit - SP6/T7, Roche). The MMP-9 RNA probe was generated from a cDNA clone encoding a 318 bp portion of the MMP-9 gene amplified using the following primers: 5'-TTT*GAGCTC*TACAGTCTGTT-TCTGGTGG-3' (Forward primer; the italicized portion designates a SacI restriction site) and 5'- ATA*GGATCC*GGCGTCAAACTCCTT-3' (Reverse primer; the italicized portion designates a BamHI restriction site). To generate this portion of the MMP-9 gene, total RNA from untreated 72 hpf embryos was isolated, DNase-treated, made into cDNA as described previously and polymerase-amplified via PCR. The 318 bp PCR product was digested using SacI and BamHI restriction enzymes and subsequently cloned into PSPT18 (Roche). The PSPT18-MMP9 construct was linearized with BamHI, and SP6

and T7 RNA polymerase were used to create sense and antisense DIG-labeled RNA probes, respectively. The MMP-13 RNA probe was generated from a cDNA clone encoding the 289 bp portion of the MMP-13 gene amplified by the primers given in Table 2.1. To generate this portion of the MMP-13 gene, total RNA from untreated 72 hpf embryos was isolated, DNase-treated, made into cDNA as described previously and polymerase-amplified via PCR. The 289 bp PCR product was then cloned into pCRII-TOPO (Invitrogen). This fragment was liberated from pCRII-TOPO by digestion with EcoRI, and the product was inserted into the EcoRI site of PSPT19 (Roche). The PSPT19-MMP13 construct was linearized with SacI, and SP6 and T7 RNA polymerase were used to create sense and antisense DIG-labeled RNA probes, respectively.

Embryos to be used for *in situ* hybridization were grown in 0.003% (0.033 mg/mL in embryo medium) phenylthiourea (Sigma) to inhibit formation of pigmentation. Prior to initiation of staining, embryos were dechorionated, euthanatized using an overdose of MS-222 (Sigma), and fixed overnight in BT-fix (4% sucrose, 4% paraformaldehyde, 0.1 M sodium phosphate, 0.15 mM calcium chloride, titrated to pH 7.3) (Westerfield 2000). The *in situ* hybridization protocol followed was a slight modification of that described by (Oxtoby and Jowett 1993). Following staining, the embryos were cleared of non-specific staining by being transferred into methanol for 10 min, soaked in isopropanol for 10 min, and then placed in 1,2,3,4-tetrahydronaphthalene (Sigma-Aldrich) for visualization.

2.5. *In Vitro* Transcription / Translation

Zebrafish antisense morpholino oligonucleotides (MOs) designed to block initiation of MMP-2, MMP-9, or MMP-13 mRNA translation were obtained from Gene Tools, LLC (Philomath, Oregon). Morpholino sequences are provided in **Table 2.2**. The control morpholino (Control-MO) served as a negative control since it targets a human β-globin intron mutation known to cause β-thalessemia. This MO has not been reported to possess any other target or generate any phenotypes in any known test system except human β-thalessemic hematopoietic cells. In order to determine the effectiveness of each morpholino, a coupled *in vitro* transcription / translation reaction of each MMP protein was carried out using a TnT® Quick Coupled Transcription / Translation System (Promega). The MMP-2 construct used in these reactions is described above and consisted of full length MMP-2 cDNA expressed in pCR-Blunt II-TOPO. Constructs consisting of full length MMP-9 or MMP-13 cDNA expressed in pCMV-SPORT6.1 were obtained from Open Biosystems (Huntsville, AL). MMP-13 cDNA was subsequently subcloned into the pTnT vector (Promega). *In vitro* transcription / translation reactions were set up as 12.5 μL reactions consisting of 125 ng cDNA, 1 μL Redivue® L-[^{35}S]methionine (GE Healthcare), and either no morpholino or 10 μM of Control-MO or MMP-MO. A control reaction containing neither MMP cDNA nor morpholino was also included. Following 90 min of incubation at 30ºC, radioactive translation products were resolved by 10% Bis-Tris polyacrylamide gel electrophoresis. The gel was fixed for 15 min, soaked in 10% glycerol for 1 h and then dried at 65ºC for 1.5 h. The dried gel was exposed to a Storage Phosphor Screen (GE Healthcare) for 24 h and imaged using a Storm 860 PhosphorImager (GE Healthcare).

2.6. Microinjection of Antisense Morpholino Oligonucleotides

Prior to injections, 1 mM stocks of each morpholino were diluted to concentrations ranging from 50 μM-300 μM (approximately 2 ng-10 ng equivalent) in 1 X Danieau buffer (58 mM NaCl, 0.7 mM KCl, 0.4 mM $MgSO_4$, 0.6 mM $Ca(NO_3)_2$, 5 mM HEPES, pH to 7.6) (Nasevicius and Ekker 2000). Embyros were injected with 4 nL of Control-MO or MMP-MO at the 1-2 cell stage as described by (Nasevicius and Ekker 2000) using a General Valve Corporation Picospritzer II. Approximately 2 h following the injection, embryos were visualized using an Olympus IX51 inverted microscope equipped with an EXFO X-Cite 120 fluorescence illumination system, and any embryos that were found to be unfertilized, damaged, and/or possess an uneven distribution of the morpholino were discarded (morpholinos were fluorescein-tagged in order to visualize and monitor injection success). Embryos that had been successfully injected with the morpholino, as judged both by even distribution throughout the cell mass (at 2 hpf, embryos are at the 64-cell stage) and a strong fluorescent signature, were maintained at 28°C in embryo medium (Westerfield 2000). Embryos were assessed every 24 h through 72 hpf and any changes in normal morphology were visualized and documented by taking representative digital photographs using a Scion CFW-1310C color digital camera interfaced to either an Olympus IX51 inverted scope or an Olympus SZ4060 zoom stereo microscope. In addition, approximately 15 embryos per treatment were collected at 72 hpf and 96 hpf for Alcian blue staining.

2.7. Collection and Treatment of Zebrafish Embryos

Embryos were collected at 3 hpf from breeding stocks of zebrafish. Treatments consisted of exposure through 24, 48, 72 or 96 hours post fertilization (hpf) to 1, 10, or 100 mg/L of dexamethasone (2.55, 25.48, or 254.81 μM equivalent) or hydrocortisone (2.76, 27.59, or 275.88 μM equivalent) alone or in conjunction with 100-250 nM mifepristone (RU486) in embryo medium. Dexamethasone and hydrocortisone were dissolved in dimethylformamide (DMF) prior to being diluted in embryo medium to the experimental concentrations. In order to generate experimental concentrations of RU486, a 1 mM stock of RU486 was first made by dissolving in 100% ethanol, and a subsequent 100 μM working stock was generated by further dilution into ethanol. Final concentrations of RU486 were achieved by diluting this working stock in embryo medium. All RU486 stocks were stored at -20ºC between uses. Controls, including a no treatment control of embryo medium alone and a solvent control consisting of ethanol and/or DMF alone were run concurrently with each treatment. When co-treatments of dexamethasone or hydrocortisone and RU486 were performed, a RU486 control consisting of the appropriate concentration of RU486 alone was included. The no treatment control embryos were monitored throughout the course of each study to confirm embryo viability. Data from any treatment in which > 10% of the solvent or no treatment control embryos exhibited developmental abnormalities were not considered for analysis. All chemicals and solvents were purchased from Sigma-Aldrich (St. Louis, MO) and possessed purities ≥ 98%.

2.8. Alcian Blue Staining

Embryos to be used for Alcian blue staining were grown in 0.003% phenylthiourea. Embryos were euthanatized using an overdose of MS-222, fixed overnight at 4°C in 4% paraformaldehyde, and then transferred to 70% ethanol to allow dehydration of the tissue for at least 24 h. Prior to staining, embryos were bleached in 30% hydrogen peroxide for approximately 2 h and then rinsed twice with 0.1% Tween-20 in phosphate buffered saline. Staining was performed overnight at room temperature using 0.1% Alcian blue 8GX (Sigma-Aldrich) that had been filtered through a 2 μm syringe filter. Following staining, embryos were placed in acidified ethanol for 2-4 h to allow for clearing of non-specific staining. Finally embryos were washed in increasing concentrations of glycerol (15 min each of 20%, 50%, 80%, and 100%) and visualized as described previously.

2.9. *In Vitro* Zymography

Lysates were prepared as described by (Crawford and Pilgrim 2005). Lysate protein concentration was determined using a Modified Lowry Protein Assay Kit (Pierce) to ensure that each reaction contained equal amounts of total protein. *In vitro* zymography reactions were set up in 96-well plates as follows: 0.5-1 μL lysate (corresponding to approximately 0.2-16 μg total protein), 10 μL of 1 mg/mL fluoresceinated type I or type IV DQ™ Collagen (Molecular Probes), and lysis buffer (150 mM NaCl, 10 mM HEPES, 2 mM DTT, 0.1% Triton X-100, pH 8.0) to bring the volume to 200 μL. EDTA (1 mM), GM6001 (100 μM; Calbiochem), MMP-9/MMP-13 Inhibitor I (10 μM; Calbiochem) MMP-2/MMP-9 Inhibitor II (100 μM; Calbiochem;

(Tamura *et al.* 1998)) were added to these reactions as inhibitors. When inhibitors were utilized, reactions consisting of lysate, lysis buffer, and inhibitor only were allowed to preincubate for 2 h to overnight prior to addition of fluoresceinated substrate. Following preincubation, reactions were incubated for 24-72 h prior to measurement. FITC fluorescence was measured using a Perkin Elmer HTS 7000 Plus Bio Assay Reader set for excitation at 492 nm and emission detection at 535 nm. All reactions were run in at least triplicate and were corrected for background fluorescence.

2.10. Statistical Analysis

Statistical analysis was performed using the SigmaStat v1.0 computer software package (Jandel Scientific). Data were evaluated either by an unpaired Student's *t*-test, One-Way Analysis of Variance (ANOVA), or Kruskal-Wallis ANOVA on Ranks. The Student-Newman-Keuls test, the Bonferroni *t*-test, or Dunnett's test were used as the multiple comparison methods. The probability level for statistical significance was $p < 0.05$.

2.11. Tables

Table 2.1. Primers used in Real-Time RT-PCR reactions. (F) forward primer; (R) reverse primer.

Gene	Primer Sequence	Product Size
β-Actin	(F) – 5'-CGAGCAGGAGATGGGAACC-3' (R) – 5'-CAACGGAAACGCTCATTGC-3'	102 bp
28S rRNA	(F) – 5'-CCTCACGATCCTTCTGGCTT-3' (R) – 5'-ATTCTGCTTCACAATGATA-3'	151 bp
MMP-2	(F) – 5'-AGCTTTGACGATGACCGCAAATGG-3' (R) – 5'-GCCAATGGCTTGTCTGTTGGTTCT-3'	224 bp
MMP-9	(F) – 5'-AACCACCGCAGACTATGACAAGGA-3' (R) – 5'-GTGCTTCATTGCTGTTCCCGTCAA-3'	89 bp
MMP-13	(F) – 5'-ATGGTGCAAGGCTATCCCAAGAGT-3' (R) – 5'-GCCTGTTGTTGGAGCCAAACTCAA-3'	289 bp

Table 2.2. Morpholino sequences targeting the 5'-untranslated region of the genes of interest as a means of blocking translation.

Gene	Morpholino Sequence
Control	5'-CCTCTTACCTCAGTTACAATTTATA-3'
MMP-2	5'-TCTGAAAAACTTAACGGACAGCATG-3'
MMP-9	5'-CGCCAGGACTCCAAGTCTCATCTTG-3'
MMP-13	5'-AAGCAGGTCTTCATGTTTCTCGTGC-3'

3.0. MATRIX METALLOPROTEINASE-13 (MMP-13) IS REQUIRED FOR ZEBRAFISH (*DANIO RERIO*) DEVELOPMENT AND IS A TARGET FOR GLUCOCORTICOIDS

3.1. Abstract

MMPs are endopeptidases that degrade the proteins of the extracellular matrix. Expression and activity of the MMPs are essential for embryogenesis, where MMPs participate in the normal extracellular matrix remodeling that occurs during tissue morphogenesis and development. Studies have demonstrated that MMP gene expression is inhibited by glucocorticoids in mammalian cell culture systems, and that exposure to glucocorticoids causes developmental abnormalities in several species. Therefore, it was proposed that glucocorticoids impede normal development through alteration of MMP expression. Zebrafish (*Danio rerio*) were used as a model to study MMP-13 expression both during normal embryogenesis and following acute exposure to two glucocorticoids, dexamethasone and hydrocortisone. MMP-13 is one of three collagenases identified in vertebrates that catalyzes the degradation of type I collagens at neutral pH. MMP-13 expression varied during zebrafish development, with peak expression at 48 hpf. Morpholino knockdown studies showed that MMP-13 expression is necessary for normal zebrafish embryogenesis. Acute exposure to dexamethasone and hydrocortisone resulted in abnormal zebrafish development including craniofacial abnormalities, altered somitogenesis, blood pooling and pericardial and yolk sac edema as well as increased MMP-13 mRNA and activity at 72 hpf. *In situ* hybridization experiments were used to confirm the increase in MMP-13 expression following glucocorticoid treatment and

showed elevated MMP-13 expression in the rostral trunk, brain, eye, heart, and anterior kidney of treated embryos. These data demonstrate that normal zebrafish embryogenesis requires MMP-13 and that dexamethasone and hydrocortisone modulate the expression of this gene, leading to increased activity and potentially contributing to subsequent dysmorphogenesis.

3.2. Introduction

The MMPs are a family of endopeptidases characterized by a requirement for Zn^{2+} and Ca^{2+} for activity. There are currently over 20 distinct MMPs that are classified based on their substrate specificities. These include the collagenases, which degrade connective tissue collagens; the type IV collagenases (gelatinases), which degrade basement membrane collagens and gelatins; the stromelysins, which have a broad substrate specificity, including proteoglycans, laminins, fibronectins, and gelatins; and a fourth group that is distinct from the other four in both sequence and substrate specificity (Bode *et al.* 1999; Brinckerhoff and Matrisian 2002). A number of physiological processes require MMP activity, including development, uterine resorption after pregnancy, ovulation, and wound healing (Brinckerhoff and Matrisian 2002). Inappropriate MMP expression and activity is associated with a variety of diseases. For example, these enzymes are responsible for the destruction of the joint during rheumatoid arthritis and for the degradation of the basement membranes and ECM during tumor invasion and metastasis (Folgueras *et al.* 2004; Hendrix *et al.* 2003; Rundhaug 2005). MMP expression is also associated with cardiovascular and pulmonary disease, as well as degenerative neurological disorders, such as multiple sclerosis (Leppert *et al.* 2001;

Tayebjee *et al.* 2005).   Although originally thought to contribute to disease progression solely through their activity toward the matrix proteins, it is becoming apparent that the MMPs have a more complex role, specifically through regulation of cell growth, apoptosis, and angiogenesis, processes which are critical for development (Lemaitre and D'Armiento 2006; Rundhaug 2005).

MMPs are regulated primarily at the levels of transcription and enzyme activity (Vincenti *et al.* 1996). The inflammatory cytokines, including IL-1 and TNF-α, produced at the site of inflammation result in stimulation of MMP gene expression (Chakraborti *et al.* 2003).   MMP gene expression is also affected by non-soluble factors such as matrix components, changes in cell shape/size, and matrix disruption.   Inhibition of MMP gene expression is observed following glucocorticoid or retinoic acid exposure in mammalian cell culture systems (Chakraborti *et al.* 2003).   Transcriptional regulation by MMPs is cell type-specific, and recent data suggest that this may be due, in part, to changes in histone acetylation associated with the MMP genes (Young *et al.* 2005).   Recently, several MMP homologs have been identified in zebrafish.   In zebrafish, MMP-2 activity is required for normal development, as is expression of TIMP-2 (Zhang *et al.* 2003a; Zhang *et al.* 2003b).   In addition, two isoforms of a novel membrane-type MMP were isolated from zebrafish (MT-MMP α and β) and determined to be necessary for normal embryogenesis (Zhang *et al.* 2003c).   Also, MMP-9 has been identified and characterized in order to study zebrafish myelopoiesis (Yoong *et al.* 2007).

Glucocorticoids are a class of steroid hormones that are produced endogenously under the control of the HPA axis (Jacobson 2005).   These hormones regulate cellular growth, differentiation, and apoptosis. Glucocorticoids are also one of the most

commonly prescribed drugs worldwide, primarily used for their anti-inflammatory or immune-suppressive effects (Bello and Garrett 1999). These compounds are used to treat asthma, dermatitis, rheumatoid arthritis, and autoimmune diseases, as well as to prevent graft rejection. Glucocorticoids are potent teratogens, with the most common defect observed being cleft palate. Most glucocorticoid-associated effects are mediated by the GR. Prior to ligand activation, the GR resides in the cytoplasm in complex with two HSP90 proteins and several immunophilins (Schaaf and Cidlowski 2002). Activation results in GR conformational changes that lead to dissociation of the GR from the accessory proteins and exposure of nuclear localization signals. Following dimerization with a second activated GR, the GR homodimer complex enters the nucleus and binds to specific cis-acting elements, the GREs in the promoter regions of target genes. In addition, the GR homodimers are capable of binding to less well defined cis-elements and repressing transcription. Glucocorticoids also inhibit gene expression through antagonism of other transcription factors, including AP-1 and NF-κB, which is considered the main mechanism of their anti-inflammatory effects (Almawi and Melemedjian 2002; Herrlich 2001).

The purpose of these studies is to determine the role of MMP-13 in normal zebrafish embryogenesis and to establish a link between MMP expression and activity and the teratogenic effects known to result from exposure to glucocorticoids. The data presented here demonstrate that MMP-13 is critical for proper embryonic development in zebrafish. In addition, these data show that the glucocorticoids dexamethasone and hydrocortisone cause dysmorphogenesis and result in increases in MMP-13 expression and activity at 72 hpf. These studies demonstrate for the first time that a collagenase,

MMP-13, was necessary for normal zebrafish embryogenesis and that the expression of this gene could be modified by exposure to glucocorticoids.

3.3. Results

3.3.1. Zebrafish exhibit MMP-13 protein sequence identity with other species

Alignment of MMP-13 protein sequences for zebrafish, mouse, and human reveals 47% identity between all three species and approximately 52% identity between zebrafish and either mouse or human (**Figure 3.1**). Protein domain structure analysis of the zebrafish MMP-13 amino acid sequence using the NCBI Protein-Protein BLAST network service suggests that it is comprised of a signal peptide, propeptide, catalytic domain, hinge region, and hemopexin domain. Of these domains, the catalytic domain exhibits the highest level of similarity between all three species (103 of 161 amino acids are identical corresponding to 64% similarity), followed by the hemopexin domain (43%), propeptide (41%), hinge region (25%), and signal peptide (16%).

3.3.2. Developing zebrafish exhibit varying MMP-13 expression levels

To determine the expression of MMP-13 mRNA occurring during normal zebrafish embryogenesis, embryos maintained in embryo medium were collected at six different time points and analyzed via quantitative RT PCR (**Figure 3.2**). All PCR data is normalized to 28S rRNA since β-Actin has been shown to change during embryogenesis (Sawai and Campos-Ortega 1997). MMP-13 mRNA expression levels are at a minimum at 12 hpf, corresponding to the beginning of the segmentation period (Kimmel *et al.* 1995). Expression of MMP-13 mRNA increases following 12 hpf, reaching maximum expression at 48 hpf, the long-pec stage (Kimmel *et al.* 1995). MMP-

13 mRNA levels at 48 hpf are significantly higher than all other time points measured. Expression levels remain elevated at 72 and 96 hpf (the protruding-mouth stage and early larval period, respectively) (Kimmel *et al.* 1995), although they are significantly less than that observed at 48 hpf.

3.3.3. MMP-13 expression is required for normal zebrafish embryogenesis

To demonstrate the importance of MMP-13 in normal zebrafish embryogenesis, a sequence-specific morpholino targeting this gene was utilized to transiently knock down expression (Nasevicius and Ekker 2000). Coupled *in vitro* transcription / translation reactions were performed to demonstrate the effectiveness of the designed morpholino to block MMP-13 protein expression. A control reaction, which was included to ensure that the rabbit reticulocyte system did not produce any aberrant protein bands and which contained neither MMP-13 cDNA nor morpholino, does not produce a band (**Figure 3.3, lane 1**). The reaction containing MMP-13 cDNA in the absence of morpholino produces a 48 kDa protein, corresponding to the active enzyme (**Figure 3.3, lane 2**). Addition of control-MO does not affect protein production (**Figure 3.3, lane 3**). However, addition of the MMP13-MO decreases protein production (**Figure 3.3, lane 4**), demonstrating that the morpholino is able to block translation of MMP-13 *in vitro*.

The MMP13-MO was injected at concentrations ranging from 10 μM to 100 μM in order to establish an effective morpholino concentration. Approximately 30-50 embryos were examined per concentration depending on injection success. Embryos injected with 10 μM exhibit no gross morphological changes and appear identical to those injected with control-MO (**Figure 3.4**). When the concentration of morpholino is

increased to 50 μM, less than 10% of the morphants exhibit an abnormal phenotype consisting of pericardial and yolk sac edema, decreased blood flow with associated blood pooling, and body axis curvature. MMP-13 morphants also have shortened and kinked tails, often with associated malformation of the actinotrichia leading to a loss of tailfin architecture. Embryos injected with 100 μM MMP13-MO exhibit similar phenotypes to those injected at 50 μM, although at a higher incidence (greater than 90% of those injected). Additional phenotypes observed at this concentration include atypical craniofacial development and epithelial hyperplasia. These data indicate that expression of MMP-13 is necessary for proper embryo development.

3.3.4. *Exposure to dexamethasone or hydrocortisone causes developmental abnormalities in zebrafish embryos*

For the purposes of these studies, hydrocortisone was selected since is it the synthetic analogue of cortisol, known to be the major corticosteroid released from teleost interrenal tissue (Prunet et al. 2006). Dexamethasone, a synthetic glucocorticoid, is generally believed to be more potent than hydrocortisone and has frequently been utilized to study the effects of this class of steroid hormones on various model systems. To demonstrate the effect of dexamethasone and hydrocortisone on zebrafish development, embryos were exposed to 1, 10, or 100 mg/L of either glucocorticoid starting at approximately 3 hpf. Embryos treated at 1 or 10 mg/L do not exhibit any gross changes compared to the vehicle control when monitored through 72 hpf (data not shown). However, exposure to 100 mg/L dexamethasone or hydrocortisone results in alterations in morphology and gross lesions by 48 hpf (**Figure 3.5A**) which become more severe by 72 hpf (**Figure 3.5B**). Alterations in morphology in dexamethasone- and hydrocortisone-

treated embryos include changes in the normal head-trunk angle, truncated body axis, and alteration in the shape and size of both the yolk sac and yolk sac extension.

Glucocorticoid-treated embryos also demonstrate altered somitogenesis, evident from the apparent compaction of the distance between somites and the divergence from the usual chevron shape of these segments. Dexamethasone-treated embryos, more so than the hydrocortisone-treated embryos, exhibit significant changes in the craniofacial region at 72 hpf. A statistically significant decrease in both body and head length, and a statistically significant increase in interocular distance are observed following exposure to 100 mg/L dexamethasone (**Table 3.1**). A number of tissue-specific lesions are observed in the embryos treated with 100 mg/L dexamethasone or hydrocortisone, such as pericardial edema, yolk sac edema, and blood pooling in the head and yolk sac extension.

3.3.5. Exposure to dexamethasone or hydrocortisone causes an induction of MMP-13 mRNA in zebrafish embryos

A dose-response experiment revealed that embryos exposed to 1 or 10 mg/L dexamethasone (but not hydrocortisone) through 72 hpf possessed increased MMP-13 transcript levels (data not shown). However, no phenotype changes were observed at any time point at either of these concentrations. Only embryos exposed to 100 mg/L through 72 hpf showed corresponding changes in phenotype and genotype at the chosen time point; therefore, this concentration was chosen for the remaining studies. Embryos exposed to 100 mg/L dexamethasone or hydrocortisone demonstrate a statistically significant ($p < 0.05$) increase in MMP-13 mRNA measured via quantitative RT-PCR at 24 and 72 hpf post-treatment (**Figure 3.6**). At 24 hpf, dexamethasone- and

hydrocortisone-treated embryos exhibit a 2.9 and 3.1 fold induction over the solvent control-treated embryos, respectively. A 2.5 and 4.9 fold induction is observed in dexamethasone- and hydrocortisone-treated embryos at 72 hpf, respectively. Hydrocortisone appears to be a more efficacious inducer of MMP-13 expression than dexamethasone at the dose tested, although a statistically significant ($p < 0.05$) difference between these two groups is observed at 72 hpf only.

In situ hybridization using a MMP-13-specific mRNA probe confirms glucocorticoid-induced MMP-13 expression (**Figure 3.7**). Low levels of expression are observed throughout the entire embryo, with the exception of the yolk sac and yolk sac extension. Sense probes yield almost no signal and confirm the specificity of the antisense probe for MMP-13 mRNA (data not shown). In dexamethasone-treated embryos, increased MMP-13 expression is observed in the brain, eye and rostral trunk. Hydrocortisone-treated embryos show increased MMP-13 expression localized to the eye, heart, anterior kidney, and the rostral trunk.

3.3.6. Collagenase activity is increased in embryos treated with dexamethasone or hydrocortisone

Gelatin zymography is typically used to examine the proteolytic activity of MMPs in cell and tissue extracts. This method employs SDS-polyacrylamide gels co-polymerized with a protein substrate of interest on which MMPs can exert their proteolytic activity. Subsequent Coomassie Blue staining of the gel following incubation reveals white bands signifying sites of proteolysis, and analysis of the molecular weight and size of these bands allows for pro- and active enzyme determination (Frederiks and Mook, 2004). A more recent method employed during these studies, *in vitro*

zymography, has been shown to be an effective means of determining MMP activity in lysates isolated from zebrafish embryos (Crawford and Pilgrim, 2005). *In vitro* zymography is a plate-based, quantitative method which uses heavily fluoresceinated native collagens as substrates for MMPs. In their native state, these substrates are only weakly fluorescent due to intermolecular quenching. However, when proteolytic cleaving relieves this quenching, fluorescent signal increases and can be picked up by a plate reader (Crawford and Pilgrim, 2005). Using this method, increased collagenase activity is observed in lysates isolated from zebrafish embryos treated with 100 mg/L dexamethasone or hydrocortisone through 72 hpf (**Figure 3.8A**). These data confirm that increased transcript levels detected via Real Time RT-PCR and *in situ* hybridization result in increased enzyme activity. Inclusion of 1 mM EDTA, a chelator of divalent cations, in these reactions results in a complete loss of activity for all treatment groups and demonstrates that the activity observed is due to a metalloenzyme (**Figure 3.8B**). Use of a MMP-13 specific inhibitor results in a statistically significant decrease in collagenolytic activity in both dexamethasone-treated (39% inhibition) and hydrocortisone-treated (83% inhibition) lysates, suggesting that the activity observed can be attributed, at least in part, to MMP-13.

3.4. Discussion

The MMPs are important mediators of tissue remodeling during development through regulation of matrix degradation, apoptosis, cell cycle and angiogenesis (Egeblad and Werb 2002; Lemaitre and D'Armiento 2006). Several MMP homologs have been identified in zebrafish, including two gelatinases (MMP-2 and MMP-9), two isoforms of

a novel membrane-type MMP (MT-MMP α and β), and a homolog to human TIMP-2 (Yoong *et al.* 2007; Zhang *et al.* 2003a; Zhang *et al.* 2003b, c). Morpholino knockdown of MMP-2, TIMP-2, and MT-MMP α and β have demonstrated that expression of these proteins is required for normal zebrafish development (Zhang *et al.* 2003a; Zhang *et al.* 2003b, c). Blocking translation of these genes resulted in an abnormal phenotype constituting various degrees of abnormal axis formation, including truncation. Additional analysis of tissue sections from embryos injected with antisense MOs targeting MT-MMP α and β suggested that although the basic tissue types had formed, the normal organization of these tissues was noticeably altered (Zhang *et al.* 2003c). The data presented here show that zebrafish MMP-13 is also critical for normal embryogenesis. However, the types of lesions observed in MMP13-MO knockdown studies differed from those reported in above-mentioned studies, perhaps signifying the involvement of different organ systems or tissue types.

MMP-13 mRNA levels vary during zebrafish embryogenesis with expression correlating with times during development requiring cell migration and tissue morphogenesis. MMP-13 mRNA expression levels were at a minimum at 12 hpf, corresponding to the beginning of the segmentation period during which a number of morphogenetic movements occur. These include the formation of the optic primordium, tail bud, Kupffer's vesicle, and anterior somites (Kimmel *et al.* 1995). A low expression level during this stage of embryogenesis suggests that this particular matrix metalloproteinase may play a minor role in early development. Alternatively, this could represent the beginning of embryonic expression of this gene following degradation of maternal transcript. Expression of MMP-13 mRNA increased from 12 to 24 hpf, a time

during which the posterior half of the somites (approximately somite seven to thirty) are being produced. This increase in expression may signify a more central role for MMP-13 in the development of the posterior embryonic mesenchyme. MMP-13 reaches maximum expression at 48 hpf, the long-pec stage. As the name implies, it is during this time that the pectoral bud becomes elongated and chondrocytes begin to differentiate within the mesenchyme to form the first cartilage (Kimmel *et al.* 1995). This is in keeping with data from mammalian systems demonstrating that MMP-13 has an important role in bone and cartilage development (Blavier and Delaisse 1995; D'Angelo *et al.* 2000; Stahle-Backdahl *et al.* 1997; Wu *et al.* 2001). Therefore, MMP-13 activity at this point may play a role in shaping what is essentially the scaffolding of the fin, allowing it to develop from a simple bud to a complex appendage. During this period, significant changes in the heart are also occurring, including the development of the pericardial cavity and movement of the atrium to acquire a location dorsal to the ventricle (Kimmel *et al.* 1995). MMP-13 levels remained elevated through 96 hpf during which time the mouth and gill structure becomes well formed, the pectoral fin continues to enlarge, and the earliest bone (cleithrum) appears (Kimmel *et al.* 1995).

The data provided above show that MMP-13 is required for normal zebrafish development. MMP-13 activity functions to degrade matrix proteins, in addition to modulating the activity of other MMPs (Leeman *et al.* 2002). Using a morpholino to knockdown MMP-13 translation in a dose-dependent manner resulted in several developmental abnormalities. Morphants exhibited a number of morphology changes relating to body axis formation, craniofacial development, and tailfin development. MMP13-null mice exhibit abnormal bone growth resulting in embryonic and adult

skeletal dysmorphogenesis (Inada *et al.* 2004), similar to the abnormalities observed in the MMP-13 morphants monitored through 72 hpf.

The data presented here show that normal zebrafish development is also affected by exposure to two glucocorticoids, dexamethasone and hydrocortisone. A relatively high concentration of glucocorticoid was needed to elicit effects in both genotype and phenotype, although use of these drugs in zebrafish embryos at micromolar levels has been reported previously (Langenau *et al.*, 2005; To *et al.*, 2007; Tseng *et al.*, 2005). Some of the defects observed, such as body axis curvature, truncated body axis and altered somitogenesis, indicate dysfunctional matrix metabolism and remodeling. Although somitogenesis was abnormal, there was no change in the actual number of somites, suggesting that glucocorticoids effect somite migration. Somites begin to appear at approximately 10.5 hpf and are produced in an anterior to posterior wave until approximately 30 pairs are created (Stickney *et al.* 2000). The first somites appear to be formed at a more rapid rate than the later ones; specifically, three per hour for the first six and two per hour thereafter (Kimmel *et al.* 1995). Altered somitogenesis could also explain the abnormal tail morphology frequently observed in glucocorticoid-treated embryos. Taking into account the rate of somitogenesis, and considering it was the posterior 14 somites that most often exhibited malformation, one could speculate that aberrant somitogenesis starts at approximately 18 to 20 hpf. Additionally, data show that MMP-13 expression is increased by glucocorticoid exposure at 24 hpf. Taken together, these data suggest that the increased MMP-13 expression induced by dexamethasone and hydrocortisone could be contributing to altered somite migration.

Both quantitative RT-PCR and *in situ* hybridization data show that dexamethasone and hydrocortisone activate expression of MMP-13 in the developing zebrafish embryo. This finding is somewhat in conflict with previous data in mammalian cell models demonstrating that MMP expression is inhibited by exposure to glucocorticoids (Vincenti *et al.* 1996). However, the majority of these data concern other MMPs and the only data for MMP-13 is limited to cultured bone cells rather than a full developing system (Canalis and Delany 2002). Still, one report using rabbit knee cartilage as a model demonstrates that glucocorticoid injection repressed MMP-13 expression in the uninjured knee, whereas it enhanced MMP-13 expression in an injured state (Kydd *et al.* 2005). This suggests that glucocorticoid regulation of MMP-13 may differ depending on the remodeling status of the tissue. Recent data from other cell types demonstrate that glucocorticoids stimulate MMP-9 expression through induction of sGITR (Lee *et al.* 2004; Lee *et al.* 2003). These data suggest that regulation of MMP expression by glucocorticoids may be more complex than originally hypothesized. Data provided here support the complexity of MMP-13 expression in the developing embryo, not only in the activation of expression, but in the specific localization of MMP-13 expression following glucocorticoid exposure. The difference observed between the localization of MMP-13 expression following hydrocortisone or dexamethasone treatment may result from the GR variant expressed in these tissues. The related teleost fish species rainbow trout (*Oncorhychus mykiss*) and Burton's mouthbrooder (*Haplochromis burtoni*)) each have two distinct GR genes (Bury *et al.* 2003; Greenwood *et al.* 2003), and alternative splicing results in functionally different GR transcripts with different transactivation properties and affinities for cortisol (Greenwood *et al.* 2003;

Takeo *et al.* 1996). A full characterization of zebrafish GR has yet to be conducted, although gene duplication events in several teleost fish species in addition to those described above have been reported (Robinson-Rechavi *et al.*, 2001a; Robinson-Rechavi *et al.*, 2001b). Interestingly, rather than functional redundancy as is often seen in mammalian species, it appears as if these duplicated genes often encode functionally different receptors (Prunet *et al.*, 2006). A search of the zebrafish genome reveals that a single GR has been identified thus far (Ensembl Gene ID ENSDARG00000025032; Gene Name zgc:113038 [ZFIN]), although it is apparent that further investigation may reveal additional isoforms similar to those described for rainbow trout and Burton's mouthbrooder. Outside of different tissue expression of the GR, it is also possible that the variability in localization of MMP-13 transcript may be due to differences in the pharmacokinetics of dexamethasone and hydrocortisone. In humans, circulating hydrocortisone (cortisol) is bound extensively to the plasma proteins cortisol-binding globulin (CBG; transcortin) and albumin (Thomson *et al.*, 2007); however, the synthetic glucocorticoid dexamethasone has a much lower affinity for these proteins. This is significant since only unbound glucocorticoid is pharmacologically active.

This laboratory has shown that glucocorticoid- induced MMP-13 expression is followed by an increase in collagenase activity. MMP-13 is only one of three identified mammalian collagenases characterized by their unique ability to degrade fibrillar collagens (types I, II and III) in their resistant triple-helical form at neutral pH (Lemaitre and D'Armiento, 2006). However, it seems likely that the increased activity observed is a result of increased MMP-13 expression. Of the collagenases, MMP-1 and MMP-13 are the primary tissue collagenases. MMP-8 (collagenase-2) is expressed primarily in

response to inflammation, and appears to have, if any, a minor role in development, as knockout MMP-8 mice develop normally (Balbin, *et al.* 2003). No MMP-1 homolog has been identified in zebrafish, and there is not, as of yet, a clear murine ortholog to MMP-1, although two candidates have been identified (Balbin, *et al.* 2001). Therefore, although there may be some contribution of other potentially unidentified zebrafish collagenases in these assays, the increase in enzyme activity demonstrated via *in vitro* zymography most likely reflects the increase in MMP-13 mRNA expression observed following glucocorticoid exposure.

The zebrafish MMP-13 amino acid sequence shows 52% identity with the human or mouse MMP-13 amino acid sequence. The highest level of similarity is in the catalytic domain of the enzyme, which mediates substrate cleavage. Both the hemopexin and propeptide domains show a high level of identity as well (41% and 43% respectively). The lowest region of identity corresponds to the signal peptide domain. The overall high degree of similarity indicates that the sequence is indeed the zebrafish homologue of mammalian MMP-13. Further, the conservation of the catalytic domain suggests that specific inhibitors of mammalian MMP-13 activity are likely to inhibit zebrafish MMP-13 as well. This is confirmed by the data given above demonstrating that the MMP-13-specific inhibitor lowered dexamethasone- and hydrocortisone-induced MMP-13 activity by 39% and 83%, respectively, in the *in vitro* zymography assays.

In summary, these data demonstrate that MMP-13 expression is critical for normal zebrafish embryonic development. These data also suggest that the developing zebrafish is an appropriate model for examining glucocorticoid-induced teratogenesis. The suitability of zebrafish as a developmental model to study glucocorticoids stems not

only from their fast gestation period, but also from the fact that they lend themselves well to the utilization of a wide array of molecular techniques. Further, this model system closely mimics the development of other species such as mice and humans, suggesting that zebrafish serve as a valuable tool for examining the effects elicited by exogenous steroids *in vivo*. This becomes especially important for glucocorticoid exposure, as the exact mechanism by which this class of compounds exerts its teratogenic effects is still unclear. These data show that dysregulation of MMP-13 at critical times in development has a significant impact on somitogenesis, organ development, and tissue architecture. Additionally, the regulation of this gene by glucocorticoids appears more complex than cell models suggest, indicating that examination of MMPs in a dynamic whole animal model will contribute to our understanding of their role in teratogenesis.

3.5. Figures

```
                              Signal Peptide
                          ──────────────────────────►◄────────
Zebrafish    1 MKTCFRLCVFITLLFSGHSSPIAPPAGGDQDTLAENYLTRLYGLPKPAQNPSPSGGEKRS
Mouse        1 .MHSAILATFFLLSWTPCWSLPLPYGDDDDDDLSEEDLVFAEHYLKSYYHPATLAGILKK
Human        1 .MHPGVLAAFLFLSWTHCRALPLPSGGDEDD.LSEEDLQFAERYLRSYYHPTNLAGILKE
Consensus    1        L  F  L      s   P  g d D L E  L          k  P    G

                                  Propeptide
                         ───────────────────────────────────►◄──────
Zebrafish   61 SDVSL...RLKEMQQFFKLKVSGKLDQETLEVMKKPRCGVPDIKAYSTFAGDYKWKKHQL
Mouse       60 STVTSTVDRLREMQSFFGLEVTGKLDDPTLDIMRKPRCGVPDVGEYNVFPRTLKWSQTNL
Human       59 NAASSMTERLREMQSFFGLEVTGKLDDNTLDVMKKPRCGVPDVGEYNVFPRTLKWSKMNL
Consensus   61 s vs       RL EMQ FF L V GKLD  TL vMkKPRCGVPD   Y F    KW k  L

                                Catalytic Domain
                         ────────────────────────────────────────────
Zebrafish  118 TYRIGNYTPDMSVAEVDDSISKALKVWADVTPLRFTRIYSGTADIMISFATGDHRDGYPF
Mouse      120 TYRIVNYTPDMSHSEVEKAFRKAFKVWSDVTPLNFTRIYDGTADIMISFGTKEHGDFYPF
Human      119 TYRIVNYTPDMTHSEVEKAFKKAFKVWSDVTPLNFTRLHDGTADIMISFGTKEHGDFYPF
Consensus  121 TYRI NYTPDMs  EV    KA KVW DVTPL FTR y G ADIMISF t  H D YPF

                                               ◄- - - - - - - - - - - - - -
Zebrafish  178 DGPNGFLAHAFPPFEGIGGDAHFDDDETFSYRSPQYYNLFLVAAHEFGHSLGLEHSQDPG
Mouse      180 DGPSGLLAHAFPPGPNYGGDAHFDDDETWT.SSSKGYNLFIVAAHELGHSLGLDHSKDPG
Human      179 DGPSGLLAHAFPPGPNYGGDAHFDDDETWT.SSSKGYNLFLVAAHEFGHSLGLDHSKDPG
Consensus  181 DGP G LAHAFPP   GGDAHFDDDET   S   YNLFlVAAHEfGHSLGL HS DPG

                ◄- - - - - - - - - - - ►            Hinge Region
                                          ─────────────────────────►◄────
Zebrafish  238 ALMYPTYVYRDVDRFVLPRDDVNGIQSLYGPNTDVNTDDSKPTPPVTPNTCDPNLVLDAV
Mouse      239 ALMFPIYTYTGKSHFMLPDDDVQGIQFLYGPGDE....DPNPKHPKTPEKCDPALSLDAI
Human      238 ALMFPIYTYTGKSHFMLPDDDVQGIQSLYGPGDE....DPNPKHPKTPDKCDPSLSLDAI
Consensus  241 ALM P Y Y    F LP DDV GIQsLYGP        D  P  P TP  CDP L LDA

Zebrafish  298 TMLRGEIMFFKNSFFWRSYPQSADVEQQLIKSFWPEAPDNIDAAFESVMQDKVFLIKGEK
Mouse      295 TSLRGETMIFKDRFFWRLHPQQVEAELFLTKSFWPELPNHVDAAYEHPSRDLMFIFRGRK
Human      294 TSLRGETMIFKDRFFWRLHPQQVDAELFLTKSFWPELPNRIDAAYEHPSHDLIFIFRGRK
Consensus  301 T LRGE M FK  FFWR  PQ  d E  L KSFWPE P  iDAA E    D F   G K

                                Hemopexin Domain
                         ───────────────────────────────────────────────
Zebrafish  358 VWALYGYDMVQGYPKSLSMFRLPKNVRKIDAVLYKEDSNSILFFANNQVYSYNEGIRQMD
Mouse      355 FWALNGYDILEGYPRKISDLGFPKEVKRLSAAVHFENTGKTLFFSENHVWSYDDVNQTMD
Human      354 FWALNGYDILEGYPKKISELGLPKEVKKISAAVHFEDTGKTLLFSGNQVWRYDDTNHIMD
Consensus  361  WAL GYD  GYPk  S   lPK V ki A    Ed    LfF  NqV sY       MD

                         ─────────────────────────────────────────────────►
Zebrafish  418 KGFPKPVEEVFPGMTGKVTAAFQYRGLNYLFSGSKMLEFGS.NNRLFRVLNNNYFLPCK
Mouse      415 KDYPRLIEEEFPGIGNKVDAVYEKNGYIYFFNGPIQFEYSIWSNRIVRVMPTNSILWC.
Human      414 KDYPRLIEEDFPGIGDKVDAVYEKNGYIYFFNGPIQFEYSIWSNRIVRVMPANSILWC.
Consensus  421 K  P   EE FPG   KV A    G  Y F G   E      NR  RV  N  L C
```

Figure 3.1. Protein sequence alignment of zebrafish, mouse, and human MMP-13 demonstrates high identity. Multiple sequence alignment was performed using MultAlin and alignment output was generated using Boxshade v.3.21. Black boxes signify identical residues within a column consensus and gray boxes signify similar residues within a column consensus. In the consensus row, capital letters indicate sequence identity between all three species and lowercase letters indicate sequence identity between zebrafish and either mouse or human. Domains determined using the NCBI Protein-Protein BLAST network service are given above the sequence and are delineated by solid arrows. The zinc binding site is delineated by a dashed arrow.
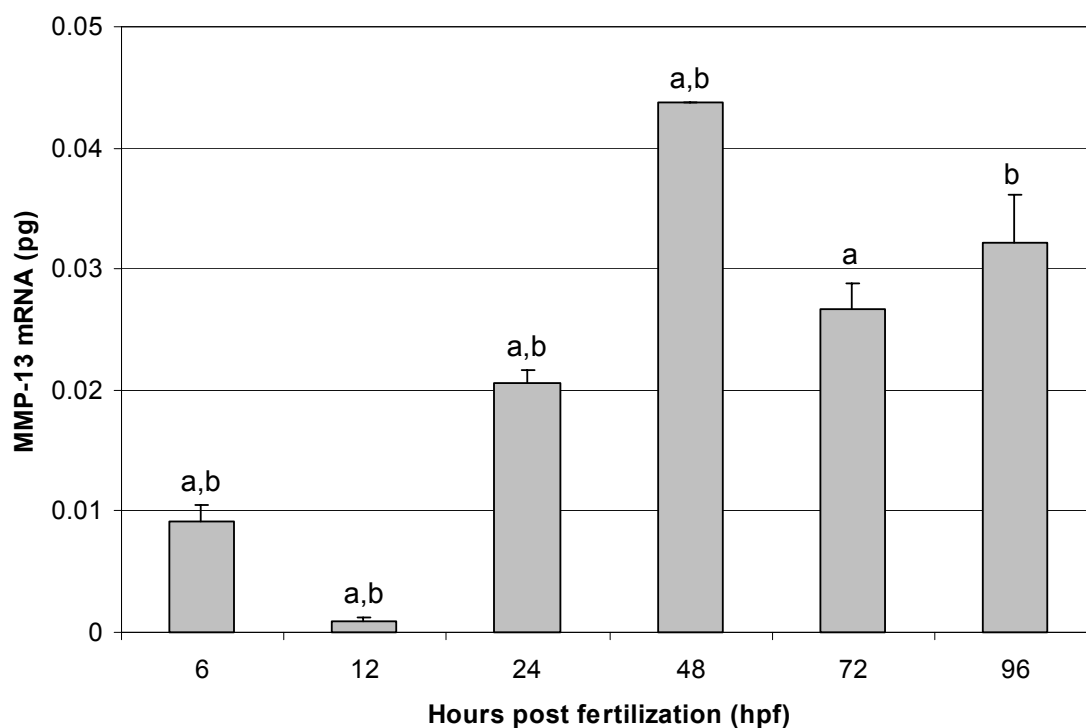
Figure 3.2. MMP-13 mRNA expression is regulated through 96 hpf. Total RNA was isolated from developing zebrafish at 6, 12, 24, 48, 72 and 96 hpf and used as template for quantitative RT-PCR using primers specific for zebrafish MMP-13 and 28S rRNA. Data were quantified by utilizing a standard curve generated from specific PCR products at concentrations ranging from 1 ng to 500 fg. Data were normalized to 28S rRNA. PCR reactions were performed in triplicate and data are representative of three separate experiments. Error bars denote standard deviation. Kruskal-Wallis One-way Analysis of Variance (ANOVA) on Ranks was used to compare changing MMP-13 mRNA levels since equal variance was not achieved. Bars labeled with the same letter (a, b) are statistically different ($p < 0.05$).

	1	2	3	4	
		▬	▬	▬	← 48 kDa
MMP-13 cDNA	–	+	+	+	
Control-MO	–	–	+	–	
MMP13-MO	–	–	–	+	
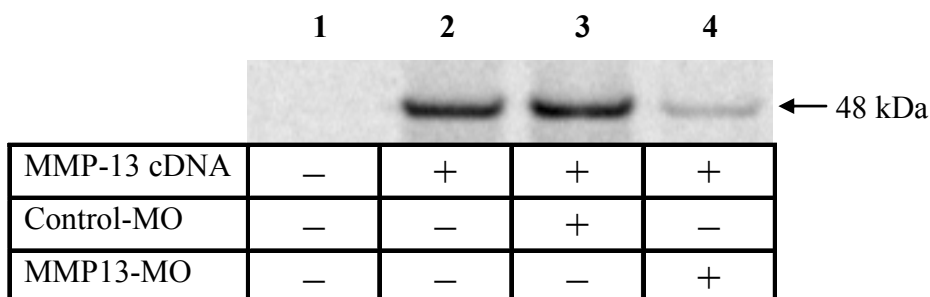
Figure 3.3. MMP13-MO is effective in preventing protein translation. A coupled *in vitro* transcription / translation reaction in the presence of [^{35}S]methionine was utilized to demonstrate the effectiveness of the MMP13-MO at blocking MMP-13 protein translation. The reactions were performed as follows: in the absence of MMP-13 cDNA and morpholino (**lane 1**), in the absence of morpholino (**lane 2**), in the presence of 10 μM control-MO (**lane 3**), and in the presence of 10 μM MMP13-MO (**lane 4**). Given the addition of MMP13-MO resulted in a decrease in the 48 kDa protein corresponding to MMP-13 active enzyme, it appears this morpholino is capable of blocking translation *in vitro*.
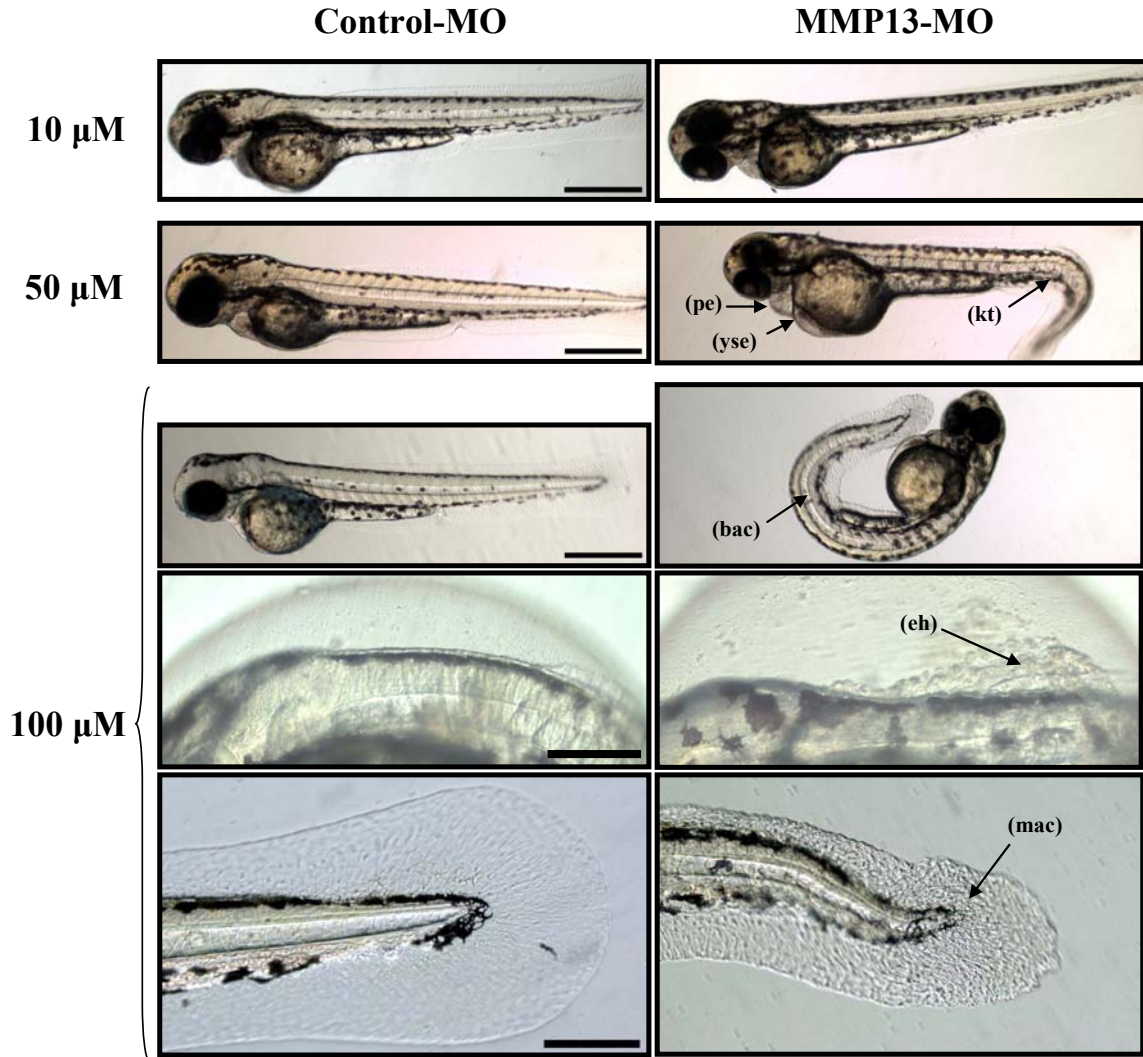
Figure 3.4. MMP-13 expression is required for normal zebrafish development. Microinjection of an MMP-13 antisense MO into 1- to 2-cell embryos was used to knockdown MMP-13 gene expression as a means of elucidating its role in embryogenesis. Control-MO injected embryos develop normally through 72 hpf and exhibit normal body structure, tailfin development and cellular integrity. Embryos injected with 50 μM or 100 μM MMP13-MO experience aberrant phenotypes at <10% or >90% incidence, respectively. These include pericardial edema **(pe)**, yolk sac edema **(yse)**, body axis curvature **(bac)**, kinked tail **(kt)**, malformation of tailfin actinotrichia **(mac)**, and epithelial hyperplasia **(eh)**, among others. Magnification: 40x (scale bar = 500 μm) or 100x (scale bar = 250 μm).
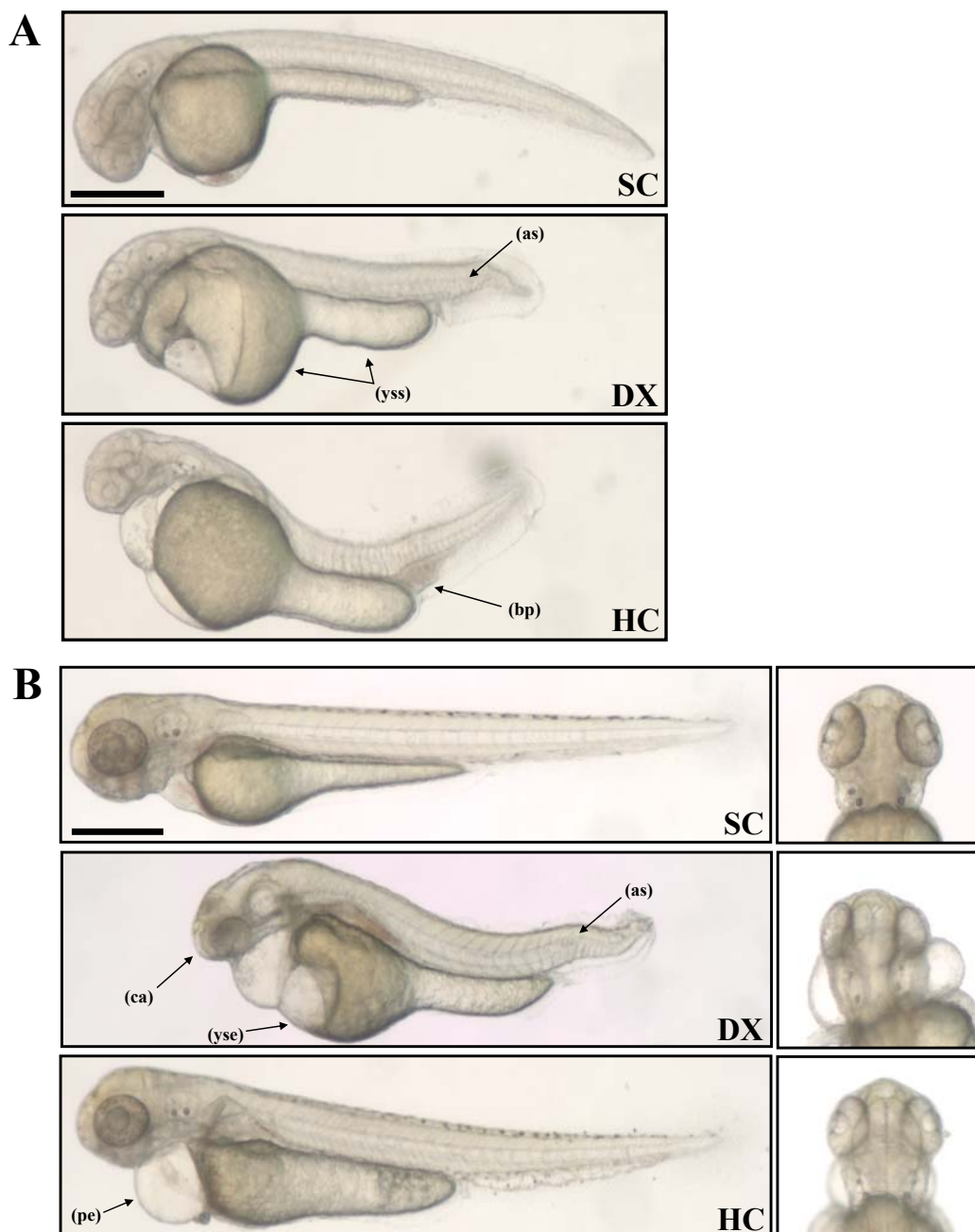
Figure 3.5. Exposure to hydrocortisone and dexamethasone results in abnormal zebrafish development. Representative photographs of embryos treated with 100 mg/L dexamethasone or hydrocortisone for **(A)** 48 h or **(B)** 72 h (whole body lateral views and dorsal head views). Dorsal views of the head are included to demonstrate changes in the craniofacial region. Changes in morphology include body axis curvature, alterations in yolk sac / yolk sac extension shape and size **(yss)**, craniofacial abnormalities **(ca)**, altered somitogenesis **(as)** and truncated body axis. Lesions included pericardial edema **(pe)**, yolk sac edema **(yse)**, and localized blood pooling **(bp)** in the head and yolk sac extension. Note: DX – dexamethasone, HC – hydrocortisone, SC – solvent control (0.2% DMF). Magnification: 40x (scale bar = 500 μm).

Figure 3.6. MMP-13 mRNA expression is increased by exposure to glucocorticoids.
Zebrafish embryos were exposed to 0.2% DMF or 100 mg/L dexamethasone or
hydrocortisone for 24, 48, or 72 hours, and RNA was isolated for use as template in
quantitative RT-PCR. Data were quantified by utilizing a standard curve generated from
specific PCR products at concentrations ranging from 1 ng to 500 fg. Data were
normalized to β-Actin. PCR reactions were performed in triplicate and data are
representative of three separate experiments. Error bars denote standard deviation. A
statistically significant increase in MMP-13 expression in glucocorticoid-treated embryos
is observed at 24 and 72 hpf as determined by an unpaired Student's *t*-test (* $p < 0.05$).
A statistically significant difference in MMP-13 mRNA levels between dexamethasone-
treated and hydrocortisone-treated embryos is seen at 72 hpf only (# $p < 0.05$). Note: DX
– dexamethasone, HC – hydrocortisone, SC – solvent control (0.2% DMF).
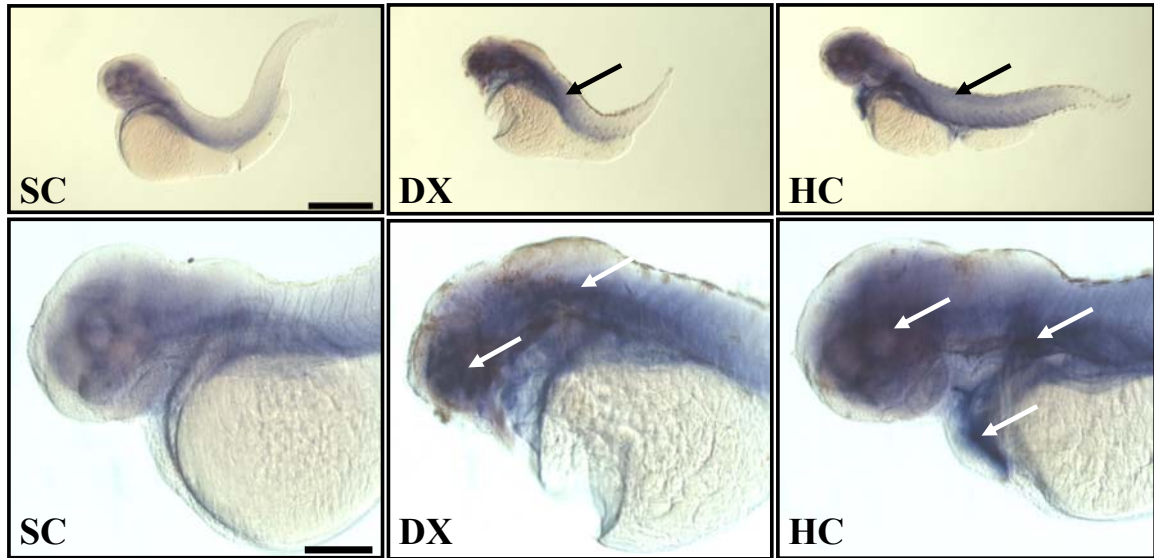
Figure 3.7. ***In situ*** **analysis of MMP-13 localization at 72 hpf confirms increased mRNA expression.** MMP-13 expression following exposure to hydrocortisone and dexamethasone was localized using *in situ* hybridization. Black arrows indicate general areas of the embryo trunk with increased expression and white arrows indicate specific organs with increased expression. Note: DX – dexamethasone, HC – hydrocortisone, SC – solvent control (0.2% DMF). Magnification: 40x (scale bar = 500 μm) or 100x (scale bar = 250 μm).
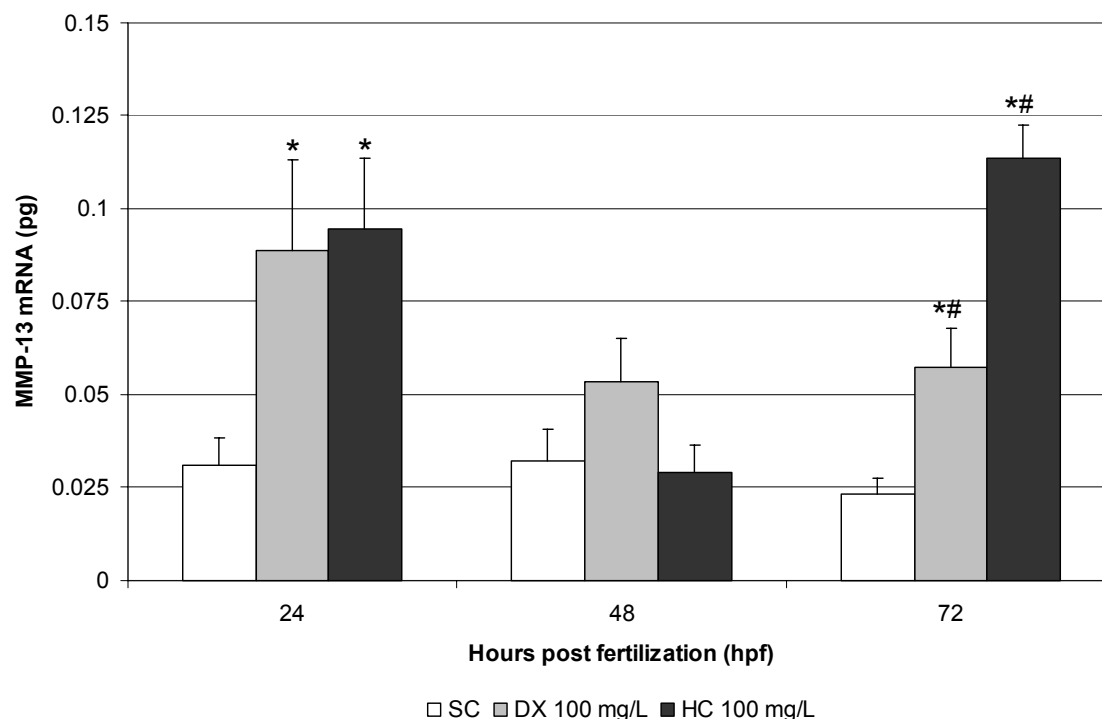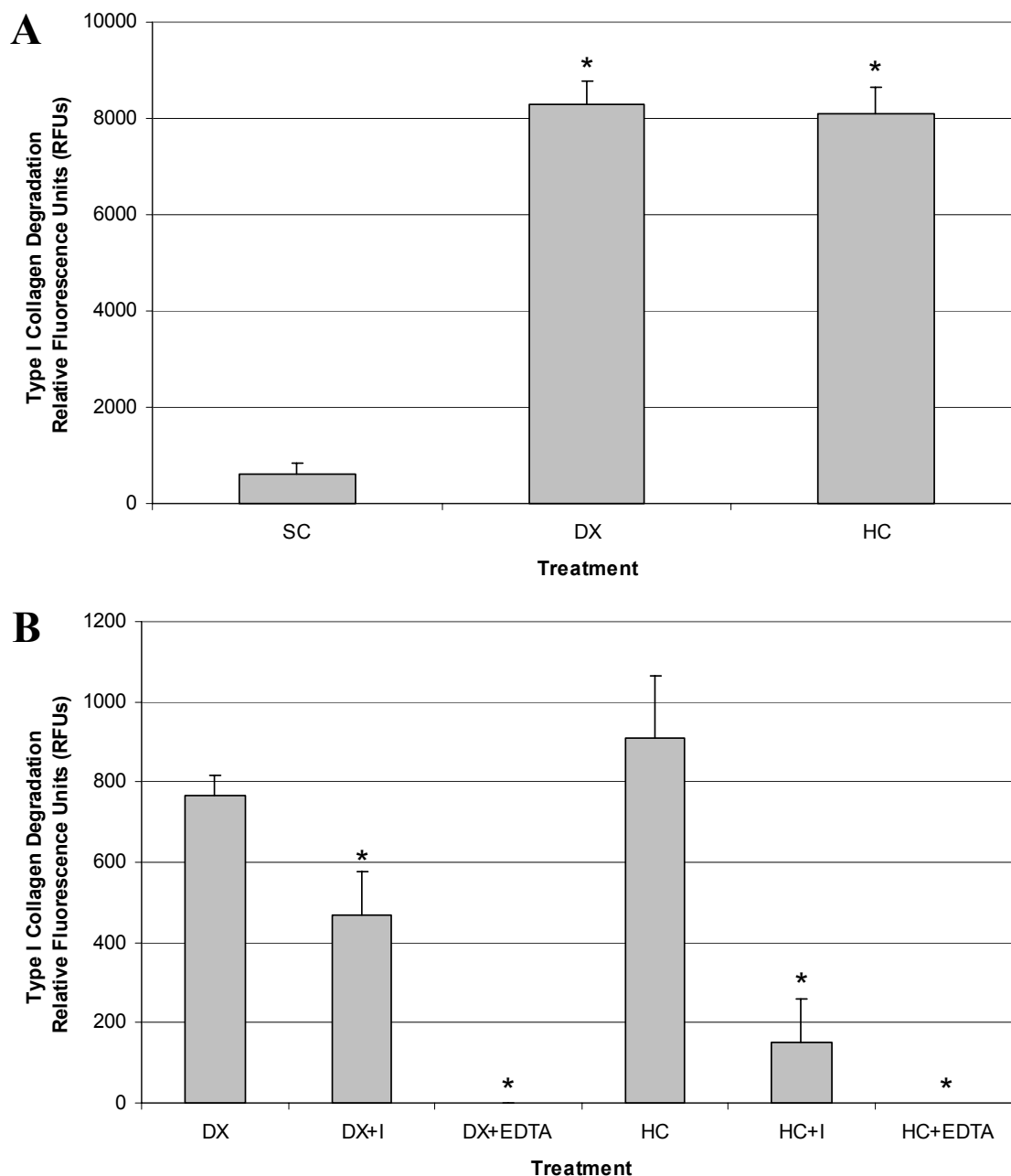
Figure 3.8. Exposure to glucocorticoids increases collagenase activity. Results of *in vitro* zymography assays conducted on zebrafish embryo lysates. **(A)** Lysates isolated from embryos treated with 100 mg/L dexamethasone (DX) or hydrocortisone (HC) through 72 hpf result in significant increases in type I collagen degradation over the solvent control (SC). **(B)** MMP-9/MMP-13 Inhibitor I (10 μM) effectively inhibits the degradation of type I collagen in treatment lysates while EDTA completely eliminates lysate activity, suggesting the activity observed can be attributed, at least in part, to MMP-13. Error bars denote standard deviation. Statistical significance was determined via an unpaired Student's *t*-test (* $p < 0.05$). Note: DX – dexamethasone, HC – hydrocortisone, I – MMP-9/MMP-13 Inhibitor I.

3.6. Tables

Table 3.1. Quantification of glucocorticoid-induced phenotypes demonstrates changes in morphology.

Parameter	Solvent Control 0.2% DMF	Dexamethasone 100 mg/L	Hydrocortisone 100 mg/L
(A) Body length	3.02±0.12	2.04±0.19*	3.05±0.12
(B) Interocular distance	0.12±0.01	0.15±0.02*	0.13±0.01
(C) Head length	0.36±0.01	0.24±0.03*	0.35±0.02
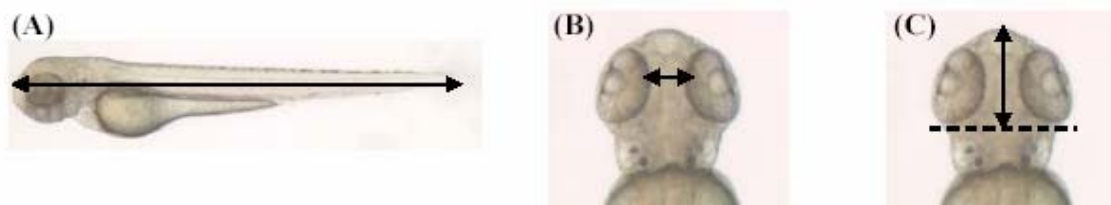
Note: Six 72 hpf embryos were measured per treatment. All measurements are in millimeters (mm). Comparison between the solvent control and treatment groups for each parameter was conducted via an unpaired Student's *t*-test. A statistically significant (* $p < 0.05$) decrease in body / head length and increase in interocular distance is observed for dexamethasone-treated embryos only.

4.0. THE ROLE OF MATRIX METALLOPROTEINASE EXPRESSION IN ZEBRAFISH (*DANIO RERIO*) CRANIOFACIAL DEVELOPMENT

4.1. Abstract

MMPs play a pivotal role in development due to their ability to remodel the extracellular matrix, a function necessary for proper cellular migration and tissue morphogenesis. Knockout mouse models suggest that these enzymes are required for normal embryogenesis since these animals show appreciable skeletal defects. Zebrafish (*Danio rerio*) serve as an ideal model system to study the molecular signaling occurring during craniofacial morphogenesis given the ease with which targeted gene knockdown can be accomplished using antisense MOs. The studies presented here showed that MMP-2 and MMP-9 expression was differentially regulated during embryogenesis, and that gene transcripts were more prevalent rostrally. Morpholino knockdown of MMP-2 resulted in deleterious alterations in phenotype related to axis formation, fin development and craniofacial development and was dose dependant. In contrast, morpholino knockdown of MMP-9 did not result in any gross morphological changes, suggesting that MMP-2 expression and activity may compensate for loss of MMP-9, as observed in murine models. To examine this, zebrafish embryos were co-injected with MMP-2 and MMP-9 morpholinos at concentrations known to produce no phenotype changes individually, and the resulting embryos exhibited severe morphant dysmorphogenesis, suggesting that MMP-2 may compensate for loss of MMP-9 function. Double knockdown morphants exhibited abnormal phenotypes similar to those observed in MMP-2 morphants. Focusing on the craniofacial region, it was shown that morpholino

knockdown of MMP-2, MMP-9 and MMP-13 resulted in a number of changes in cartilage formation and migration, including quantifiable alterations in both the mandibular and hyoid arches concurrently. These studies demonstrate that appropriate expression of MMP-2, MMP-9, and MMP-13 is necessary for proper zebrafish craniofacial morphogenesis. This work represents the first time craniofacial patterning following MMP knockdown has been studied in the zebrafish, and provides valuable insights into the role MMPs play in this process.

4.2. Introduction

The MMPs are a family of zinc-dependent endopeptidases that cleave extracellular matrix ECM components, cellular receptors, and non-matrix proteins such as growth factors and cytokines (reviewed in (Brinckerhoff and Matrisian 2002; Chakraborti *et al.* 2003; McCawley and Matrisian 2001; Visse and Nagase 2003)). These metalloenzymes play a vital role in a number of physiological and pathological processes. Central among these is development, where MMPs participate in events related to angiogenesis, cellular migration and aggregation, and apoptosis (Lemaitre and D'Armiento 2006; Stamenkovic 2003). The importance of MMPs in development has been demonstrated via the creation of knockout mice, which display defects related mainly to altered skeletogenesis. MMP-2 (gelatinase A) null mice exhibit abnormal long bone and craniofacial development, progressive loss of bone mineral density (with associated decreases in osteoblast and osteoclast numbers), and articular cartilage destruction (Itoh *et al.* 1997; Mosig *et al.* 2007). MMP-9 (gelatinase B) deficient mice display an abnormal pattern of skeletal growth plate vascularization and ossification

resulting in progressive lengthening of the growth plate (Vu *et al.* 1998). MMP-13 (collagenase 3) knockout mice have increases in tibial and femoral trabecular bone density, defects in growth plate cartilage and primary ossification centers, and delayed endochondral ossification (Inada *et al.* 2004; Stickens *et al.* 2004). MT1-MMP-deficient mice possess defects in endochondral and intramembranous bone formation resulting in craniofacial dysmorphia, osteopenia, arthritis, dwarfism, soft-tissue fibrosis, and eventual death within a few weeks of birth (Holmbeck *et al.* 1999; Zhou *et al.* 2000).

Several MMP homologs have been identified and characterized in zebrafish, including MMP-13 (see studies described above in Section 3.0), MMP-2, MMP-9, two isoforms of a novel membrane-type MMP (MT-MMP α and β), and a tissue inhibitor of metalloproteinase 2 (TIMP-2) (Yoong *et al.* 2007; Zhang *et al.* 2003a; Zhang *et al.* 2003b, c). Targeted gene knockdown using antisense MOs demonstrates that MMP-2, MT-MMP α and β, MMP-13, and TIMP-2 are all required for normal development since blocking translation of these genes results in aberrant phenotypes in embryos consisting of a truncated anterior-posterior axis, body axis curvature, and craniofacial defects, among others (Zhang *et al.* 2003a; Zhang *et al.* 2003b, c).

The purpose of these studies is to demonstrate that the expression of the gelatinases MMP-2 and MMP-9 and the collagenase MMP-13 are required for proper zebrafish craniofacial patterning. Craniofacial development in vertebrate embryos involves coordinated cell movement and communication between the neural crest, mesodermal mesenchyme, and surrounding epithelia (reviewed in (Le Douarin *et al.* 1994)). These processes have previously been characterized in mouse (Chai and Maxson 2006; Hall 1980; Trainor and Tam 1995) and avian models (Couly and Le Douarin 1990;

Noden 1983, 1988), while craniofacial development studies in zebrafish have emerged more recently (Kimmel *et al.* 1998; Kimmel *et al.* 2001; Neuhauss *et al.* 1996; Piotrowski *et al.* 1996; Schilling *et al.* 1996; Yelick and Schilling 2002). The results presented here demonstrate that MMP-2 and MMP-9 expression is regulated during embryogenesis and higher levels of expression occur rostrally. MMP-2 knockdown resulted in altered axis formation, fin development and craniofacial development and was dose dependant. However, morpholino knockdown of MMP-9 did not result in any gross morphological changes. These data suggest that, in the developing zebrafish, MMP-2 activity may be compensating for loss of MMP-9. To examine this, zebrafish embryos were co-injected with both MMP-2 and MMP-9 morpholinos at concentrations known to produce no phenotype changes individually. The resulting embryos exhibited severe dysmorphogenesis, suggesting that MMP-2 may compensate for loss of MMP-9 function. Further, knockdown of MMP-2, MMP-9, and MMP-13 resulted in changes in cartilage formation and migration, including quantifiable alterations in both the mandibular arch (lower jaw length) and hyoid arch (ceratohyal cartilage length), indicating that appropriate expression of these enzymes is critical for normal craniofacial patterning.

4.3. Results

4.3.1. Developing zebrafish exhibit varying MMP-2 and MMP-9 expression levels

To determine the expression of MMP-2 and MMP-9 during zebrafish embryogenesis, MMP-2 (**Figure 4.1A**) and MMP-9 (**Figure 4.1B**) mRNA levels were analyzed using quantitative RT-PCR at 6, 12, 24, 48, 72 and 96 hpf. PCR data were normalized to 28S rRNA since β-Actin has been shown to change during zebrafish embryogenesis (Sawai and Campos-Ortega 1997). Expression of MMP-2 is minimal at 6

hpf, and then gradually increases through 96 hpf, the early larval period. The only time points that are not statistically different ($p < 0.05$) from one another are 6 hpf versus 12 hpf, and 48 hpf versus 72 hpf. MMP-9 expression differs from that of MMP-2. Specifically, levels decrease between 6 hpf and 12 hpf, corresponding to the shield stage and the beginning of the segmentation period, respectively. MMP-9 transcript levels subsequently increase through 48 hpf, the long-pec stage, where expression is highest among the developmental time points examined. This high level of expression is followed by a significant decrease at 72 hpf and a recovery at 96 hpf (the protruding-mouth stage and early larval period, respectively). The 6 hpf expression level is not significantly different ($p < 0.05$) from those measured at 24, 48 or 96 hpf. Similarly, the amount of MMP-9 transcript measured at 48 hpf is not significantly different ($p < 0.05$) from that measured at 96 hpf.

In situ hybridization of embryos at 72 hpf using a MMP-2-specific mRNA probe (**Figures 4.2A**, and **4.2B**) reveals that transcript is expressed throughout the entire embryo, with the exception of the yolk-sac and yolk-sac extension. Higher levels of expression appear to be localized rostrally (**Figure 4.2B**) in the craniofacial region and anterior kidney. Distinct lines of expression are seen in the neurocranial cartilage and midbrain. Similarly, higher levels of MMP-9 expression (**Figure 4.2D**) are located rostrally in the craniofacial region (neurocranial cartilage), anterior kidney, heart, and midbrain. However, MMP-9 transcript also appears to be localized to the medial trunk of the embryo, superior to the yolk-sac extension (**Figure 4.2C**).

4.3.2. Morpholino knockdown of MMP-2 causes developmental abnormalities and co-injection of gelatinase morpholinos demonstrates the ability of MMP-2 to compensate for loss of MMP-9 function

To determine whether expression of MMP-2 and MMP-9 are necessary for normal zebrafish craniofacial development, sequence-specific morpholinos targeting these genes were utilized (Nasevicius and Ekker 2000). To demonstrate the effectiveness of the MMP morpholinos to block MMP-2 and MMP-9 protein expression, coupled *in vitro* transcription / translation reactions were performed prior to microinjection. A control reaction, which contained neither MMP-2 or MMP-9 cDNA nor morpholino, was included in each set of reactions to ensure that the rabbit reticulocyte system did not produce aberrant protein bands. The MMP-2 control reaction and the MMP-9 control reaction do not produce a band (**Figure 4.3A and 4.3B, lane 1**). The reactions containing MMP-2 or MMP-9 cDNA in the absence of morpholino produce 66 or 86 kDa proteins, respectively, corresponding to the active enzymes (**Figure 4.3A and 4.3B, lane 2**). Addition of Control-MO does not affect protein production (**Figure 4.3A and 4.3B, lane 3**). However, addition of either the MMP2-MO or MMP9-MO decreases protein production (**Figure 4.3A and 4.3B, lane 4**), demonstrating that the morpholinos utilized in these experiments are capable of blocking *in vitro* MMP-2 or MMP-9 translation, respectively.

Previous data demonstrate that MMP-2 is necessary for normal zebrafish embryogenesis by showing that injection of a MMP-2 antisense MO consistently results in a truncated anterior-posterior axis when monitored through 72 hpf (Zhang *et al.* 2003b). To confirm these findings in the zebrafish strain (AB) used by this laboratory,

these injections were repeated, in addition to using morpholinos specific to MMP-9. Morpholinos were injected at concentrations ranging from 50 μM to 200 μM to establish a dose-response, with 30-50 embryos examined per concentration. Embryos injected with 50 μM MMP2-MO exhibit no gross morphological changes and appear identical to those injected with Control-MO (**Figure 4.4A**). Increasing the morpholino concentration causes a dose-dependent increase in mortality, with 200 μM MMP2-MO resulting in the death of 80% of embryos injected. Surviving morphants injected with 200 μM MMP-2 morpholino display truncated axes, pericardial edemas, stunted fin development, and altered craniofacial development including stunted eye development. MMP-2 morphants injected at 100 μM possess similar lesions, although the severity of these lesions is noticeably less. An additional change noted in these embryos is modified tailfin actinotrichia. Injection of up to 200 μM MMP9-MO does not result in a decrease in embryo viability, as demonstrated by low mortality and gross abnormalities in morphants at all concentrations tested. The morphological changes observed in MMP-9 morphants are generally mild and consist of slight pericardial edema and slight modification of tailfin actinotrichia.

In mammalian models, MMP-2 and MMP-9 can compensate for one another's expression (Agrawal *et al.* 2006; Lambert *et al.* 2003; Vu *et al.* 1998). To determine whether MMP-2 expression is compensating for the loss of MMP-9 expression in the zebrafish, concentrations of MMP2-MO and MMP9-MO ranging from 50 μM to 200 μM were co-injected into 1-2 cell embryos and monitored through 72 hpf. A dose-response relationship in terms of both mortality and severity of morphant abnormalities is apparent **(Figure 4.4B)**. Co-injection of 50 μM MMP2-MO and 50 μM MMP9-MO results in

only 2% mortality and abnormalities in 16% of the embryos injected (consisting mainly of modified tailfin actinotrichia). Maintaining the dose of MMP2-MO at 50 μM and increasing the dose of MMP9-MO to 100 μM results in only a slight increase in mortality (13%), but an appreciable increase in abnormalities observed (65%). The types of morphological changes observed expand to include pericardial and yolk-sac edemas, localized hemorrhaging, truncated axes, altered craniofacial development, and stunted pectoral fin growth. Modified tailfin actinotrichia continue to be the most common morphological trait observed, although the changes occurring here are noticeably more severe. Doubling the MMP9-MO concentration injected (200 μM) results in both modification of the tailfin actinotrichia and tail kinking. Doubling the MMP2-MO concentration (100 μM) and maintaining the MMP9-MO concentration (200 μM) causes a precipitous increase in mortality (92%). Of the surviving morphants, 67% exhibit a complete loss of structural organization and epithelial hyperplasia. Embryos injected with 300 μM Control-MO, corresponding to the highest combined concentration of MMP-2 and MMP-9 morpholinos, experience no deaths and only 4% of morphants display slight changes in morphology (data not shown). These data suggest that MMP-2 is capable of compensating for loss of function experienced by developing embryos following knockdown of MMP-9. Since co-injection of 50 μM MMP2-MO and 200 μM MMP9-MO produces the highest percentage of abnormalities with the lowest percentage of deaths, it was chosen as the concentration to be used for the morphometrics study.

4.3.3. Embryos co-injected with MMP2-MO and MMP9-MO exhibit altered craniofacial morphogenesis

Studies have demonstrated that MMP-2 knockout mice have abnormal craniofacial development as well as a number of other skeletal defects (Itoh *et al.* 1997; Mosig *et al.* 2007). Zebrafish MMP-2 morphants have also been shown to possess severe developmental abnormalities, although craniofacial defects were not reported (Zhang *et al.* 2003b). Further, MMP-9 knockout mice exhibit an atypical pattern of skeletal growth plate vascularization and ossification (Vu *et al.* 1998). To characterize the craniofacial changes observed following MMP-2/MMP-9 double knockdown, morphants (72 hpf and 96 hpf) were stained with Alcian blue following injection with MMP2-MO (50 μM) and/or MMP9-MO (200 μM). Following staining procedures, morphometric measurements were carried out on the craniofacial cartilage of 96 hpf embryos. These consisted of measuring intercranial distance (ID), lower jaw length (LJL), and ceratohyal cartilage length (CCL). Intercranial distance measurements were performed by measuring the distance between the medial edge of each eye. Lower jaw length and ceratohyal cartilage length measurements were performed by measuring the distance from a reference line drawn between the posterior edges of the hyosymplectic cartilage to the anterior edge of the Meckel's cartilage and ceratohyal cartilage, respectively.

At 72 hpf, Alcian blue staining reveals a partially developed craniofacial cartilage architecture (**Figure 4.5A**), the most obvious components of which are the neurocranial cartilages (ethmoid plate, trabecula, and parachordal cartilage) and several of the pharyngeal cartilages (hyosymplectic, palatoquadrate, ceratohyal, and Meckel's cartilage). Embryos injected with Control-MO, MMP2-MO, or MMP9-MO individually

show no difference in the formation of either the neurocranial or pharyngeal cartilages. However, embryos co-injected with the MMP2-MO and MMP9-MO exhibit stunted growth of all cartilage types. The only structure readily visible in the MMP-2/MMP-9 morphants is the trabecula.

Alcian blue staining of 96 hpf morphants demonstrates a more well developed cartilaginous head skeleton (**Figure 4.5B**). The repetitive fragments of the ceratobranchial cartilage, which eventually form the gill arches in mature zebrafish (Schilling *et al.* 1996), are now visible. Visual observation reveals no difference between the morphants injected with Control-MO, MMP2-MO, or MMP9-MO individually. Further, morphometric measurement of intercranial distance, lower jaw length, or ceratohyal cartilage length reveals no significant ($p < 0.05$) differences. Embryos co-injected with MMP2-MO and MMP9-MO show evidence of underdeveloped cartilage formation. Specifically, the lateral fragments of ceratohyal cartilage fail to fuse entirely and the ceratobranchial cartilage is essentially non-existent. Further, the anterior migration and growth of the lower jaw is impaired. This is supported by the fact that morphometric measurement of these morphants show a significant ($p < 0.05$) decrease in both the lower jaw length and ceratohyal cartilage length (**Figure 4.5C**).

4.3.4. Embryos injected with MMP13-MO exhibit altered craniofacial morphogenesis

Collagenases play a central role in cranial morphogenesis (Holmbeck 2005), and reports indicate that MMP-13 is necessary for normal development in mice (Inada *et al.* 2004; Stickens *et al.* 2004) and zebrafish (see studies described above in Section 3.0). Therefore, the effect of loss of MMP-13 gene expression on zebrafish craniofacial patterning using the MMP13-MO described previously was also characterized. At 72

hpf, embryos injected with 100 μM MMP13-MO display delayed cartilage formation since there is no cartilage observable following Alcian blue staining of these morphants (**Figure 4.6A**). The overall head size of MMP-13 morphants is also decreased. At 96 hpf, this trend continues, with MMP-13 morphants exhibiting underdeveloped cartilage formation (**Figure 4.6B**). Apparent differences between the MMP-13 morphants and the embryos injected with Control-MO (100 μM) include misshaped lower jaw and ceratohyal cartilages, as well as a complete absence of ceratobranchial cartilage in MMP-13 morphants. This change in pharyngeal cartilage "shape" is potentially the result of altered cartilage growth and migration since morphometric measurements reveal that MMP-13 morphants have both a significantly ($p < 0.05$) smaller lower jaw length and ceratohyal cartilage length compared to embryos injected with Control-MO (**Figure 4.6C**). Ventral views of MMP-13 morphants also reveal changes in trabecula/ethmoid plate shape and an apparent absence of hyosymplectic cartilage.

4.4. Discussion

The need for appropriate MMP expression and function during zebrafish embryogenesis has been demonstrated for several metalloproteinases including MMP-13 (see studies described above in Section 3.0) MMP-2, and MT-MMP α and β, (Zhang *et al.* 2003b, c). Blocking translation of these genes resulted in abnormal phenotypes ranging from whole-body effects such as truncated or curved body axis, to localized lesions such as pericardial and yolk-sac edema, blood pooling, and malformed tailfin actinotrichia. The results provided above support the need for proper expression of the gelatinases MMP-2 and MMP-9 and suggest that MMP-2 may be able to compensate for

loss of MMP-9 function. Examination of craniofacial patterning following knockdown of these gelatinases, as well as the collagenase MMP-13, reveal that these MMPs also play a critical role in the proper migration and growth of craniofacial cartilages.

Similar to the qualitative pattern of MMP-2 expression described previously by (Zhang *et al.* 2003b), quantitative RT-PCR data presented above showed MMP-2 expression levels gradually increasing from 6 hpf (shield stage) to 96 hpf (early larval period). These data suggest a fundamental role for MMP-2 in late embryonic/early larval development, during which time the pectoral fin, neurocranium, and pharyngeal arches are undergoing extensive chondrification and growth (Hall and Miyake 1992; Kimmel *et al.* 1998; Schilling and Kimmel 1997). This is supported by previously published data showing morpholino blockage of MMP-2 translation only effects development following gastrulation, indicated by non-responsiveness of several axis specification markers at 10 hpf (Zhang *et al.* 2003b).   Further, type IV collagen (a gelatinase substrate) degradation has been demonstrated to increase with zebrafish embryo age as measured via *in vitro* zymography (Crawford and Pilgrim 2005). In terms of MMP-9, recent data show that MMP-9 transcript was detected via qualitative RT-PCR in unfertilized eggs, thus indicating maternal origin, and continued to be expressed at varying levels through 7 dpf (Yoong *et al.* 2007).   When monitored via quantitative RT-PCR, MMP-9 mRNA expression was found to differ from that of MMP-2. A sharp decrease in transcript levels from 6 hpf to 12 hpf suggest the degradation of maternal transcript and beginning of zygotic expression, respectively. MMP-9 transcript levels then increased through 48 hpf, the long-pec stage, where they reached a maximum among all time points measured. As the name implies, it is during this time that the pectoral bud becomes elongated and

chondrocytes initiate differentiation within the fin mesenchyme (Kimmel *et al.* 1995).

Chondrocyte differentiation within the pharyngeal arches is also occurring at 48 hpf (Hall

and Miyake 1992; Schilling and Kimmel 1997). From this point, there was a significant

decrease in transcript levels at 72 hpf, followed by an increase at 96 hpf to a level roughly

equivalent to the 48 hpf value. The pattern of expression observed for MMP-9 nearly

mimicked that found previously in this laboratory for the collagenase MMP-13 (see

studies described above in Section 3.0). It is likely MMP-13 plays a role in cartilage

development given both that procollagen is the primary protein synthesized in

chondrocytes and type II collagen comprises the majority of ECM cartilage (Lang *et al.*

2006). MMP-13 is capable of cleaving multiple types of interstitial collagens, including

type II, at a site three-fourths from the N-terminus (Visse and Nagase 2003). Although

type II collagen is not a specific substrate for MMP-9, other collagen types such as IV, V,

XI, and XIV, are substrates for this MMP (Chakraborti *et al.* 2003). Outside of substrate

specificity, it should be noted that MMP-13 has the capability of initiating the activation

of several MMPs, including MMP-9 (Chakraborti *et al.* 2003). Therefore, co-expression

of MMP-9 and MMP-13 in the developing zebrafish embryo may result in local

activation of MMP-9.

In situ hybridization demonstrated that high levels of MMP-2 and MMP-9

transcript were localized rostrally, although some expression was observed in the embryo

trunk as well. Distinct areas of expression were seen in the craniofacial region and

midbrain. The presence of these gelatinases in the craniofacial region may be associated

with the thin layers of matrix collagens and proteoglycans known to be synthesized and

secreted by mature chondrocytes (Schlombs *et al.* 2003). In terms of their presence in the

midbrain, accumulating evidence suggests the participation of gelatinases in a diverse array of developmental processes within the nervous system including neuron and glia differentiation, peripheral neuron outgrowth, and oligodendrocyte process extension and migration (Luo 2005). Comparisons of expression levels between the two gelatinases revealed that MMP-2 was more abundant, corresponding well with quantitative RT-PCR data showing approximately 40-fold greater amounts of MMP-2 transcript versus MMP-9 transcript at 72 hpf. The widespread expression of MMP-2 could potentially be explained by the fact that the promoter of this gene has several characteristics of a constitutive (housekeeping) promoter (Chakraborti *et al.* 2003; Huhtala *et al.* 1990). MMP-2 *in situ* data given here was consistent with that reported previously (Zhang *et al.* 2003b), which showed MMP-2 was expressed along the entire anterior-posterior axis at 24 hpf. The MMP-9 *in situ* data presented above diverged somewhat from previously reported data showing MMP-9 to be expressed in a population of circulating white blood cells that likely represented zebrafish heterophils (Yoong *et al.* 2007). However, similar to the expression pattern observed in the study described here, these MMP-9 positive cells were found to be concentrated in the head region of the embryo at 72 hpf. The disparity in staining pervasiveness could therefore represent either differences in staining / clearing methodologies or antisense probe design.

Morpholino knockdown data show that MMP-2 was required for normal zebrafish development and that a dose-response relating to the morpholino concentration injected can be achieved. Embryos injected with increasing concentrations of MMP-2 exhibited dose-dependent increases in both mortality and phenotype severity. The phenotype achieved utilizing 200 μM MMP2-MO mimicked that observed previously (Zhang *et al.*

2003b) including a severely truncated axis, pericardial edema, and altered craniofacial morphogenesis. Several phenotypes reported in MMP-2 null mice correspond with those observed in zebrafish MMP-2 morphants such as smaller stature and abnormal craniofacial development (Itoh *et al.* 1997; Mosig *et al.* 2007). Interestingly, the dose required to achieve this phenotype (~6.5 ng) was roughly 3.5 times less than the 24 ng implemented in a previously reported study (Zhang *et al.* 2003b). This could simply be due to strain differences in the zebrafish used for microinjection, which has been observed previously in this laboratory (data not shown).

Since injection of up to 200 μM MMP9-MO was well tolerated, with mortality and abnormality occurring in approximately 10% of morphants, it was proposed that knockdown of MMP-9 was not functionally significant because the actions of MMP-2 could potentially compensate for this loss. This possibility seemed probable given that the physiological function of these two gelatinases is similar (Visse and Nagase 2003). Further, quantitative RT-PCR and *in situ* hybridization data showing that the relative abundance of MMP-9 in the developing embryo is less than MMP-2 support this hypothesis. To test this hypothesis, various concentrations of these morpholinos ranging from 50 μM - 200 μM were co-injected, and a dose-response in terms of both mortality and phenotype severity was established. Concentrations of each gelatinase morpholino known to produce little or no change in phenotype, when co-injected, resulted in increased mortality and appreciable changes in morphology. Therefore, MMP-2 appears able to compensate for loss of MMP-9 function. A study examining development of MMP-9 null mice support this possibility, showing that changes in these animals was limited to a 10% shorter long bone length and alteration of endochondral bone formation

that resolved three weeks postnatally (Vu *et al.* 1998). It was proposed that this resolution was the result of upregulation of compensatory pathways. Similar approaches used in the morpholino studies described here (i.e. double knockout) have been adopted in mouse studies looking at choroidal neovascularization related to age-related macular degeneration (Lambert *et al.* 2003) and experimental autoimmune encephalomyelitis (Agrawal *et al.* 2006). Researchers found that both the incidence and severity of choroidal neovascularization were attenuated in MMP-2/MMP-9 double knockout mice compared to single gene-deficient mice or corresponding wild-type controls (Lambert *et al.* 2003). Similarly, MMP-2/MMP-9 double knockout mice were required to inhibit dystroglycan cleavage and prevent leukocyte infiltration, thereby conferring resistance to experimental autoimmune encephalomyelitis (Agrawal *et al.* 2006).

Dose-dependent alterations in axis formation, actinotrichia development, and craniofacial development were observed following co-injection of MMP-2 and MMP-9 morpholinos. Actinotrichia, present during the late embryonic/early larval period, are unsegmented collagenous fin rays that eventually form the segmented bony fin rays of adult zebrafish (Kimmel *et al.* 1995). A number of collagen types are known to exist in the fin rays (reviewed in (Mari-Beffa *et al.* 2007)), so modification of these structures following co-injection of MMP-2 and MMP-9 morpholinos implicates these MMPs in matrix remodeling during development or following injury. In fact, MT1-MMP and MMP-2 have been shown to play a central role during fin regeneration in zebrafish (Bai *et al.* 2005).

Given the role the ECM plays in neural crest cell migration (Henderson and Copp 1997; Perris 1997; Perris and Perissinotto 2000) and cartilage morphogenesis (Kimmel *et*

al. 1998; Kimmel *et al.* 2001), altering the function of enzymes responsible for ECM remodeling during embryogenesis could potentially result in changes in craniofacial patterning. Alcian blue staining revealed significant decreases in lower jaw length and ceratohyal cartilage length in MMP-2/MMP-9 double knockdown embryos. Differences in cartilage formation were discernable starting at 72 hpf, suggesting a progressive effect rather than cartilage degradation or dedifferentiation. Single gelatinase knockdown morphants were not significantly different from controls. MMP-13 morphants exhibited severe alterations in the shape of the neurocranial and pharyngeal cartilages, as well as a loss of certain cartilage types including the hyosymplectic and ceratobranchial. Neither MMP-2/MMP-9 double knockdown morphants nor MMP-13 morphants possessed significant differences in intercranial distance compare to controls. The zebrafish eyes are derived from a single field of cells in the anterior neural plate, and any changes in intercranial distance would suggest alterations in neural plate growth (Varga *et al.* 1999). Since no difference was observed, perhaps these MMPs play a minor role in neural plate growth compared to that of neurocranial and pharyngeal cartilages. Overall, these data suggest that the expression of several MMP types is required for proper craniofacial patterning during embryogenesis. Loss of MMP function during the formation of the embryonic head skeleton could potentially alter either migration of neural crest cells or cartilage differentiation and growth, both of which involve interaction with the ECM.

Collectively, these data show that MMP-2 and MMP-9 were differentially expressed during zebrafish embryogenesis. Appropriate expression of MMP-2, MMP-9, and MMP-13 were necessary for proper zebrafish embryogenesis, and altering expression of these genes resulted in severe lesions and dysmorphogenesis of both the embryo trunk

and craniofacial region. Further, a potential relationship between MMP-2 and MMP-9 whereby MMP-2 is able to compensate for loss of MMP-9 function was identified. Although previous work has demonstrated the importance of several MMPs in zebrafish embryogenesis, this work represents the first time craniofacial cartilage patterning was thoroughly examined as an endpoint following MMP gene knockdown. However, additional studies are required to fully characterize the changes in cartilage migration and formation, and to determine the mechanism by which these changes are occurring. Large scale mutagenesis screens for mutations affecting the early development in zebrafish embryos have been performed, and a number of mutations targeting cartilage-associated ECM elements have been identified (Neuhauss *et al.* 1996; Piotrowski *et al.* 1996; Yelick and Schilling 2002). Further examination of such mutants may reveal the exact role MMPs play in neural crest cell migration and chondrocyte maturation. The large number of molecular techniques available, including a fully sequenced genome, combined with the fact that morphants exhibiting craniofacial dysmorphogenesis recapitulate what is seen in murine knockout models, suggests zebrafish would serve as a valuable model in examining human skeletal disorders. It is known that three fourths of all congenital birth defects in humans involve craniofacial malformations (Chai and Maxson 2006). Therefore, from a clinical perspective, using this model to define the genetic, molecular, and physiological events occurring during the complex process of craniofacial development may aid in identifying targets for *in utero* therapy of such ailments as cleft lip and palate.

4.5. Figures
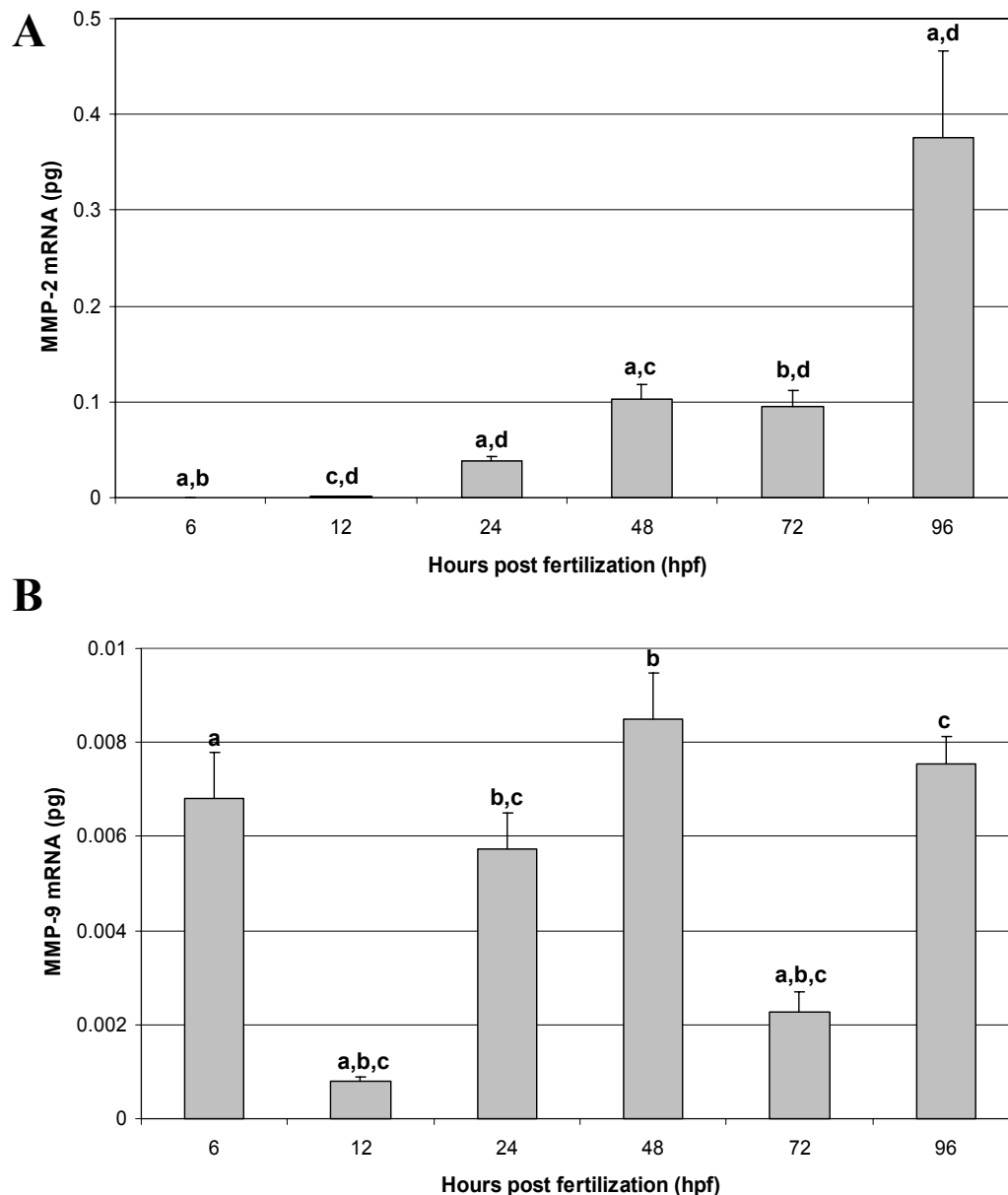
Figure 4.1. Zebrafish exhibit varying levels of MMP-2 and MMP-9 transcript during development. Total RNA was isolated from developing zebrafish at 6, 12, 24, 48, 72 and 96 hpf and used as template for quantitative RT-PCR using primers specific for zebrafish 28S rRNA, **(A)** MMP-2 and **(B)** MMP-9. Data were quantified by utilizing a standard curve generated from specific PCR products at concentrations ranging from 1 ng to 500 fg. Data were normalized to 28S rRNA, which served as the loading control. PCR reactions were performed in triplicate and data are representative of three separate experiments. Error bars denote standard deviation. Changing MMP transcript levels were compared pairwise via an unpaired Student's t-test. Bars labeled with the same letter (a,b,c,d) are statistically different ($p < 0.05$).

Figure 4.2. *In situ* **analysis of MMP-2 or MMP-9 localization at 72 hpf reveals widespread expression.** Expression of **(A,B)** MMP-2 or **(C,D)** MMP-9 at 72 hpf was examined using *in situ* hybridization of RNA probes. Black (40x) and white (100x) arrows indicate increased areas of expression. Magnification: 40x (A,C; scale bar = 250 μm) or 100x (B,D; scale bar = 100 μm).
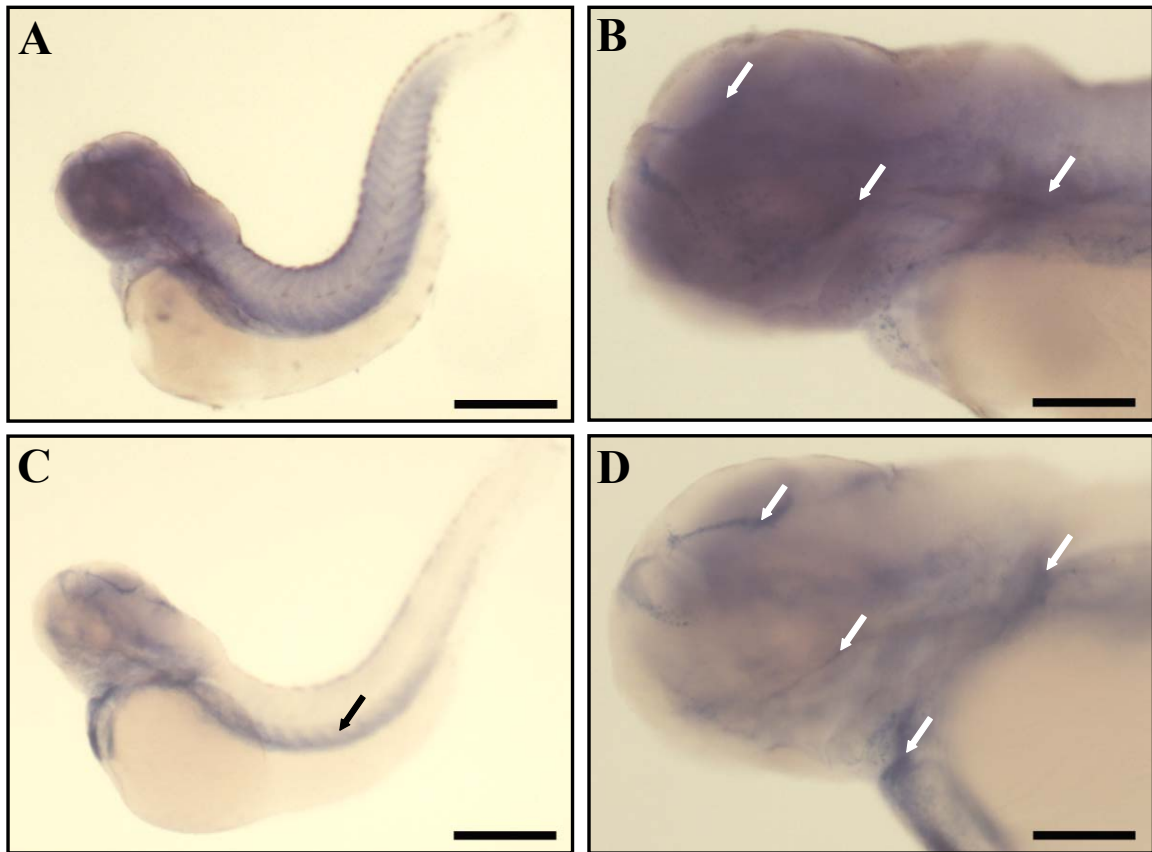
A

	1	2	3	4
MMP-2 cDNA	–	+	+	+
Control-MO	–	–	+	–
MMP2-MO	–	–	–	+

← 66 kDA

B

	1	2	3	4
MMP-9 cDNA	–	+	+	+
Control-MO	–	–	+	–
MMP9-MO	–	–	–	+

← 86 kDA
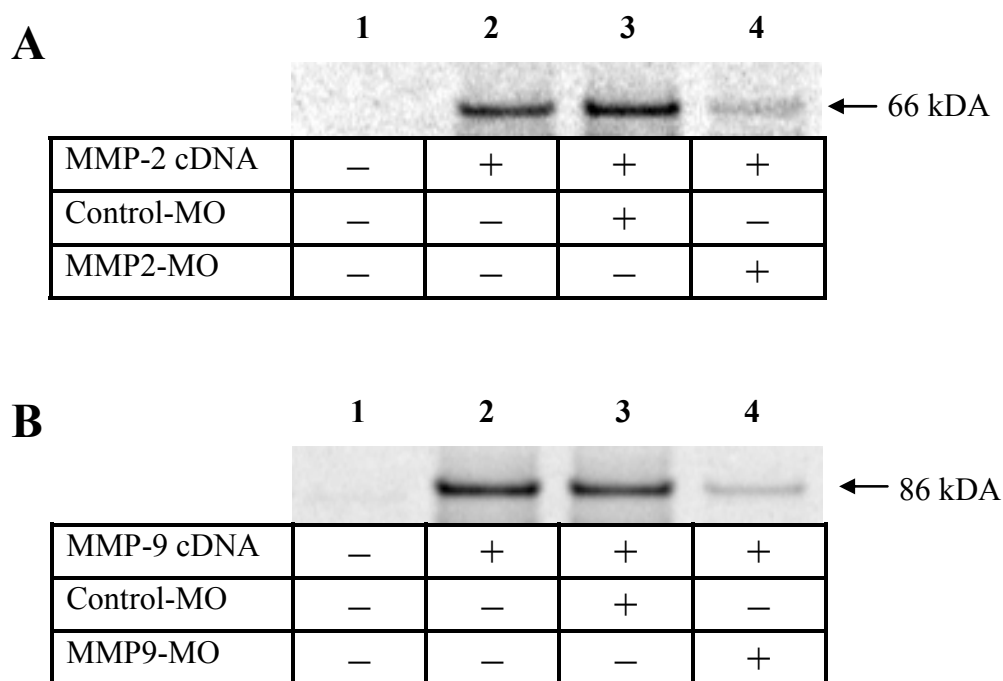
Figure 4.3. MMP2-MO and MMP9-MO are effective in preventing protein translation *in vitro*. A coupled *in vitro* transcription / translation reaction in the presence of [^{35}S]methionine was utilized to demonstrate the effectiveness of either the **(A)** MMP2-MO or **(B)** MMP9-MO at blocking *in vitro* protein translation. The reactions were performed as follows: in the absence of MMP cDNA and morpholino (**lane 1**), in the absence of morpholino (**lane 2**), in the presence of 10 μM Control-MO (**lane 3**), and in the presence of 10 μM MMP-MO (**lane 4**). Given that the addition of MMP2-MO or MMP9-MO resulted in a decrease in the 66 kDa or 86 kDa protein corresponding to MMP-2 or MMP-9 active enzyme, respectively, it appears these morpholinos are capable of blocking translation *in vitro*.

Figure 4.4. Continued on next page.

B

Cont-MO 300 µM

MMP2-MO 50 µM+
MMP9-MO 50 µM

MMP2-MO 50 µM+
MMP9-MO 100 µM

MMP2-MO 50 µM+
MMP9-MO 200 µM

MMP2-MO 100 µM+
MMP9-MO 200 µM

Figure 4.4. Continued on next page.

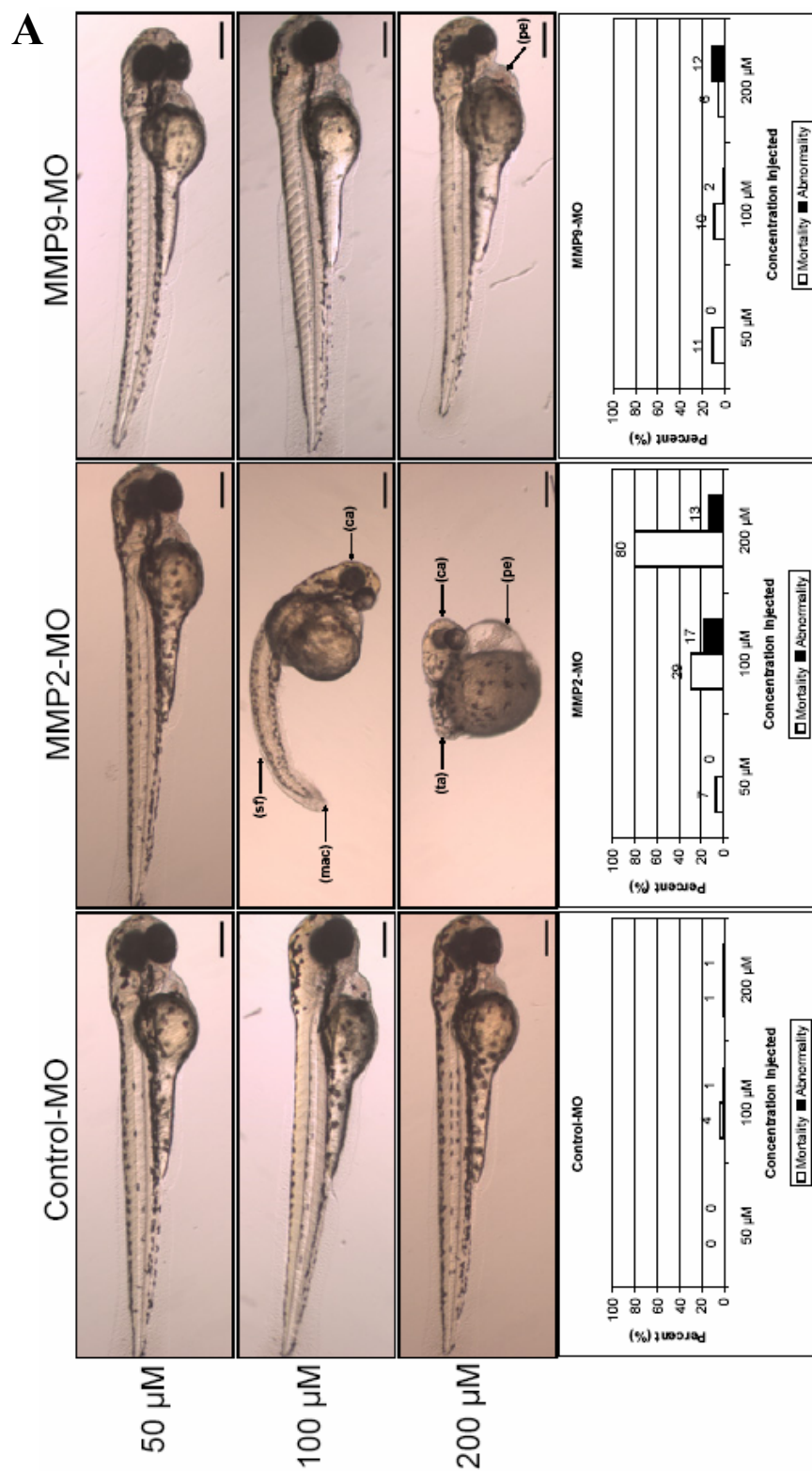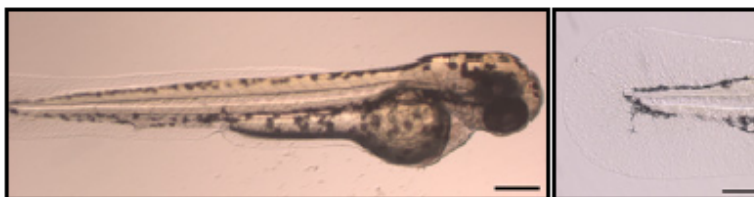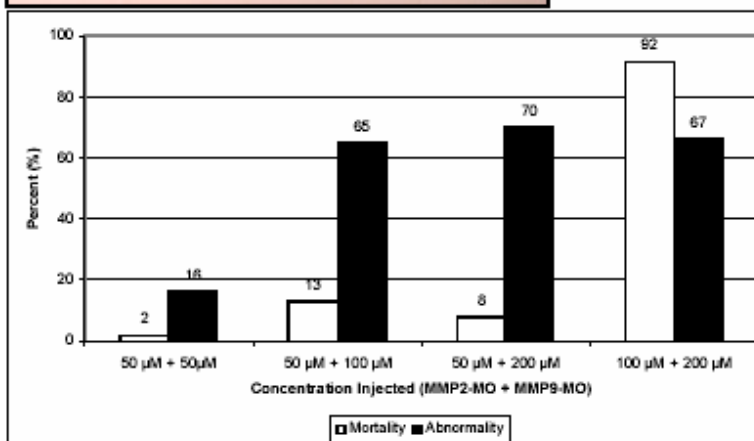**Figure 4.4. MMP-2 morphants exhibit developmental abnormalities and gelatinase morpholino co-injection demonstrates the ability of MMP-2 to compensate for loss of MMP-9 function.** Microinjection of MMP-2 and/or a MMP-9 MO into 1- to 2-cell embryos was used to knockdown gene expression as a means of elucidating their role in embryogenesis. **(A)** Control-MO, MMP2-MO, and MMP9-MO were injected at 50, 100 or 200 μM and both representative photographs and mortality / abnormality percentages are provided for each concentration (72 hpf). Control-MO injected embryos develop normally. Embryos injected with 100 or 200 μM MMP2-MO or 200 μM MMP9-MO exhibit abnormal phenotypes including: craniofacial abnormalities **(ca)**, modified actinotrichia **(mac)**, pericardial edema **(pe)**, stunted fin development **(sf)**, and truncated axis **(ta)**. **(B)** MMP2-MO and MMP9-MO were co-injected at varying concentrations to explore the possibility of MMP-2 compensating for loss of MMP-9 function. Photographs of the whole embryo (40x), details of the tailfin (100x), and a table detailing the mortality / abnormality percentages are provided for each concentration (72 hpf). Control-MO injected embryos develop normally. Increasing the co-administered concentrations allows for the establishment of a dose-response in terms of mortality and severity (but not percentage) of abnormalities observed. Representative abnormal phenotypes include: craniofacial abnormalities **(ca)**, epithelial hyperplasia **(eh)**, kinked tail **(kt)**, loss of structural organization **(ls)**, modified actinotrichia **(mac)**, pericardial edema **(pe)**, and yolk-sac edema **(yse)**. Magnification: 40x (whole embryo; scale bar = 250 μm) or 100x (tailfin; scale bar = 100 μm).

A

| Control-MO | MMP2-MO | MMP9-MO | MMP2-MO+ MMP9-MO |

B

| Control-MO | MMP2-MO | MMP9-MO | MMP2-MO+ MMP9-MO |
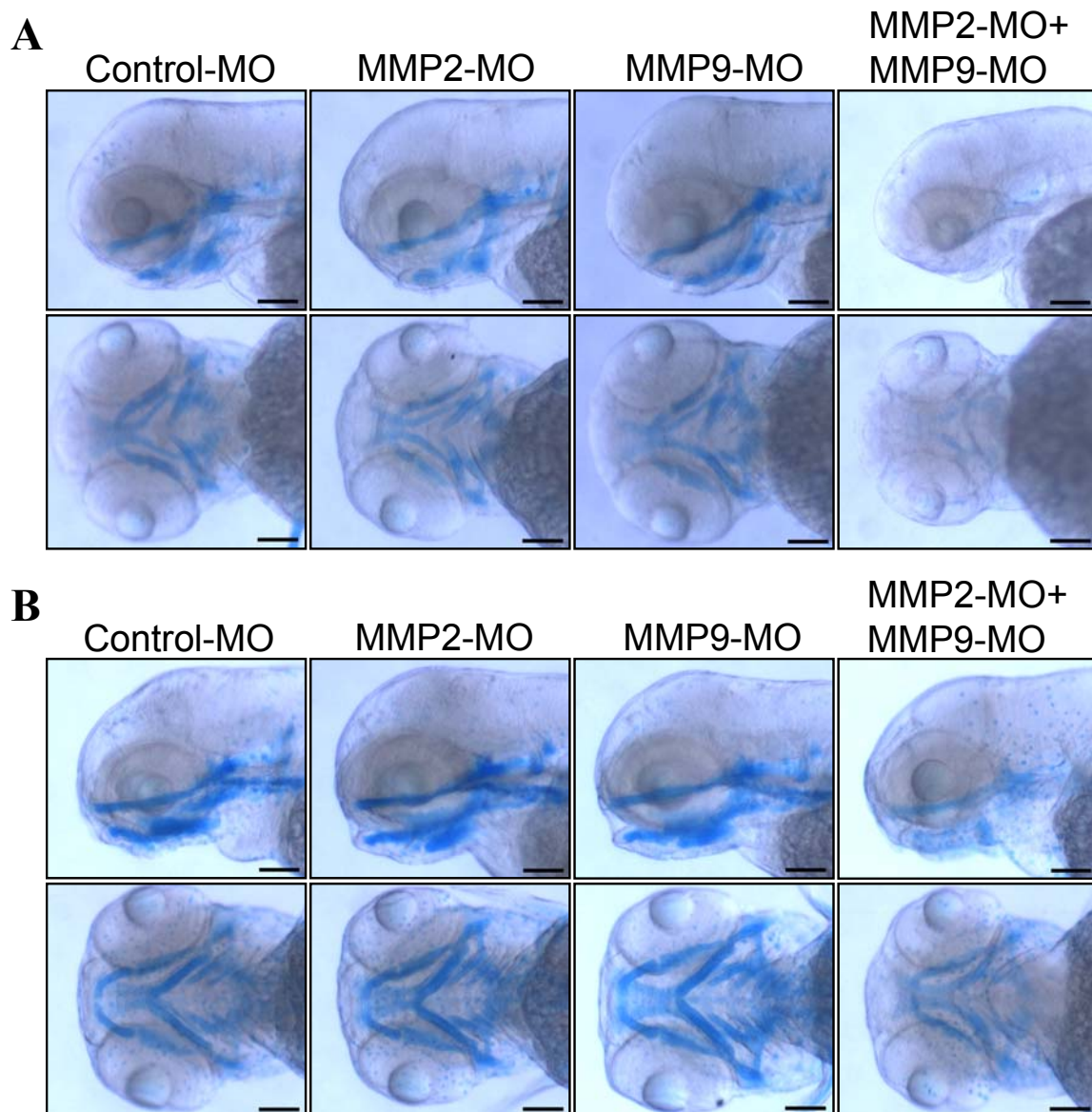
Figure 4.5. Continued on next page.
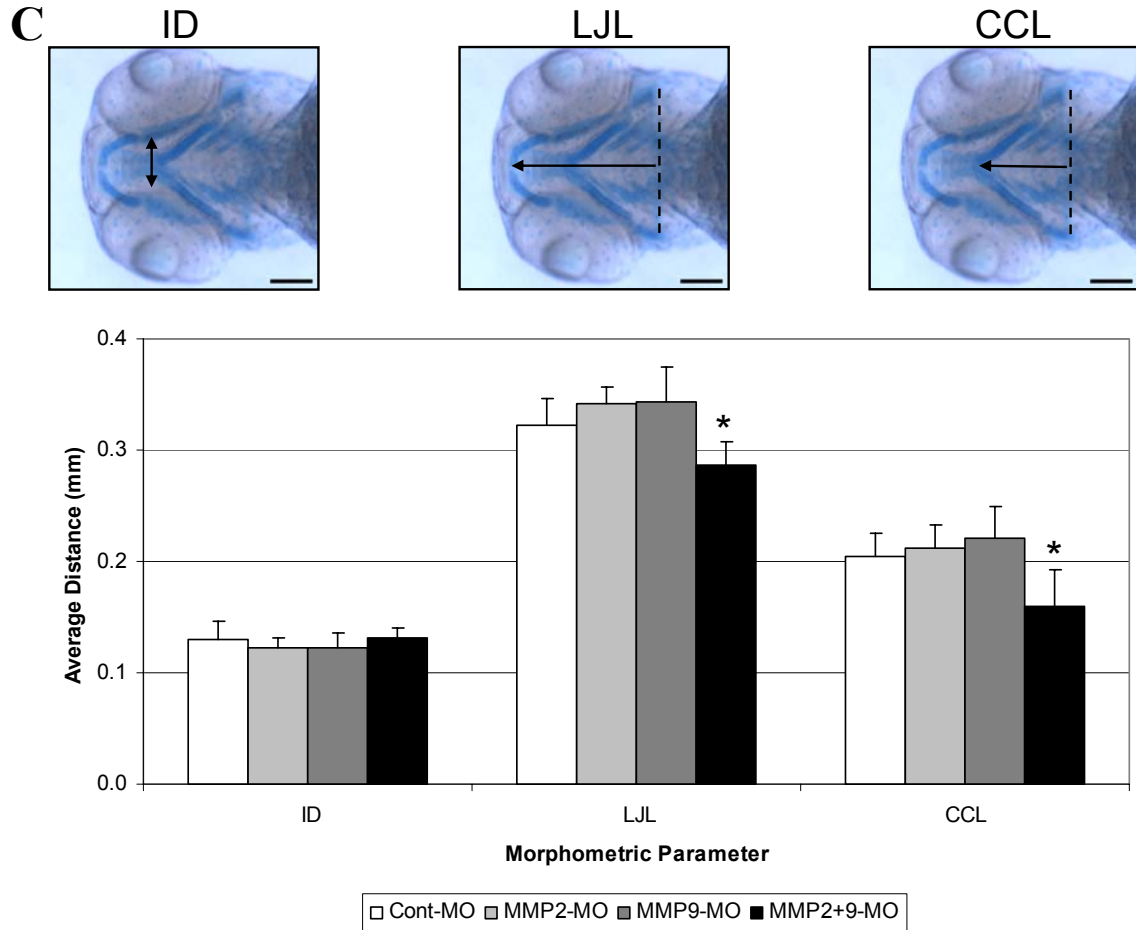
Figure 4.5. Altered craniofacial morphogenesis results from co-injection of MMP2-MO and MMP9-MO. Embryos injected with MMP2-MO (50 μM) or MMP9-MO (200 μM) alone or in conjunction were stained with Alcian blue at **(A)** 72 hpf or **(B)** 96 hpf in order to better characterize the observed changes in craniofacial development. **(C)** Following staining at 96 hpf, morphometric measurements were performed in order determine differences in intercranial distance (ID), lower jaw length (LJL), or ceratohyal cartilage length (CCL). Error bars denote standard deviation. Co-injection of MMP2-MO and MMP9-MO results in a significant decrease in LJL and CCL, but not ID (* $p < 0.05$). Injection of MMP2-MO or MMP9-MO alone does not result in any significant change in these parameters as compared to embryos injected with Control-MO (250 μM). Magnification: 63x (scale bar = 100 μm).
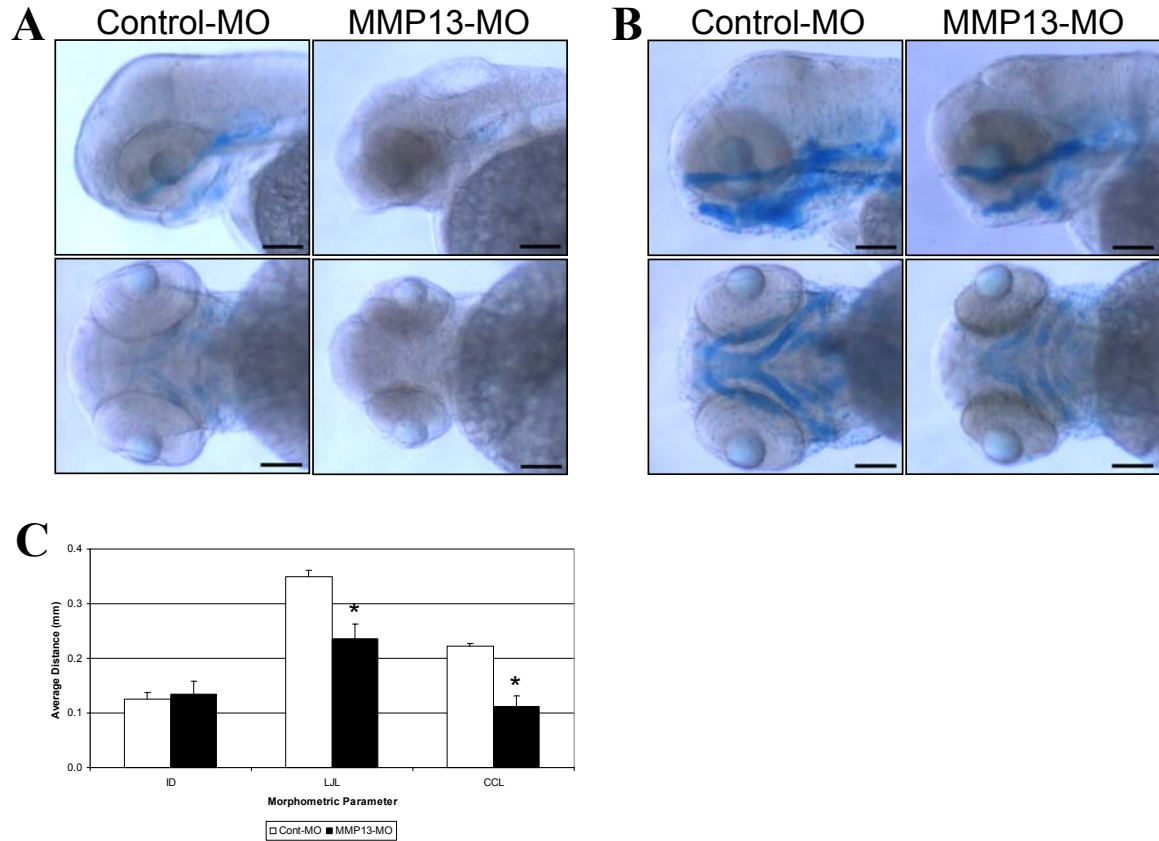
Figure 4.6. Altered craniofacial morphogenesis is observed in MMP-13 morphants. Embryos injected with Control-MO (100 μM) or MMP13-MO (100 μM) were stained with Alcian blue at **(A)** 72 hpf or **(B)** 96 hpf in order to better characterize the observed changes in craniofacial development known to result from morpholino microinjection. **(C)** Following staining at 96 hpf, morphometric measurements were performed in order to determine differences in intercranial distance (ID), lower jaw length (LJL), or ceratohyal cartilage length (CCL). Error bars denote standard deviation. MMP-13 morphants exhibit significant decreases in LJL and CCL, but not ID (* $p < 0.05$). Magnification: 63x (scale bar = 100 μm).

5.0. MATRIX METALLOPROTEINASES ARE ACTIVATED IN DEVELOPING ZEBRAFISH (*DANIO RERIO*) EMBRYOS FOLLOWING EXPOSURE TO DEXAMETHASONE OR HYDROCORTISONE VIA A GLUCOCORTICOID RECEPTOR-DEPENDENT MECHANISM

5.1. Abstract

Teratogenic effects are observed following long-term administration of glucocorticoids, although short-term glucocorticoid therapy is still utilized to reduce fetal mortality, respiratory distress syndrome and intraventricular hemorrhage in preterm infants. However, the mechanism of glucocorticoid-induced teratogenicity is unknown. It is possible that glucocorticoid-induced teratogenesis is mediated through the GR and results from altering the expression and activity of the MMPs. During embryogenesis, degradation of the extracellular matrix to allow for proper cellular migration and tissue organization is a tightly regulated process requiring appropriate temporal and spatial expression and activity of the MMPs. Studies have demonstrated that MMP gene expression can be either inhibited or induced by glucocorticoids in a variety of model systems. Using the zebrafish (*Danio rerio*) as a model of development, the data presented here demonstrate that embryonic exposure to the glucocorticoids dexamethasone or hydrocortisone increased expression of two gelatinases, MMP-2 (~1.5-fold) and MMP-9 (7.6 to 9.0-fold), at 72 hpf. Further, gelatinase activity was increased approximately 3-fold at 72 hpf following glucocorticoid treatment, and changes in craniofacial morphogenesis were also observed. Co-treatment of zebrafish embryos with each glucocorticoid and the GR antagonist RU486 resulted in attenuation of

glucocorticoid-induced increases in MMP expression (52-84% decrease) and activity (41-94% decrease). Furthermore, the abnormal craniofacial phenotype observed following glucocorticoid exposure was less severe following RU486 co-treatment. These studies demonstrate for the first time that in the embryonic zebrafish, dexamethasone and hydrocortisone are working through the GR, and that activation of this receptor can modulate expression of MMPs.

5.2. Introduction

Glucocorticoids play a central role in vertebrate physiology and are involved in numerous regulatory mechanisms associated with development, bone replication, bone differentiation, apoptosis, metabolism, circadian cell cycle rhythmicity, and the stress response, among others (Canalis and Delany 2002; Dickmeis *et al.* 2007). Glucocorticoids act as immunosuppressive and anti-inflammatory agents, and are therefore widely utilized to treat autoimmune and inflammatory disorders, transplant rejection, and lymphoproliferative diseases (Almawi *et al.* 2002). These compounds are also potent teratogens whose antenatal use has been linked to fetal growth restriction and cleft palate (Abbott 1995; Mandl *et al.* 2006), and intrauterine programming of metabolic, neuroendocrine and cardiovascular disorders in adult life (Seckl 2004). The exact mechanism by which glucocorticoids exert their teratogenic effects is unknown, although the work presented above has demonstrated that these compounds are capable of up-regulating MMP-13 (see studies described above in Section 3.0). Further, glucocorticoids have been shown to stimulate MMP-9 expression through induction of sGITR in murine macrophages (Lee *et al.* 2004; Lee *et al.* 2003). These results sit in

direct opposition to what has been found in mammalian cell models *in vitro*, where MMP expression is inhibited by exposure to glucocorticoids (Canalis and Delany 2002; Chakraborti *et al.* 2003; Vincenti *et al.* 1996). Despite contradictory findings, these studies suggest MMPs may be pivotal in eliciting glucocorticoid-induced teratogenicity and support further examination of the mechanism.

ECM remodeling is essential for a number of physiological processes including embryonic development, reproduction, tissue resorption, wound healing, and apoptosis (Brinckerhoff and Matrisian 2002; Hulboy *et al.* 1997; Nagase and Woessner 1999). Central to these processes are MMPs, a group of over 20 zinc-dependent endopeptidases responsible for precise and regulated ECM degradation. Dysregulation and excessive expression of MMPs have been tied to a number of pathological disorders such as osteo- and rheumatoid arthritis, emphysema, multiple sclerosis, bacterial meningitis, and tumor invasion and metastasis (D'Armiento *et al.* 1992; Folgueras *et al.* 2004; Hendrix *et al.* 2003; Leppert *et al.* 2001; Rundhaug 2005). Recent work using zebrafish has focused on the role of MMPs during embryonic development. This laboratory and others have shown that MMP-2, MMP-9, MMP-13, and membrane-type MMP α and β are required for normal zebrafish embryogenesis (Zhang *et al.* 2003b, c).

Most glucocorticoid-associated effects are mediated through the GR, which belongs to the nuclear receptor superfamily and acts as a ligand-dependent transcription factor (Evans 2005). A single glucocorticoid receptor has been identified in zebrafish thus far, although other related teleosts have two distinct GR genes (Bury *et al.* 2003; Greenwood *et al.* 2003). The teleost GR is unique from the mammalian GR in that the DNA-binding domain contains nine additional residues between the two zinc fingers.

These nine amino acid inserts are remarkably conserved among teleostean fish species (Stolte *et al.* 2006; Terova *et al.* 2005) and appear to be the result of alternative splicing (Stolte *et al.* 2006). It has been suggested that since these residues promote greater DNA affinity in the GR, they could have been selected to serve the large spectrum of cortisol functions in fish (Lethimonier *et al.* 2002). Such GR splice variants have been characterized in the rainbow trout (Bury *et al.* 2003) and Burtons' mouthbrooder (Greenwood *et al.* 2003; Takeo *et al.* 1996) thus far, and it is believed this could result in separate biological functions for each receptor variant (Prunet *et al.* 2006).

The purpose of these studies is to examine the effects of the glucocorticoids dexamethasone and hydrocortisone on MMP-2 and MMP-9 expression and activity and to establish a causal link between activation of the GR and changes in MMP-2, MMP-9, and MMP-13 levels. Further, a better characterization of the craniofacial defects known to result from exposure of embryonic zebrafish to these glucocorticoids is desired. The gelatinases MMP-2 (gelatinase A) and MMP-9 (gelatinase B) readily degrade gelatins (denatured collagens) and intact collagen type IV, and the collagenase MMP-13 (collagenase 3) cleaves native interstitial collagens I, II, and III (Chakraborti *et al.* 2003). These particular MMPs were selected because they have been shown to be vital for normal development in both zebrafish and mice (Inada *et al.* 2004; Itoh *et al.* 1997; Mosig *et al.* 2007; Stickens *et al.* 2004; Vu *et al.* 1998). The data presented here demonstrate that dexamethasone and hydrocortisone cause increases in MMP-2 and MMP-9 expression and activity in the zebrafish embryo at 72 hpf, with resultant changes in craniofacial morphogenesis. Co-treatment with glucocorticoids and the GR antagonist RU486 results in attenuation of the increases in MMP-2, MMP-9, and MMP-13

expression and activity normally observed following glucocorticoid treatment, as well as a partial rescue of the abnormal craniofacial phenotype. These results confirm that in the embryonic zebrafish, dexamethasone and hydrocortisone are working through the GR, and that activation of this receptor can modulate expression of MMPs.

5.3. Results

5.3.1. Exposure to dexamethasone or hydrocortisone causes an induction of MMP-2 and MMP-9 mRNA in zebrafish embryos

Previous studies from this laboratory show that zebrafish embryos respond to dexamethasone and hydrocortisone in a dose-dependent manner, and that a dose of 100 mg/L is required to generate responses in both MMP-13 mRNA expression level and phenotype. Embryos exposed to 100 mg/L dexamethasone or hydrocortisone demonstrate a significant ($p < 0.05$) increase in MMP-2 and MMP-9 mRNA at 72 hpf measured via quantitative RT-PCR (**Figure 5.1**). Dexamethasone- and hydrocortisone-treated embryos exhibit a 1.6- and 1.5-fold induction in MMP-2 mRNA over solvent-control treated embryos, respectively. A 9.0- and 7.6-fold induction in MMP-9 mRNA is also observed in dexamethasone- and hydrocortisone-treated embryos, respectively. *In situ* hybridization of 72 hpf embryos using either a MMP-2-specific or MMP-9-specific mRNA probe confirms the glucocorticoid-induced increase in expression observed via quantitative RT-PCR (**Figure 5.2**). Sense probes for each of these MMPs yield essentially no signal and confirm the specificity of the antisense probes (data not shown). For MMP-2, solvent control embryos express transcript throughout their entire body, with the exception of the yolk sac and yolk sac extension. Slightly higher levels of MMP-2 expression appear to be localized rostrally and distinct areas of expression are

observed in the neurocranial cartilage, midbrain, heart, and anterior kidney. Following

treatment with either dexamethasone or hydrocortisone, increased transcript levels are

seen in both the medial and lateral aspects of the trunk, the anterior kidney, the heart, and

several areas associated with the head specifically. These include the neurocranial

cartilage, pharyngeal cartilage, and midbrain. MMP-9 transcript in solvent control

embryos is also localized rostrally, although some expression is seen in medial trunk

superior to the yolk sac extension. Glucocorticoid-treated embryos exhibit increased

MMP-9 expression levels throughout their entire trunk and head. Given the intensity of

signal present in the embryo head following treatment with dexamethasone or

hydrocortisone, it is difficult to distinguish explicit areas of increased expression.

5.3.2. Gelatinase activity is increased in embryos treated with dexamethasone or hydrocortisone

In vitro zymography is a quantitative assay that has been shown to be an effective

means of determining MMP activity in lysates isolated from zebrafish embryos

(Crawford and Pilgrim 2005). Fluorescein-conjugated type IV collagen serves as a

substrate for MMP-2 and MMP-9, and its degradation is measured by the release of the

fluorescein which is indicative of gelatinase activity. Approximately 3-fold higher

gelatinase activity is observed in lysates isolated from zebrafish embryos treated with

either dexamethasone or hydrocortisone (**Figure 5.3A**). These data confirm that

increased transcript levels detected via quantitative RT-PCR and *in situ* hybridization

result in increased protein levels and subsequent enzyme activity. Given the broad

substrate specificity of many of the MMPs, the inclusion of several inhibitors was

implemented as a means of ascribing the gelatinase activity observed to MMP-2 and

MMP-9. The divalent cation chelator EDTA (1 mM) causes a complete loss of activity in all treatment groups which indicates that the activity observed is due to a metalloenzyme (**Figure 5.3B**). Inclusion of GM6001, a potent, broad-spectrum inhibitor of MMPs, causes a significant ($p < 0.05$) decrease in type IV collagen degradation by lysates isolated from both dexamethasone-treated (47% inhibition) and hydrocortisone-treated (91% inhibition) embryos. Similarly, use of an inhibitor specific for MMP-2 and MMP-9 (MMP-2/MMP-9 Inhibitor II) results in a 34% decrease in activity by dexamethasone-treated embryo lysates and a 62% decrease in activity by hydrocortisone-treated embryo lysates. All inhibitors were also effective in decreasing activity in solvent control lysates (data not shown). These data suggest that the activity observed can be attributed, at least in part, to the gelatinases MMP-2 and MMP-9.

5.3.3. RU486 prevents increases in MMP mRNA and activity following exposure to dexamethasone or hydrocortisone

It is generally accepted that the effects of glucocorticoids in fish are mediated through the GR (Prunet *et al.* 2006); therefore, the GR antagonist RU486 was utilized to determine whether or not glucocorticoid-induced increases in MMP expression could be inhibited. This compound has been used successfully to study GR-related functions in other teleost models such as osmoregulation, urea excretion, and metabolism (DiBattista *et al.* 2006; Marshall *et al.* 2005; Rodela and Wright 2006; Veillette *et al.* 2007; Veillette *et al.* 1995). Exposure of the embryos to dexamethasone or hydrocortisone through 72 hpf again causes an increase in MMP-2, MMP-9, and MMP-13 mRNA (**Figures 5.4A**, **5.4B**, and **5.4C**, respectively). Co-treatment of embryos with glucocorticoid and RU486, however, causes a significant ($p < 0.05$) decrease in MMP-2, MMP-9, and MMP-13

mRNA levels compared to embryos treated with glucocorticoid alone. In fact, MMP mRNA levels following co-treatment are comparable to those observed in solvent control and RU486 control embryos.

In vitro zymography assays examining type I collagen (MMP-13) and type IV collagen (MMP-2 and MMP-9) degradation confirm the results achieved using quantitative RT-PCR. Specifically, an increase in type I (**Figure 5.5A**) and type IV (**Figure 5.5B**) collagen degradation is observed in lysates isolated from embryos treated with dexamethasone or hydrocortisone. This increase in activity is attenuated in lysates isolated from zebrafish embryos co-treated with glucocorticoid and RU486. These data confirm that dexamethasone and hydrocortisone are acting through the GR, and that a link exists between activation of the GR and MMP expression and activity.

5.3.4. *Dexamethasone and hydrocortisone cause aberrant craniofacial cartilage development*

In order to better characterize the changes known to occur in the craniofacial region following exposure to dexamethasone or hydrocortisone, Alcian blue staining was performed. Alcian blue is a copper-containing phthalocyanine dye that stains glycosaminoglycans, a central component of cartilage and bone (Terry *et al.* 2000). Following staining procedures, morphometric measurements were carried out on the craniofacial cartilage of 96 hpf embryos. These consisted of measuring intercranial distance (ID), lower jaw length (LJL), and ceratohyal cartilage length (CCL). Intercranial distance measurements were performed by measuring the distance between the medial edge of each eye. Lower jaw length and ceratohyal cartilage length measurements were performed by measuring the distance from a reference line drawn

between the posterior edges of the hyosymplectic cartilage to the anterior edge of the Meckel's cartilage and ceratohyal cartilage, respectively.

At 72 hpf, all treatment groups exhibit a well-developed neurocranial cartilage architecture (**Figure 5.6A**). In the solvent control embryos, partially developed pharyngeal cartilages are observable, with the hyosymplectic, palatoquadrate, and ceratohyal cartilages being the most apparent. Partially developed hyosymplectic and palatoquadrate cartilages are also observed in dexamethasone- and hydrocortisone-treated embryos, although the level of chondrification of these cartilage types is appreciably less. Alcian blue staining of 96 hpf embryos reveals a more well-developed cartilaginous head skeleton, with all treatments possessing neurocranial, pharyngeal, and ceratobranchial cartilage types (**Figure 5.6B**). However, morphometric measurements of intercranial distance, lower jaw length, and ceratohyal cartilage length reveal quantifiable differences between treatment groups (**Figure 5.6C**). In particular, dexamethasone-treated embryos possess significantly ($p < 0.05$) smaller ceratohyal cartilage lengths, and hydrocortisone-treated embryos have both significantly ($p < 0.05$) decreased ceratohyal and lower jaw lengths. In addition, a noticeable increase in the angle between the ethmoid plate and Meckel's cartilage is seen in glucocorticoid-treated embryos. Taken collectively, these data indicate that dexamethasone and hydrocortisone are capable of causing quantifiable changes in craniofacial parameters during zebrafish embryogenesis, possibly through a mechanism involving impairment of the anterior growth and migration of the pharyngeal cartilages.

5.3.5. RU486 partially rescues abnormal craniofacial phenotypes observed following treatment with dexamethasone or hydrocortisone

As demonstrated previously, RU486 is capable of preventing glucocorticoid-induced increases in MMP mRNA and activity levels. Given these data, it was proposed that RU486 would also be able to rescue the abnormal craniofacial phenotypes known to result from exposure to dexamethasone or hydrocortisone. Alcian blue staining of 96 hpf embryos demonstrate that a partial rescue in glucocorticoid-induced phenotype can be achieved by co-treatment with RU486 (**Figure 5.7**). Embryos co-treated with either dexamethasone or hydrocortisone and RU486 possess lower jaw lengths and ceratohyal cartilage lengths closer to the controls compared to their counterparts treated with dexamethasone or hydrocortisone alone. Further, the angle between the ethmoid plate and Meckel's cartilage in these embryos is similar to that observed in the controls. However, morphometric measurements reveal that statistical significance between co-treated and glucocorticoid-only groups is not achieved even though a general trend implying phenotype rescue is apparent.

5.4. Discussion

Despite being known teratogens, glucocorticoids are one of the most commonly prescribed drugs worldwide due to their anti-inflammatory and immunosuppressant properties (Bello and Garrett 1999). Short-term antenatal glucocorticoid therapy is also generally implemented as a means of reducing fetal mortality, respiratory distress syndrome and intraventricular hemorrhage in preterm infants (Sloboda *et al.* 2005). Several medical conditions during pregnancy, such as asthma, systemic lupus

erythematosus, allografts, and inflammatory bowel disease require long-term administration of glucocorticoids (Pacheco *et al.* 2007). Well-described deleterious side effects from long-term glucocorticoid administration during pregnancy include growth restriction in fetuses (Seckl 1994) and intrauterine programming of cardiovascular, metabolic and neuroendocrine disorders in adult life (Seckl 2004). Also, retrospective studies of women receiving pharmacologic doses of glucocorticoids during pregnancy reveal instances of cleft palate, hydrocephalus, gastroschisis, stillbirths and premature births (AHFS 2000). Since the exact mechanism by which glucocorticoids elicit teratogenicity is unknown, the studies described here focused on the role of MMPs and the ability of glucocorticoids to modulate levels of these metalloenzymes during embryogenesis.

Glucocorticoids are key endocrine factors in teleosts, where they are involved in regulation of numerous physiological functions related to metabolism, growth, reproduction, osmoregulation, respiration, and the immune and stress responses (Prunet *et al.* 2006). The major circulating glucocorticoid in teleosts is cortisol (i.e. hydrocortisone) (Prunet *et al.* 2006; Vijayan *et al.* 2003), and was selected for use in the studies described here. The second glucocorticoid examined in these studies, dexamethasone, is a more potent GR agonist as demonstrated by *in vitro* experiments in rainbow trout, during which dexamethasone activated both variants of the GR at lower concentrations than cortisol (Bury *et al.* 2003). In addition, other reports demonstrate that dexamethasone was able to elicit a stronger reporter gene activation than similar concentrations of cortisol (Greenwood *et al.* 2003; Takeo *et al.* 1996).

Data presented here demonstrated that expression of MMP-2 and MMP-9 were increased at 72 hpf following glucocorticoid treatment compared to solvent controls as determined by quantitative RT-PCR and *in situ* hybridization. Recent data in murine macrophages show that glucocorticoids stimulate MMP-9 expression through induction of soluble glucocorticoid-induced tumor necrosis factor receptor (Lee *et al.* 2004; Lee *et al.* 2003). Also, previous studies conducted in this laboratory have shown that dexamethasone and hydrocortisone are able to induce MMP-13 levels during zebrafish embryogenesis. Additional quantitative RT-PCR data presented here suggest that base levels of MMP-2 mRNA were approximately 12-fold to 46-fold higher than MMP-9 mRNA at equivalent time points. This finding was supported by mRNA localization studies using *in situ* hybridization, which revealed MMP-2 mRNA was widespread throughout the entire embryo, whereas MMP-9 mRNA expression was restricted to the head and medial portion of the embryo trunk. Similar differences in expression of these gelatinases have been shown by this laboratory previously, and it has been proposed that the widespread expression of MMP-2 could be explained by the fact that the promoter of this gene has several characteristics of a constitutive (housekeeping) promoter (Chakraborti *et al.* 2003; Huhtala *et al.* 1990) . Induction of MMP-9 was more prevalent as indicated by 7.6- to 9-fold higher levels of MMP-9 mRNA versus controls, compared to approximately 1.5-fold higher MMP-2 mRNA. This was substantiated by *in situ* hybridization, which showed increased MMP-9 mRNA following glucocorticoid treatment to the point where individual areas of signal could not be identified due to overall intensity. MMP-2 mRNA signal also increased following treatment with dexamethasone or hydrocortisone, but to a lesser degree. These findings oppose those

reported for several *in vitro* mammalian cell models, which show that glucocorticoid treatment results in a downregulation of MMPs (Vincenti *et al.* 1996). However, these studies dealt with a single cell type rather than a whole animal model as described here, and are therefore not directly comparable.

The data presented above show that glucocorticoid-induced MMP-2 and MMP-9 expression is followed by an increase in gelatinase activity as determined by *in vitro* zymography. Fluoresceinated type IV collagen was used for this assay, which serves as substrate for both MMP-2 and MMP-9. Utilization of a MMP-2/MMP-9-specific *N*-sulfonylamino acid inhibitor (Tamura *et al.* 1998) resulted in a 34% and 62% decrease in activity by dexamethasone- and hydrocortisone-treated embryo lysates, respectively, constituting a partial inhibition of type IV collagenase activity. The fact that only a partial inhibition was achieved may be representative of the decreased effectiveness of this inhibitor against zebrafish MMPs, or may indicate that other MMPs present in these lysates have gelatinolytic activity. Type IV collagens are also substrates for MMP-3, MMP-7, MMP-10, MMP-12, and MMP-26 (Chakraborti *et al.* 2003). Of these, only homologs to MMP-3 and MMP-7 have been identified in zebrafish, although a full characterization of these genes has yet to be conducted. While there may be some contribution of other potentially unidentified zebrafish type IV collagenases in these assays, the ability of the MMP-2/MMP-9-specific inhibitor to at least partially inhibit type IV collagenase activity demonstrated that the increases in activity observed following glucocorticoid treatment were most likely the result of MMP-2 and MMP-9.

As described previously, a number of developmental abnormalities and localized lesions in zebrafish embryos result from exposure to dexamethasone and hydrocortisone.

These abnormalities included changes in the normal head-trunk angle, truncated body axis, changes in the shape and size of the yolk-sac and yolk-sac extension, altered somitogenesis, and abnormal craniofacial morphogenesis. In order to better characterize the changes known to occur in the craniofacial region during zebrafish embryogenesis following exposure to dexamethasone and hydrocortisone, Alcian blue staining was performed. These data show that dexamethasone and hydrocortisone were capable of altering parameters associated with both mandibular and hyoid arches, suggesting impairment of the anterior growth and migration of the pharyngeal cartilages. Specifically, dexamethasone-treated embryos possessed significantly ($p < 0.05$) smaller ceratohyal cartilage lengths, and hydrocortisone-treated embryos had both significantly ($p < 0.05$) decreased ceratohyal and lower jaw lengths. An increase in the angle between the ethmoid plate and Meckel's cartilage was also observed in glucocorticoid-treated embryos. These differences were discernable starting at 72 hpf, suggesting a progressive effect rather than cartilage degradation or dedifferentiation. No differences in intercranial distance were observed between control and glucocorticoid-treated embryos. Zebrafish eyes are derived from a single field of cells originating in the anterior neural plate, so any alterations in intercranial distance would imply changes in neural plate growth. However, since no differences were observed following treatment despite the prevalence of MMP-2 and MMP-9 in the head mesenchyme, perhaps these MMPs play minor roles in neural plate growth. In this laboratory, previous exposures of zebrafish embryos to identical concentrations of dexamethasone (100 mg/L) caused significant ($p < 0.05$) increases in intercranial (interocular) distance at 72 hpf. This may indicate that changes in intercranial distance following glucocorticoid treatment are variable. Interestingly, the

changes observed in the neurocranial and pharyngeal cartilages following glucocorticoid treatment were similar to those observed in zebrafish embryos following knockdown of MMP-2, MMP-9, and MMP-13 using antisense MOs. Since the ECM plays critical roles in both neural crest cell migration (Henderson and Copp 1997; Perris 1997; Perris and Perissinotto 2000) and cartilage morphogenesis (Kimmel *et al.* 1998; Kimmel *et al.* 2001), it appears that dysregulation of the MMPs responsible for remodeling the ECM contributes to abnormal craniofacial patterning.

RU486 is a GR receptor antagonist that binds directly to the GR, eliciting a transconformational change in the DNA-binding domain and a subsequent inability to bind to the GRE in the promoter region of target genes (Cadepond *et al.* 1997; Mahajan and London 1997). RU486 has also been shown to competitively displace cortisol from high affinity binding sites (Pottinger 1990). Data provided above demonstrate that co-treatment of zebrafish embryos with dexamethasone or hydrocortisone and RU486 inhibited the glucocorticoid-associated induction of MMP transcript shown to occur at 72 hpf. RU486 caused a 52%-84% reduction in MMP expression depending on the MMP type and treatment, with an average reduction of 63% across all MMPs. RU486 was also able to prevent glucocorticoid-associated increases in type I or type IV collagen degradation, indicating that the ability of this compound to block transcription results in a reduction in active protein as well. RU486 caused a 41%-89% reduction in type I collagen degradation and a 60%-94% reduction in type IV collagen degradation depending on the treatment. Finally, RU486 was able to decrease the severity of craniofacial changes that typically occur following glucocorticoid treatment, namely changes in lower jaw length, ceratohyal cartilage length, and in the angle between the

ethmoid plate and Meckel's cartilage. Morphometric measurements revealed that statistical significance between co-treated and glucocorticoid-only groups was not achieved, although a general trend implying phenotype rescue was apparent. This may have simply been a function of increased variability due to treatment with multiple compounds (indeed, the standard deviation among treatment groups is noticeably greater). Regardless, these data imply that RU486 is an effective GR antagonist in this model system and serves as a valuable tool in examining glucocorticoid-induced changes in MMP expression and activity.

Collectively, these data demonstrate that the glucocorticoids dexamethasone and hydrocortisone are capable of activating MMP-2 and MMP-9 expression and activity at 72 hpf. Developmentally, this induction results in altered craniofacial morphogenesis related to migration and growth of pharyngeal cartilages. This was essentially a recapitulation of the deleterious changes in the human palate (cleft palate) known to result from gestational exposure to glucocorticoids. Therefore, the teratogenic effects resulting from prolonged treatment with glucocorticoids during gestation may stem from the ability of these compounds to cause dysregulation of MMPs, subsequently leading to altered craniofacial cartilage migration and growth. Use of the GR antagonist RU486 resulted in attenuation of the increases in MMP expression and activity normally observed following glucocorticoid treatment, as well as a partial rescue in the abnormal craniofacial phenotype. These results suggest that in the embryonic zebrafish, dexamethasone and hydrocortisone function through the GR, and that activation of this receptor can modulate expression of MMPs. Further study is required to determine mechanistically how the GR and MMPs are interacting, although the data presented here

identify MMPs as a potential *in utero* therapeutic target for the prevention of glucocorticoid-induced teratogenic effects.

5.5. Figures
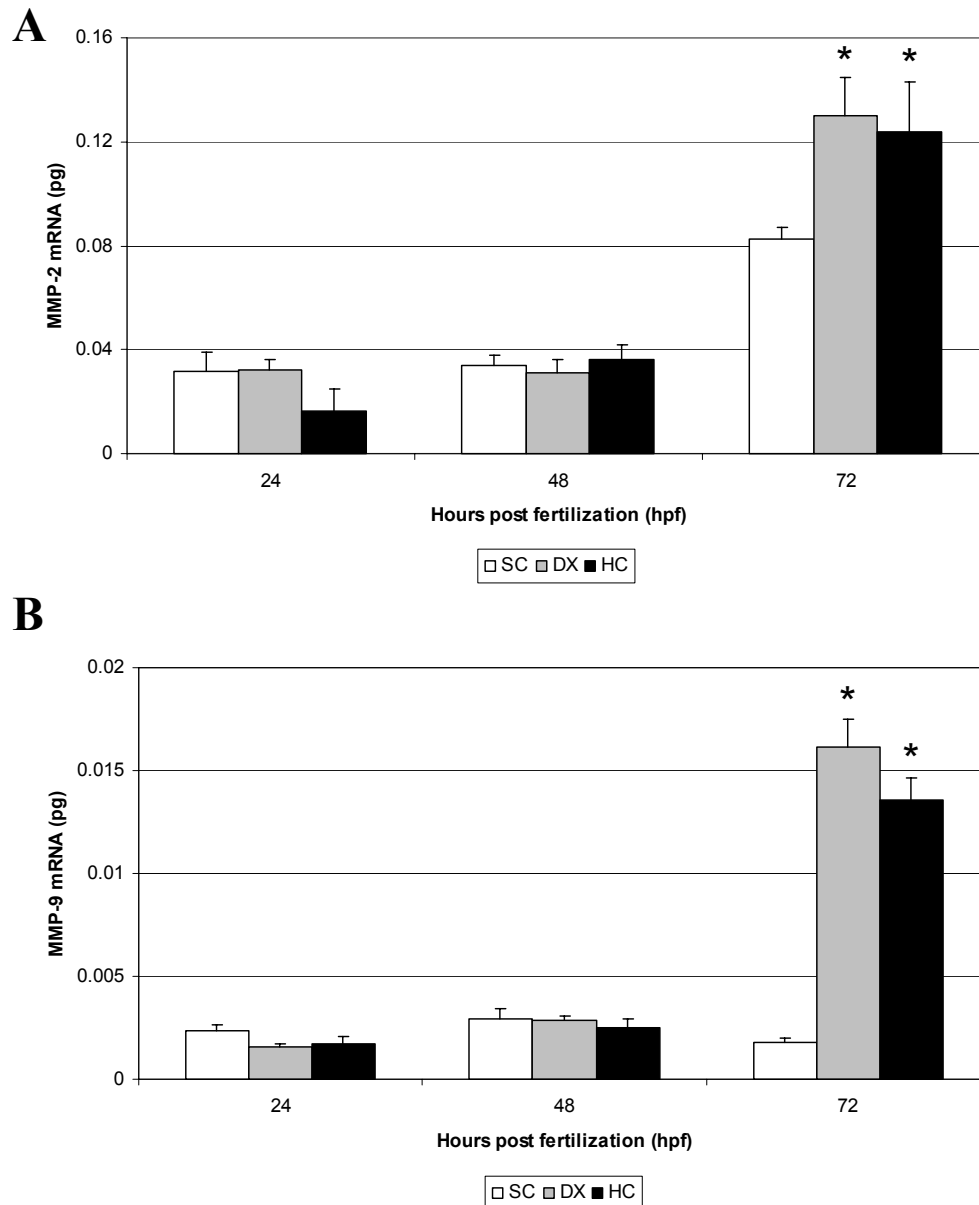
Figure 5.1. MMP-2 and MMP-9 mRNA expression is increased by exposure to glucocorticoids. Zebrafish embryos were exposed to treatments for 24, 48, or 72 hours, and RNA was isolated for use as template in quantitative RT-PCR. Data were quantified by utilizing standard curves generated from specific PCR products at concentrations ranging from 1 ng to 500 fg. Data were normalized to β-Actin. PCR reactions were performed in triplicate and data are representative of three separate experiments. Error bars denote standard deviation. A statistically significant increase in **(A)** MMP-2 and **(B)** MMP-9 expression in glucocorticoid-treated embryos is observed at 72 hpf as determined via One-Way ANOVA and Dunnett's test (* $p < 0.05$). DX, dexamethasone; HC, hydrocortisone; SC, solvent control.
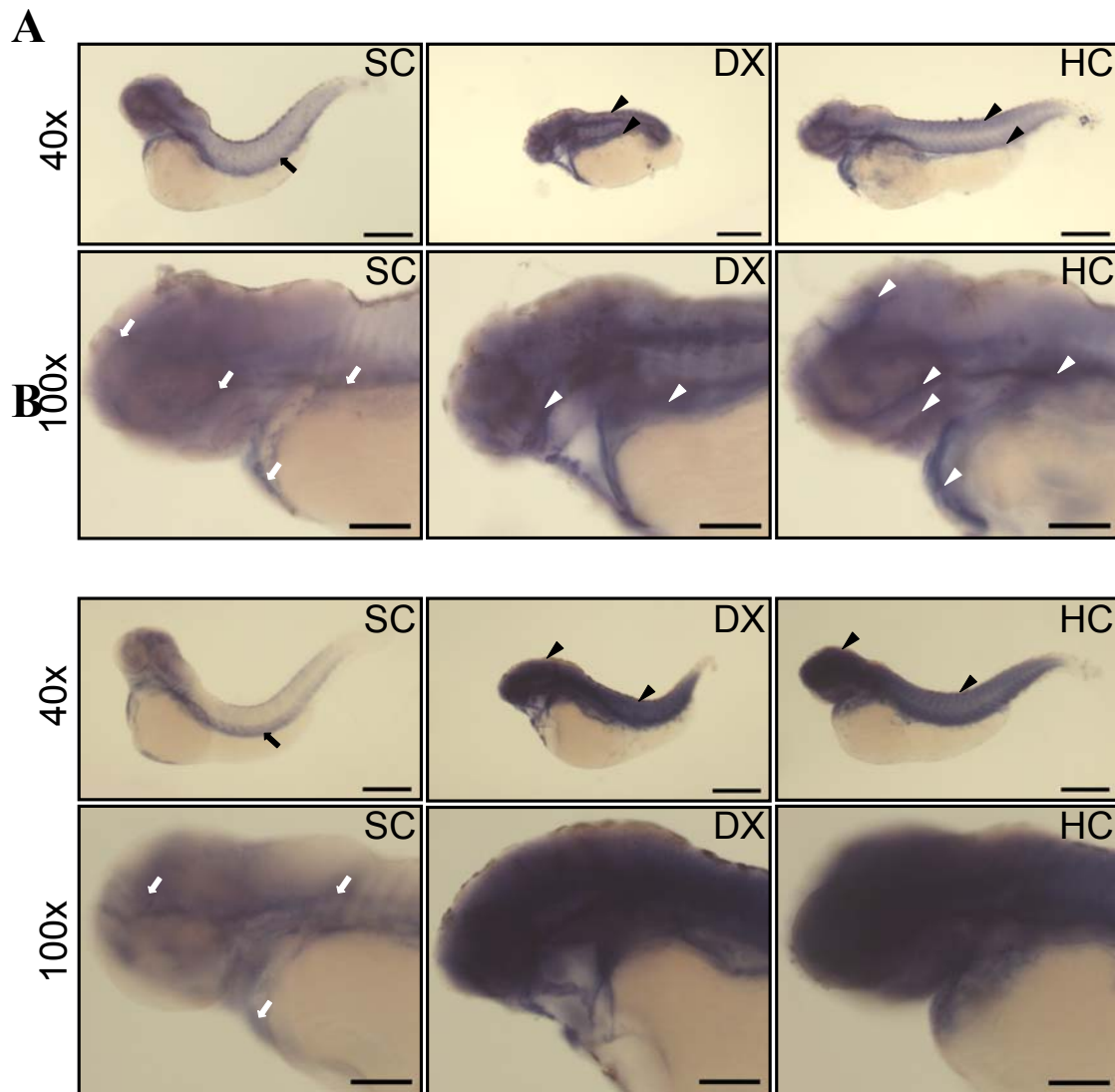
Figure 5.2. *In situ* **analysis of MMP-2 and MMP-9 localization confirms increased mRNA expression.** **(A)** MMP-2 and **(B)** MMP-9 expression following exposure to dexamethasone and hydrocortisone was localized using *in situ* hybridization at 72 hpf. Black (40x) and white (100x) arrows indicate general areas of expression in control embryos. In MMP-2 and MMP-9 control embryos, expression is observed in the trunk, neurocranial cartilage, midbrain, heart, and anterior kidney. Black (40x) and white (100x) arrowheads indicate increased areas of expression following treatment with dexamethasone or hydrocortisone. For MMP-2, these areas of increased expression include the medial and lateral aspects of the trunk, anterior kidney, heart, neurocranial cartilage, pharyngeal cartilage, and midbrain. For MMP-9, increased expression is noted throughout the head and trunk. Magnification: 40x (scale bar = 250 μm) or 100x (scale bar = 100 μm).
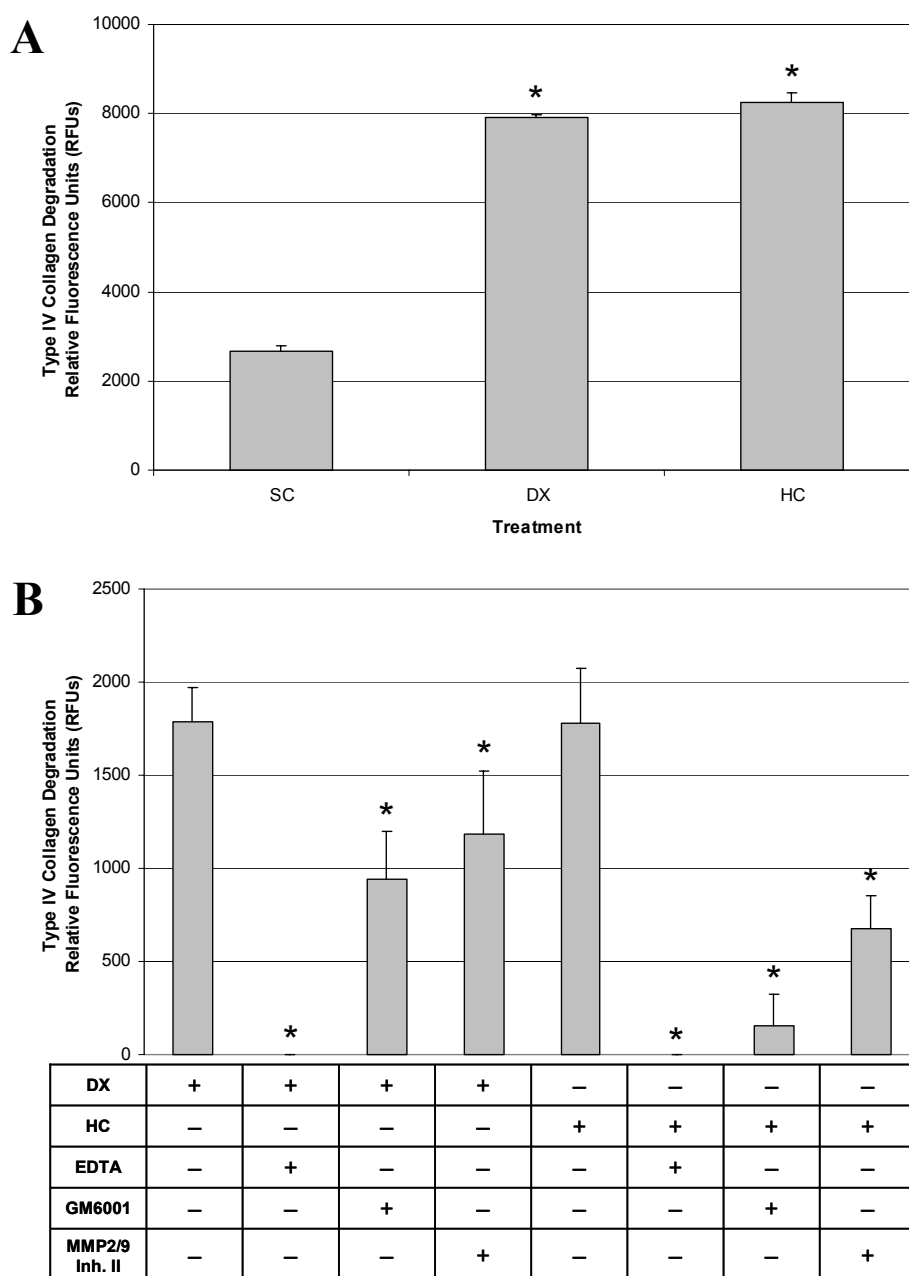
Figure 5.3. Exposure to glucocorticoids increases gelatinase activity. Results of *in vitro* zymography assays conducted on zebrafish embryo lysates using type IV collagen as an indicator of gelatinase activity. **(A)** Lysates isolated from embryos treated with dexamethasone or hydrocortisone through 72 hpf result in significant increases in type IV collagen degradation over the solvent control. **(B)** GM6001 (100 μM) and MMP-2/MMP-9 Inhibitor II (100 μM) effectively inhibit the degradation of type IV collagen in treatment lysates while EDTA (1 mM) completely eliminates lysate activity, suggesting the activity observed can be attributed, at least in part, to the gelatinases MMP-2 and MMP-9. Error bars denote standard deviation. Statistical significance was determined via One-Way ANOVA and Dunnett's test (* $p < 0.05$).
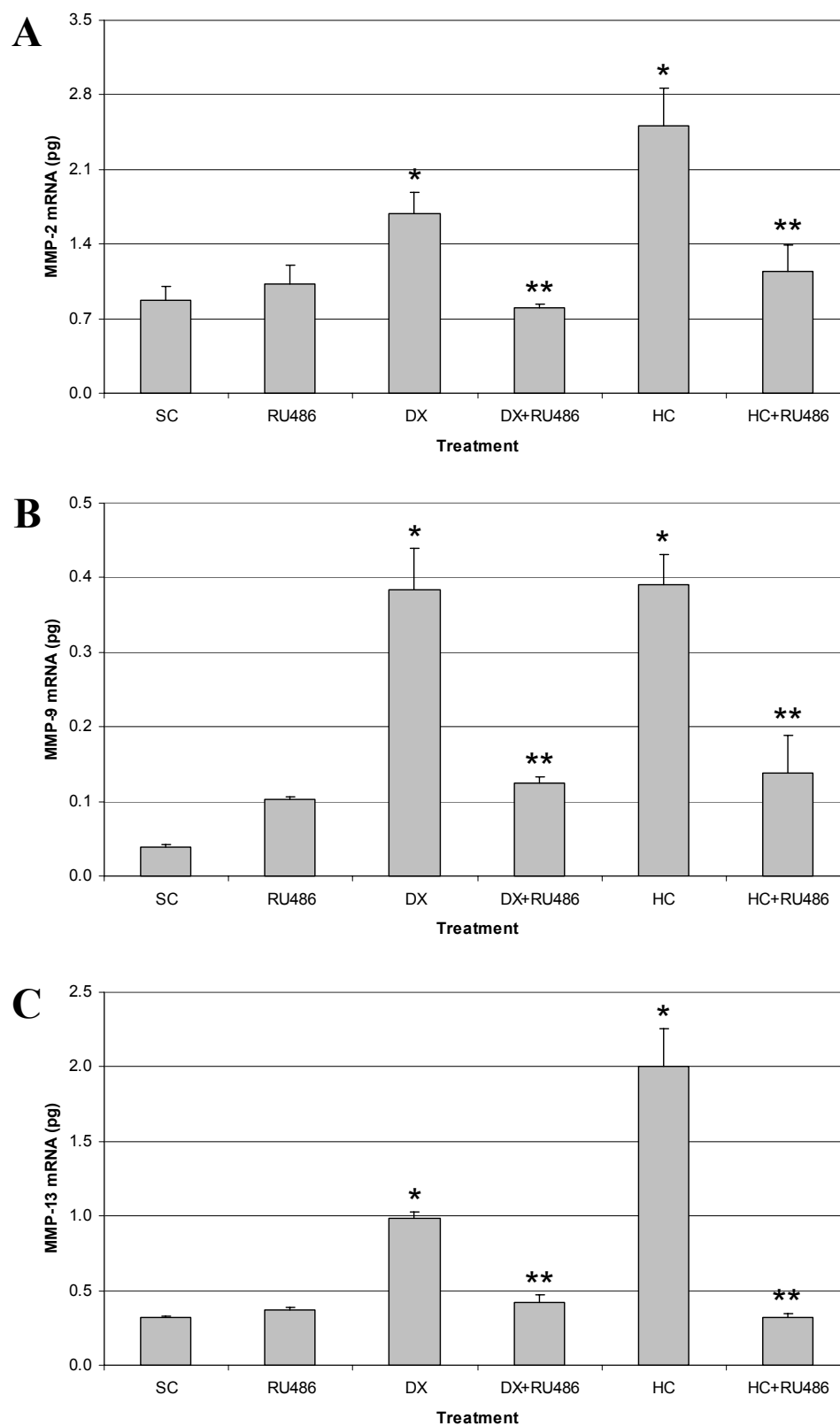
Figure 5.4. Continued on next page.

Figure 5.4. RU486 prevents increases in MMP transcript following exposure to glucocorticoids. Zebrafish embryos were exposed to treatment for 72 hours and RNA was isolated for use as template in quantitative RT-PCR. Data were quantified by utilizing standard curves generated from specific PCR products at concentrations ranging from 1 ng to 500 fg. Data were normalized to β-Actin. PCR reactions were performed in triplicate and data are representative of three separate experiments. A statistically significant increase (* $p < 0.05$) in **(A)** MMP-2 **(B)** MMP-9 and **(C)** MMP-13 expression is observed following glucocorticoid treatment as compared to the solvent control. A statistically significant decrease (** $p < 0.05$) in expression levels is observed following co-treatment with RU486. The RU486-only control does not show any change in expression levels compared to the solvent control. Statistical significance was determined via One-Way ANOVA and the Bonferroni *t*-test. Error bars denote standard deviation.

A

SC	+	–	–	–	–
DX	–	+	+	–	–
HC	–	–	–	+	+
RU486	–	–	+	–	+

B

SC	+	–	–	–	–
DX	–	+	+	–	–
HC	–	–	–	+	+
RU486	–	–	+	–	+
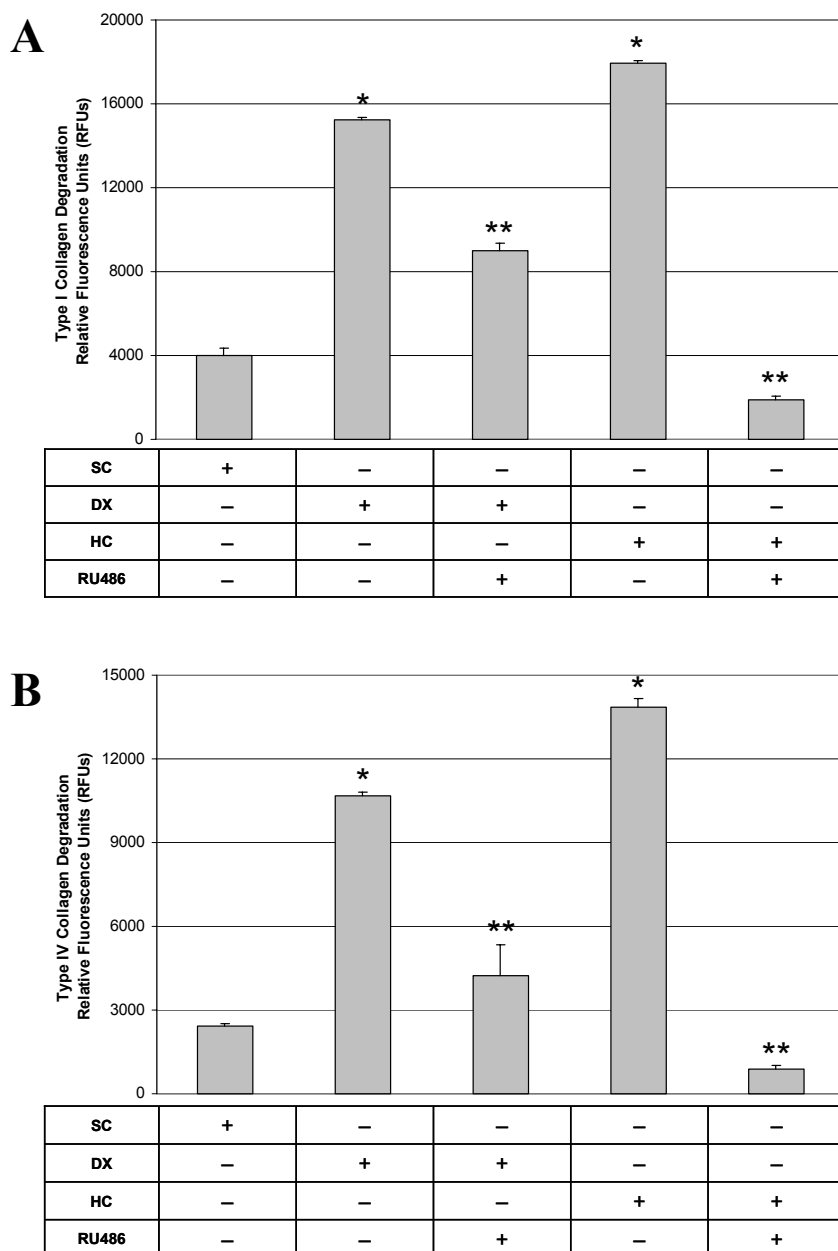
Figure 5.5. RU486 prevents increases in activity following exposure to glucocorticoids. Results of i*n vitro* zymography assays conducted on zebrafish embryo lysates to examine effects of RU486 on **(A)** type I collagen and **(B)** type IV collagen degradation. Type I collagen degradation is representative of collagenase (MMP-13) activity and type IV collagen degradation is representative of gelatinase (MMP-2, MMP-9) activity. A statistically significant increase (* $p < 0.05$) in type I or type IV collagen degradation is seen following glucocorticoid treatment compared to the solvent control. A statistically significant decrease (** $p < 0.05$) in activity levels is observed following co-treatment with RU486. Statistical significance was determined via One-Way ANOVA and the Bonferroni *t*-test. Error bars denote standard deviation.

A

B
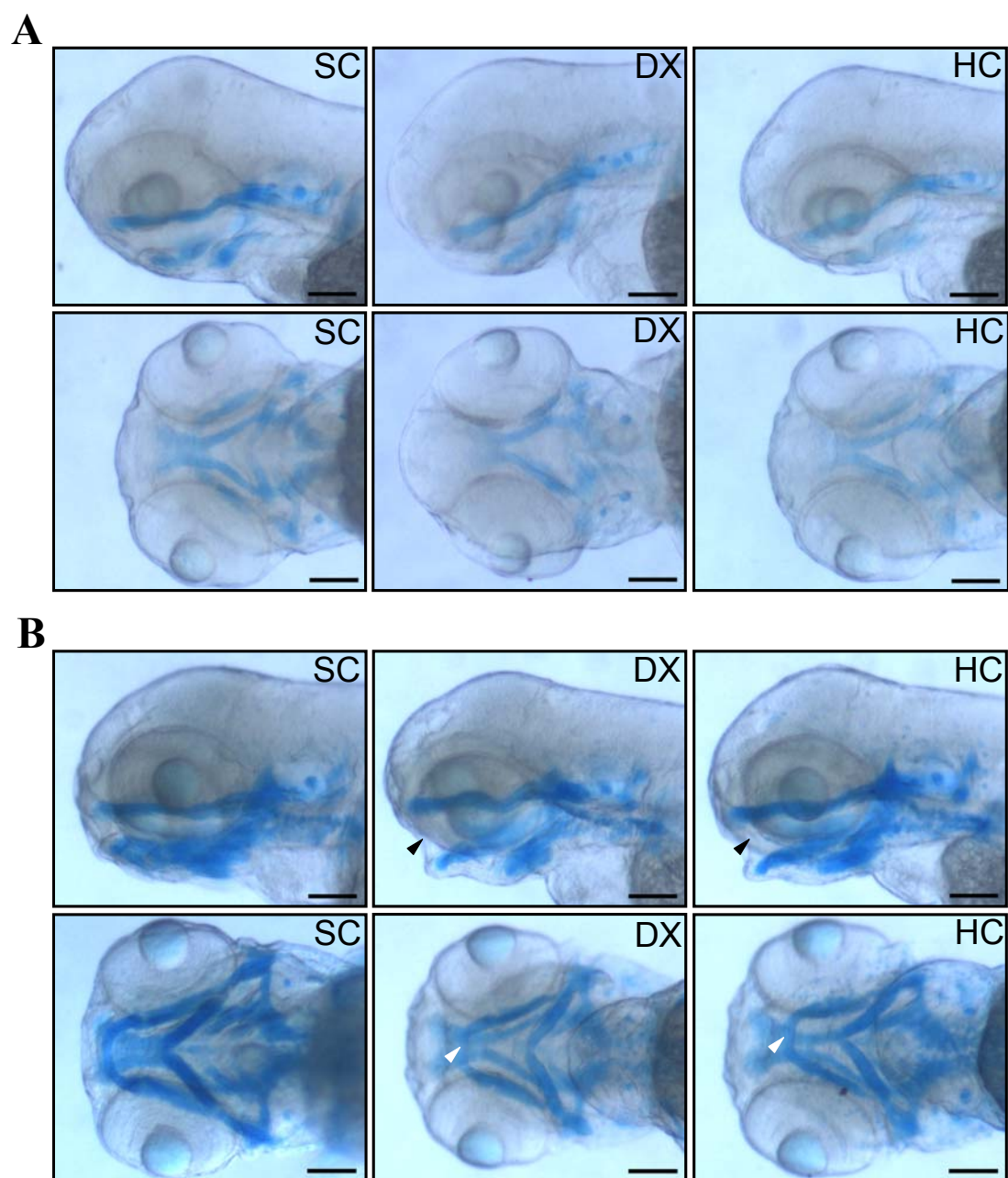
Figure 5.6. Continued on next page.
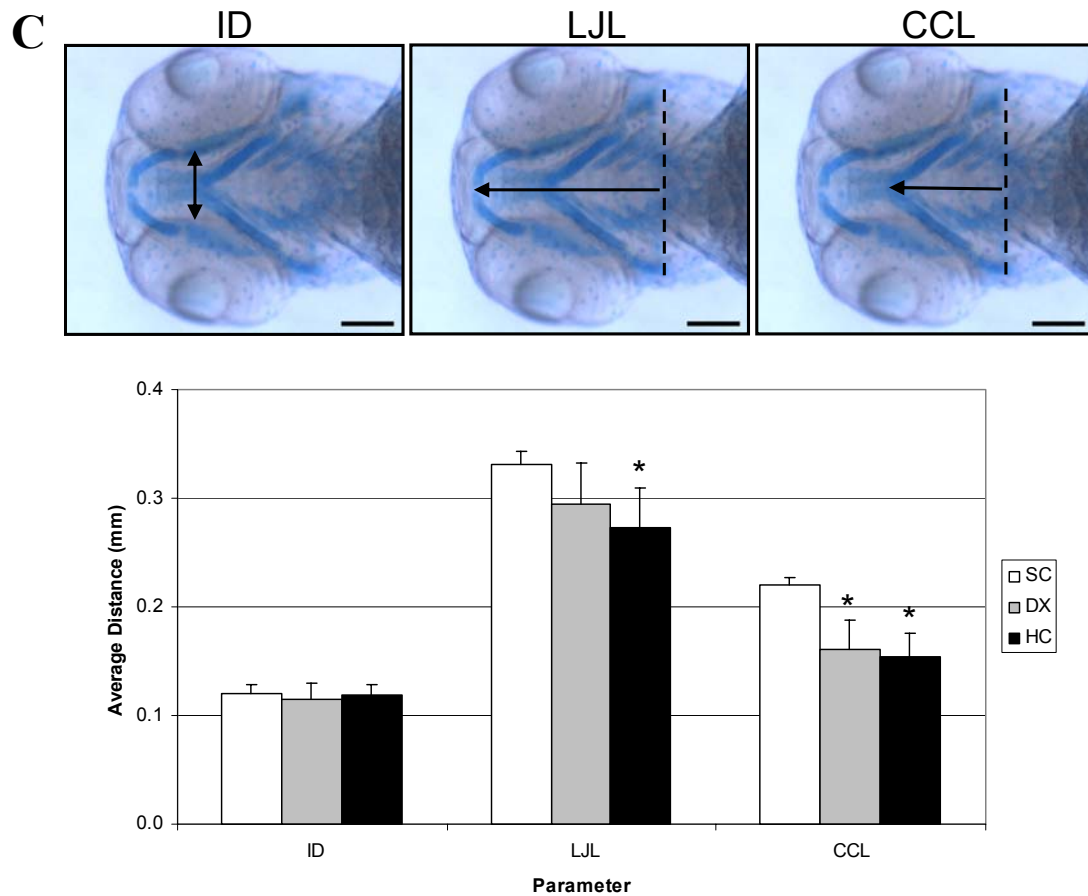
Figure 5.6. Altered craniofacial morphogenesis results from exposure to glucocorticoids. Treated embryos were stained with Alcian blue at **(A)** 72 hpf or **(B)** 96 hpf in order to better characterize the observed changes in craniofacial development following exposure to glucocorticoids. Black arrowheads indicate observed increases in the angle between the ethmoid plate and Meckel's cartilage. White arrowheads indicate observed changes in mandibular arch. **(C)** Following staining at 96 hpf, morphometric measurements were performed in order to determine differences in ID, LJL, and CCL. A statistically significant (* $p < 0.05$) decrease in LJL and CCL is observed in dexamethasone-treated embryos. Hydrocortisone-treated embryos possess a significantly smaller LJL only. Statistical significance was determined via One-Way ANOVA and Dunnett's test. Error bars denote standard deviation. Magnification: 63x (scale bar = 100 μm). ID, intercranial distance; LJL, lower jaw length; CCL, ceratohyal cartilage length.
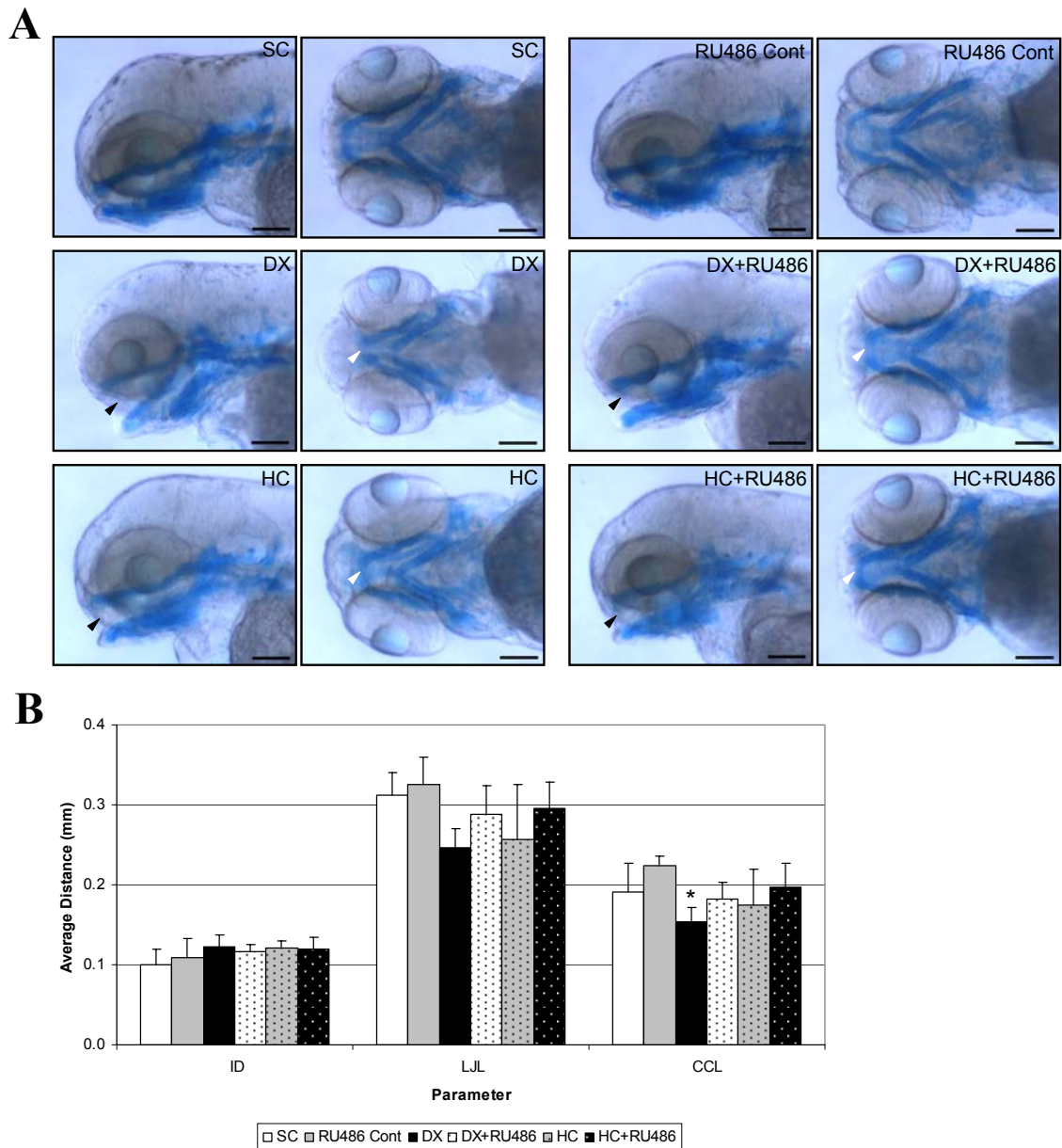
Figure 5.7. RU486 partially rescues abnormal craniofacial phenotypes observed following treatment with glucocorticoids. **(A)** Embryos were stained with Alcian blue at 96 hpf following treatment with dexamethasone or hydrocortisone alone or in conjunction with RU486. Black arrowheads indicate differences in the angle between the ethmoid plate and Meckel's cartilage. White arrowheads indicate differences in the migration of the mandibular arch. **(B)** Morphometric measurements reveal that DX treatment resulted in statistically significant decreases in CCL versus the RU486 Control (* $p < 0.05$) only. Statistical significance was determined via One-Way ANOVA and the Bonferroni t-test. Error bars denote standard deviation. Magnification: 63x (scale bar = 100 μm).

6.0. DISCUSSION

The objective of the studies presented here was to determine the role of MMP-2, MMP-9, and MMP-13 in normal zebrafish embryogenesis and to establish a causal link between activation of the GR via dexamethasone and hydrocortisone and changes in MMP expression and activity. Examining this topic is important for several reasons. First, from a human health perspective, short-term antenatal glucocorticoid therapy continues to be used to reduce fetal mortality, respiratory distress syndrome and intraventricular hemorrhage in preterm infants, despite questions of their efficacy and safety during certain periods of gestation (AHFS 2000; Sloboda *et al.* 2005). In addition, there exists a number of conditions during pregnancy that may require long-term administration of glucocorticoids, including asthma, chronic obstructive pulmonary disease, systemic lupus erythematosus, allografts, and inflammatory bowel disease (Pacheco *et al.* 2007). Given the lipophilicity and ability of this class of compounds to cross cellular membranes, fetal exposure is a reasonable concern. This is supported by studies showing long-term administration of glucocorticoids during pregnancy can result in fetal growth restriction and intrauterine programming of cardiovascular, metabolic and neuroendocrine disorders in adult life (Seckl 1994, 2004). Additional acute effects include cleft palate, hydrocephalus, gastroschisis, stillbirth, and premature birth (AHFS 2000). Of particular interest is cleft palate, which has been shown in embryonic mice as well, following exposure to hydrocortisone (Abbott 1995). However, the mechanism by which glucocorticoids cause teratogenicity is unknown. Therefore, the research presented here aimed to identify potential mechanisms in order to contribute to a better understanding of the efficacy and safety of antenatal glucocorticoid use. Adequate

reproductive studies with glucocorticoids have not been performed in humans, and the danger of these compounds can extend to neonates as well. Glucocorticoids can be distributed into the mother's milk, thereby causing suppressed growth and reduced endogenous glucocorticoid production in nursing infants (AHFS 2000).

This work is also important from an environmental health perspective as indicated by the recent public interest in pharmaceuticals in the environment (PIE). Pharmaceuticals and other personal care products have been receiving increasing attention from environmental and health agencies in both Europe and North America due to reports that they are ubiquitous in the environment (Daughton and Ternes 1999; Heberer 2002; Jones *et al.* 2002; Kolpin *et al.* 2002). Pharmaceuticals are of particular concern since they often possess similar types of physicochemical behavior characteristic of harmful xenobiotics – namely, the ability to pass membranes and biopersistence (Sanderson *et al.* 2004). Glucocorticoids are extensively prescribed for dermatological ailments such as atopic dermatitis and psoriasis due to their anti-inflammatory and immunosuppressant properties. Topical corticosteroids are also used for multiple dermatological conditions during pregnancy. Although systemic absorption has been demonstrated following topical administration (Pacheco *et al.* 2007), it is likely the large majority of topically applied drug is washed off and subsequently enters the sewage system. Systemically administered glucocorticoids may also be excreted unmetabolized in the urine or bile (AHFS 2000), while metabolites may be converted back to active compound via bacterial action (Jones *et al.* 2001). Recent studies have shown that many pharmaceuticals are incompletely eliminated at sewage treatment plants and thus, are able to enter surface water and ground (i.e. drinking) water (Jones *et al.* 2004). This situation

is aggravated by the fact that the majority of the general public dispose of unused drugs down the drain or via domestic refuse (Jones *et al.* 2001). Although evidence suggesting that pharmaceuticals exist in the environment in sufficient amounts to cause significant harm to humans has yet to be presented, some of these compounds – specifically, synthetic estrogens such as ethinylestradiol used in oral contraceptive pills – have been shown to have long-term sublethal effects on aquatic species and plants (Lyssimachou and Arukwe 2007). Appropriately, questions are being posed regarding the potential long term environmental fate and effects of pharmaceutical compounds, including glucocorticoids.

Despite an extensive literature base of studies examining environmental concentrations of various pharmaceuticals in sewage effluent, surface water, and drinking water, none contain data for dexamethasone or hydrocortisone. However, one study was identified which used quantitative structure-activity relationships (QSAR) to rank ~3000 pharmaceutical compounds in 51 classes relative to hazard potential toward algae, daphnids, and fish (Sanderson *et al.* 2004). This *in silico* prediction technique implemented the U.S. Environmental Protection Agency (USEPA) EPIWIN package, an interface program that transfers a single SMILES notation to ten separate structure estimation programs. EPIWIN has been extensively validated and is used by the USEPA, Danish, Canadian, and Dutch environmental protection agencies (Moore *et al.* 2003; Sanderson *et al.* 2004; Zeeman *et al.* 1995). The specific estimation program cited in the study described above was ECOSAR, which estimates aquatic toxicity based on structural similarity to chemicals for which aquatic toxicity data is available. ECOSAR predicted corticosteroids to be one of the most hazardous therapeutic classes, along with

cardiovascular, gastrointestinal, antiviral, anxiolytic sedatives, hypnotics and antipsychotics, and thyroid pharmaceuticals (Sanderson *et al.* 2004). Using this study as an example, the BIOWIN estimation program within EPIWIN was utilized to generate predictions for dexamethasone and hydrocortisone specifically. BIOWIN estimates the probability that a chemical under aerobic conditions with mixed cultures of microorganisms will biodegrade rapidly or slowly. It utilizes fragment constants developed using multiple linear and non-linear regressions. Using BIOWIN, both dexamethasone and hydrocortisone were predicted to "not biodegrade fast" in linear and non-linear models. Further, dexamethasone and hydrocortisone were predicted to undergo primary biodegradation in weeks and ultimate biodegradation in months. Primary biodegradation indicates significant alteration in the parent compound structure and ultimate biodegradation signifies complete degradation of the compound to carbon dioxide and water. Therefore, although empirical evidence that these compounds are present in the environment is not available currently, it appears they are capable of biopersistence based on *in silico* modeling.

Previous work examining the role of MMPs in zebrafish development has shown that various types of MMPs, including a gelatinase (MMP-2) and two isoforms of a membrane-type MMP (MT-MMP α and β), were required for proper zebrafish embryo development (Zhang *et al.* 2003b, c). The first hypothesis was based on these studies, which stated that MMP-2, MMP-9, and MMP-13 would be required for normal zebrafish embryogenesis, and that knocking down these genes via antisense MOs would result in abnormal phenotypes. There was some overlap between the studies conducted in this laboratory and those in the published literature in terms of the role of MMP-2 and MMP-

9. Specifically, it has been demonstrated previously that MMP-2 was required for normal zebrafish embryogenesis and that knocking down this gene via antisense MOs resulted in poorly defined somites, craniofacial defects, pericardial edema and a truncated anterior-posterior axis (Zhang *et al.* 2003b). However, confirmation of these findings in the zebrafish strain (AB) used in this laboratory was necessary. In addition, studies have already been conducted on MMP-2 and MMP-9 using *qualitative* RT-PCR and *in situ* hybridization to look at gene expression patterns and transcript localization, respectively. However, levels of gene expression for MMP-2 and MMP-9 have never been examined on a *quantitative* basis. Further, *in situ* analysis for MMP-2 has only been completed through 24 hpf (Zhang *et al.* 2003b). Therefore, another goal was to explore gene expression patterns for MMP-2 and MMP-9 on a quantitative basis using quantitative RT-PCR. Additionally, the potential existed to provide a better understanding of where MMP-2 and MMP-9 transcript was being expressed during late embryogenesis by performing *in situ* hybridization studies on 72 hpf embryos.

To examine whether MMP-2, MMP-9, and MMP-13 were required for normal zebrafish embryogenesis, this laboratory first looked at whether or not these particular MMPs were actually expressed during key stages of development. Quantitative RT-PCR revealed that MMP-2, MMP-9, and MMP-13 were all present during zebrafish embryogenesis (0-96 hpf), albeit with variable expression patterns (Figures 3.2 and 4.1). MMP-2 levels gradually increased from 6 hpf to 96 hpf, suggesting that there was little maternal transfer of MMP-2 transcript and that this MMP may play a larger role in late embryonic/early larval development. These results were similar to the qualitative pattern of MMP-2 expression described previously, which showed a gradual increase in

transcript signal from the 16-cell stage to 24 hpf (Zhang *et al.* 2003b). Interestingly, MMP-9 and MMP-13 exhibited similar expression patterns. Both showed high levels of maternal transcript transfer, a minimum level of expression at 12 hpf and maximum expression at 48 hpf. There are a large number of changes occurring at 48 hpf, most notably pectoral fin elongation and chondrocyte differentiation within the fin and pharyngeal arches (Hall and Miyake 1992; Kimmel *et al.* 1995; Schilling and Kimmel 1997). These MMPs most likely contribute to these processes as a result of their ability to cleave multiple collagen types (Chakraborti *et al.* 2003; Visse and Nagase 2003). Also, recall that activated MMPs can serve as pro-MMP activators *in vivo* (Cowell *et al.* 1998). For example, MMP-13 is capable of initiating activation of both MMP-2 and MMP-9 (Chakraborti *et al.* 2003). Therefore, if expressed proximally in the developing zebrafish embryo, this similarity in expression pattern could be the result of local activation of MMP-9 by MMP-13. In fact, *in situ* hybridization experiments showed that both MMP-9 and MMP-13 were highly expressed in the head mesenchyme (Figures 3.7 and 4.2). These comparable expression patterns could also be the result of activation by other MMPs, such as MMP-3 or MMP-10, both of which have been shown to activate MMP-9 and MMP-13 (along with MMP-1, MMP-7, and MMP-8) (Chakraborti *et al.* 2003). However, further study would be required to determine when and where these additional MMPs are expressed during zebrafish embryogenesis.

Use of antisense MOs to block translation of MMP-2, MMP-9, and MMP-13 demonstrated that these MMPs are required for normal zebrafish embryogenesis. MMP-2 morphants exhibited a severely truncated anterior-posterior axis, pericardial edema, and altered craniofacial morphogenesis (Figure 4.4), all of which are phenotypes that have

been reported previously, confirming that differences between the zebrafish strains utilized were minimal (Zhang *et al.* 2003b). In addition, these phenotypes are consistent with the phenotypes described for MMP-2 knockout mice, namely smaller stature and craniofacial abnormalities (Itoh *et al.* 1997; Mosig *et al.* 2007). Injection of MMP9-MO alone was well tolerated, and phenotype changes were only observed following co-injection of MMP2-MO (Figure 4.4). This was believed to be due to the ability of MMP-2 to compensate for loss of MMP-9 function, and was supported by this laboratory's data showing that the relative abundance of MMP-9 transcript in the developing zebrafish embryo was one to two orders of magnitude less than MMP-2 as determined via quantitative RT-PCR (Figure 4.1). In addition, several other studies reported in the literature have shown that compensatory pathways between these gelatinases may directly influence the endpoint or phenotype of interest (Agrawal *et al.* 2006; Lambert *et al.* 2003; Vu *et al.* 1998). MMP-13 morphants exhibited epithelial hyperplasia, pericardial and yolk sac edema, body axis curvature, and kinked tails (Figure 3.4). The phenotype changes related to axis formation (i.e. body axis curvature, kinked tails) are representative of MMP-13 knockout mice, which have been shown to display skeletal dysmorphogenesis (Inada *et al.* 2004). The ability of zebrafish morphants to mimic what is seen in knockout mice truly speaks to the usefulness of this model system in studying development and, given their fully sequenced genome, suggests zebrafish would serve as a valuable model to complement the mouse studies that are typically conducted.

For all of the morphants generated during these studies, changes in craniofacial structure and tailfin actinotrichia were observed. Alcian blue staining of craniofacial cartilage revealed that both MMP-2/MMP-9 double knockdown morphants and MMP-13

morphants possessed significantly decreased lower jaw and ceratohyal cartilage lengths (Figures 4.5 and 4.6). MMP-13 morphants also exhibited severe alterations in the shape of the neurocranial and pharyngeal cartilages, as well as loss of hyosymplectic and ceratobranchial cartilages. The ECM plays a critical role in craniofacial patterning, an event which involves both neural crest cell migration and cartilage morphogenesis (Henderson and Copp 1997; Kimmel *et al.* 1998; Kimmel *et al.* 2001; Perris 1997; Perris and Perissinotto 2000). Therefore these data suggest that loss of function in the MMP enzymes responsible for remodeling the ECM could potentially alter either migration of neural crest cells or cartilage differentiation and growth, resulting in aberrant craniofacial patterning. Modification of tailfin actinotrichia following MMP knockdown (Figure 4.4) is most likely the result of altering the remodeling status of one or more of the collagen types known to exist in the fin (Mari-Beffa *et al.* 2007). Taken collectively, these studies support the hypothesis that MMP-2, MMP-9, and MMP-13 are required for normal zebrafish embryogenesis.

There exists contradictory findings between multiple *in vitro* and *in vivo* models in terms of regulation of MMPs by glucocorticoids, and it appears that the ability of this class of steroid hormones to cause gene upregulation or downregulation may vary depending on cell type, MMP type, the presence of additional receptor molecules, or tissue remodeling status. These studies demonstrating that MMP gene expression is differentially regulated by glucocorticoids, taken along with the fact that glucocorticoids are known teratogens, led us to the second hypothesis. Specifically, that exposure to the glucocorticoids dexamethasone or hydrocortisone would alter MMP-2, MMP-9, and MMP-13 expression and activity in developing zebrafish embryos, thereby leading to

abnormal development. The studies described here represent the first time regulation of MMPs by glucocorticoids was examined in an *in vivo* model of development.

Initial dose-response experiments used concentrations of dexamethasone and hydrocortisone ranging from 1 to 100 mg/L. A dose of 100 mg/L (equivalent to ~255 μM dexamethasone and ~276 μM hydrocortisone) was eventually designated as the concentration with which to conduct all future studies since it consistently generated changes in both genotype and phenotype following acute exposure. This is a relatively high concentration, although use of these compounds in zebrafish embryos at micromolar levels has been reported previously (Langenau *et al.* 2005; To *et al.* 2007; Tseng *et al.* 2005). In considering PIE specifically, it is highly unlikely that this concentration of glucocorticoid would be found in surface water or drinking water. However, one must consider the potential long-term effects low-level, chronic exposure may have on both humans and various aquatic species. Given the lipophilic nature of these compounds, exposures to minute quantities of drug over an extended period of time could result in significant bioaccumulation of parent compound and/or metabolites. It is also possible that environmentally relevant concentrations of glucocorticoids, when mixed with similar concentrations of other pharmaceuticals, could have additive or synergistic adverse effects. The question of whether this dose could potentially reach a developing fetus is complicated and would depend on both route of administration (intravenous, inhalation, topical, etc.) and the initial pharmacologic dose administered. The human adrenal cortex produces approximately 20 mg of cortisol daily, however pharmacologic doses (i.e. any dosage greater than this physiologic dose of 20 mg) can be as high as 15-30 mg/kg daily depending on glucocorticoid type (AHFS 2000). Topical doses of glucocorticoids can

actually be much higher, although the amount absorbed systemically is considerably less than the dose administered due to the barrier function of skin. Regardless, these studies were designed as proof-of-principle and a dose of 100 mg/L was effective at generating the phenotypic and genotypic endpoints needed to study the interaction of glucocorticoids and MMPs.

Both dexamethasone and hydrocortisone were capable of inducing MMP-2, MMP-9, and MMP-13 gene expression at 72 hpf as determined via quantitative RT-PCR (Figures 3.6 and 5.1). These results were confirmed via *in situ* hybridization studies, which demonstrated that increased expression of these MMPs typically occurred in the medial and lateral aspects of the trunk, heart, anterior kidney, and various components of the head mesenchyme including the neurocranial and pharyngeal cartilages (Figures 3.7 and 5.2). It is interesting to note that for MMP-13, the localization of transcript signal varied depending on glucocorticoid treatment. This may be indicative of differences in the GR variant expressed in specific tissues of the developing embryo, or may simply be due to differences in the pharmacokinetics of dexamethasone and hydrocortisone. Variability in MMP-2 transcript locale was not observed and it was not possible to distinguish explicit differences in the transcript locale for MMP-9 due to the intensity of signal following glucocorticoid treatment. Glucocorticoid-induced MMP-2, MMP-9, and MMP-13 expression was followed by an increase in gelatinase and collagenase activity, respectively, as determined via *in vitro* zymography (Figures 3.8 and 5.3). Various inhibitors including the divalent cation chelator EDTA, the broad-spectrum MMP inhibitor GM6001, and two *N*-sulfonylamino acid inhibitors specific for MMP-2, MMP-9, and MMP-13 were used to demonstrate that these increases in activity were due to the

MMPs of interest. It should be noted that increases in transcript amounts do not always correlate with increase in protein levels. Therefore, showing increased metalloproteinase activity was extremely important since it provided direct evidence that the upregulation of MMP-2, MMP-9, and MMP-13 following glucocorticoid treatment resulted in functionally significant enzyme levels.

These increases in MMP expression and activity following glucocorticoid treatment resulted in numerous developmental abnormalities including altered somitogenesis, changes in the normal head-trunk angle, truncated body axis, pericardial and yolk sac edemas, alteration in yolk sac/yolk sac extension shape and size, and abnormal craniofacial morphogenesis (Figure 3.5 and Table 1). Since changes in the craniofacial region were consistently observed and served as an easily quantifiable endpoint, Alcian blue staining was utilized in order to provide a more detailed characterization of the modifications that were occurring in craniofacial cartilage following treatment. These assays revealed that dexamethasone and hydrocortisone were capable of altering parameters associated with both the mandibular and hyoid arches, particularly ceratohyal cartilage length, lower jaw length, and ethmoid plate/Meckel's cartilage orientation (Figure 5.6). These results suggested a potential impairment of the anterior growth and migration of the pharyngeal cartilages and pointed to dysregulation of the MMPs (subsequently leading to aberrant ECM remodeling) as a contributor to this abnormal craniofacial patterning.

These data corroborate the second hypothesis and demonstrate the complexity with which glucocorticoids regulate MMP expression and activity. An interesting detail that came out of these studies was the fact that some of the changes in phenotype

observed following antisense MO knockdown mimicked those observed with glucocorticoid treatment, particularly the changes observed in the neurocranial and pharyngeal cartilages. This indicates that it is not necessarily the overexpression or lack of MMPs that definitively causes these changes. Rather, merely dysregulation of MMP expression and activity can lead to appreciable modifications in development, most likely the result of alterations in ECM remodeling. This seems probable given that cell invasion through the ECM is a driving force for developmental processes (Mignatti and Rifkin 1993). This phenomenon has been shown in studies conducted in zebrafish MT-MMP α and β as well, whereby overexpression or MO knockdown of these MMP isoforms led to identical phenotypes consisting of various degrees of abnormal axis formation, including a truncated axis (Zhang *et al.* 2003c).

It is generally well accepted that glucocorticoid-associated effects in mammals and fish are mediated through the GR. Multiple GR genes have been identified in rainbow trout and Burton's mouthbrooder (Bury *et al.* 2003; Greenwood *et al.* 2003), and although only one GR gene has been characterized in the zebrafish thus far, it is likely that further examination will yield additional isoforms. That being said, the third hypothesis stated that glucocorticoid-induced teratogenesis would be mediated through the GR and that co-treatment with RU486 would attenuate changes in MMP expression and activity. RU486 is a GR receptor antagonist that acts by attaching directly to the receptor, subsequently eliciting a transconformational change in the DNA-binding domain that prevents the GR from binding to the GRE in the promoter region of target genes (Cadepond *et al.* 1997; Mahajan and London 1997). In addition, RU486 has been shown to competitively displace cortisol from high affinity binding sites (Pottinger 1990).

The data presented here demonstrate that co-treatment of zebrafish embryos with dexamethasone or hydrocortisone and RU486 inhibited the glucocorticoid-associated induction of MMP expression known to occur at 72 hpf (Figure 5.4). Co-treatment also resulted in attenuation of increases in type I or type IV collagen degradation, indicating the ability of RU486 to prevent formation of active protein by blocking transcription (Figure 5.5). Finally, RU486 was able to decrease the severity of craniofacial changes that typically occur following glucocorticoid treatment (Figure 5.7). These data imply that the actions of dexamethasone and hydrocortisone in the developing zebrafish are mediated through the GR, and by antagonizing receptor interactions, it is possible to prevent induction of MMP-2, MMP-9, and MMP-13, thereby confirming the third hypothesis. However, further study is required in order to determine the exact mechanism(s) by which the GR and MMPs interact. Given the extensive amount of literature available regarding the promoter regions of the various MMPs, it does not seem likely that zebrafish MMP promoters contain GREs, although luciferase reporter studies could be conducted to confirm this absence. It is possible that these glucocorticoids could be acting through some soluble factor such as the sGITR. Recall that glucocorticoids have been shown to stimulate MMP-9 expression through induction of sGITR in murine macrophages (Lee *et al.* 2004; Lee *et al.* 2003). As a better understanding of the true complement of MMPs present and active in zebrafish becomes available, so will an appreciation of the complexity of their regulation. In terms of regulation by glucocorticoids specifically, this will be aided by a better characterization of both the number and differential expression patterns of various GR isoforms in the zebrafish.

Collectively, these studies show that MMP-2, MMP-9, and MMP-13 are required for proper zebrafish embryogenesis and that by changing their expression through glucocorticoid exposure, it is possible to elicit teratogenic effects. Therefore, in humans, glucocorticoid-induced teratogenicity could partially be due to modulation of MMP levels *in vivo*. From a clinical perspective, using this model to define the genetic, molecular, and physiological events occurring during the complex process of craniofacial development may aid in identifying targets for *in utero* therapy of such ailments as cleft lip and palate. This is significant since three fourths of all congenital birth defects involve craniofacial malformations (Chai and Maxson 2006). A large number of synthetic MMP inhibitors (MMPIs) have been developed based on the amino acid sequence of the cleavage site for various ECM substrates. MMPI classes include hydroxamic acid derivatives, tetracycline derivatives, carboxylates, organoborates, and dithiolates (Ganea *et al.* 2007). Synthetic MMPIs have exhibited efficacy in animal models of cancer and arthritis, although clinical trials have not been as successful (reviewed in (Coussens *et al.* 2002)). Approximately 82% of those MMPI candidates implemented in clinical trials have been discontinued, and from the seven compounds remaining in trial, only one (Periostat®) has been approved (Ganea *et al.* 2007). This limited success has been attributed to poor bioavailability, low specificity, high toxicity, and administration to patients only with advanced stages of disease (whereas preclinical models received MMPIs at early disease stages) (Coussens *et al.* 2002; Ganea *et al.* 2007). Despite these setbacks, use of MMPIs to prevent the initiation and progression of diseases associated with aberrant ECM remodeling continues to be an attractive therapeutic strategy.

7.0. REFERENCES

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8.0. CURRICULUM VITAE

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Education

Sept. 2002 – Jan. 2008	**Rutgers, The State University of New Jersey & University of Medicine and Dentistry of New Jersey** Doctor of Philosophy Joint Degree in Toxicology January 2008
Sept. 2000 – May 2003	**Rutgers, The State University of New Jersey** Master of Science Degree in Environmental Science May 2003
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Research Experience

Sept. 2004 – Aug. 2007	**Rutgers, The State University of New Jersey & University of Medicine and Dentistry of New Jersey** Graduate Assistant
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Work Experience

June 2001 – May 2007	**Bristol-Myers Squibb Company** Intern – Occupational and Environmental Toxicology, Corporate Quality, Environmental, Health and Safety Department
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Publications

Hillegass, J.M., Villano, C.M., Cooper, K.R., and White, L.A. 2007.   Matrix metalloproteinase-13 (MMP-13) is required for zebrafish (*Danio rerio*) development and is a target for glucocorticoids. *Toxicological Sciences* 100 (1): 168-179.

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