# CYCLOOXYGENASE-1 DERIVED PROSTAGLANDIN E2 ($PGE_2$) SIGNALING IN EARLY DEVELOPMENT

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CELL AND DEVELOPMENTAL BIOLOGY

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Dissertation under the direction of

Professors Lilianna Solnica-Krezel and Raymond N. DuBois

Prostaglandin G/H synthases (PGHS), commonly referred to as cyclooxygenases (COX-1 and COX-2), catalyze a key step in the synthesis of biologically active prostaglandins (PGs), the conversion of arachidonic acid (AA) into prostaglandin $H_2$ ($PGH_2$). PGs have important functions in a variety of physiologic and pathologic settings, including inflammation, cardiovascular homeostasis, reproduction, and carcinogenesis. However an evaluation of prostaglandin function in early mammalian development has been difficult due to the maternal contribution of prostaglandins from the uterus. The emergence of zebrafish as a model system has begun to provide some insights into the roles of this signaling cascade during vertebrate development. In zebrafish, COX-1 derived prostaglandins are required for two distinct stages of development, namely during gastrulation and segmentation. During gastrulation, $PGE_2$ signaling promotes cell motility, without altering the cell shape or directional migration of gastrulating mesodermal cells via the G-protein coupled prostaglandin E2 receptor (EP4). During segmentation, COX-1 signaling is also required for posterior mesoderm development,

including the formation of vascular tube structures, angiogenesis of intersomitic vessels, and pronephros morphogenesis. We propose that deciphering the role for prostaglandin signaling in zebrafish development could delineate mechanistic details underlying various disease processes that result from perturbation of this pathway and uncover novel potential therapeutic targets.

To my late grandmother, Soo-Ae Cho,
whose sacrifice and dedication inspires me daily

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# ABBREVIATIONS

PG, prostaglandin;

COX, prostaglandin endoperoxide synthase;

Ptges, microsomal $PGE_2$ synthase;

$PGE_2$, prostaglandin $E_2$;

$PGI_2$, prostaglandin $I_2$;

$PGF_{2\alpha}$, prostaglandin $F_{2\alpha}$;

WT, wild-type;

MO, morpholino;

AP, anterior-posterior;

EP, prostaglandin $E_2$ receptor;

Indo, Indomethacin;

MPO: myeloperoxidase;

E1A: beta-globin E1

ICM: intermediate cell mass

# CHAPTER I

## INTRODUCTION

Prostanoids, comprised of prostaglandins (PGs) and thromboxanes (TXs), are oxygenated metabolites of C20 polyunsaturated fatty acids including arachidonic and eicosapentaenoic acids that are released from membrane phospholipids. Prostaglandins have been shown to play important roles in various physiological processes in invertebrates (Rowley et al., 2005; Stanley, 2000), vertebrates (Cha et al., 2005; Evans and Gunderson, 1998; Grosser et al., 2002; Yang et al., 2002) and mammals (Langenbach et al., 1999; Narumiya et al., 1999), indicating that these bioactive lipid molecules are evolutionarily conserved. Until now, most of the studies evaluating prostaglandin signaling have come from mammalian models. Analyses of genetic knockouts of cyclooxygenases and prostaglandin signaling components have led to significant progress in this field. This introduction will summarize the recent advances in the field of prostaglandin biology with a particular focus on the developmental role of these lipid mediators. In addition, we propose that zebrafish may provide a very useful model system to elucidate the precise role of prostaglandin signaling pathways and their biological endpoints in early development.

### Historical perspective

#### *Discovery of prostanoids*

Seminal work in understanding the prostaglandin biosynthetic pathway elucidated the mechanism by which aspirin exerts its anti-inflammatory, analgesic, and antipyretic actions. In 1971, Sir John Vane showed that aspirin and other non-steroidal anti-inflammatory drugs (NSAIDs) inhibit the activity of the enzyme now known as cyclooxygenase (COX), leading to the formation of prostaglandins (PGs) that cause inflammation, swelling, pain, and fever (Vane, 1971). Since this discovery there has been a fervent interest in understanding how cyclooxygenase signaling functions in both physiological and pathophysiological conditions.

Long before we knew how aspirin worked, Kurzroc and Lieb first determined there was some biologic activity in semen when they observed violent contractions of the uterus following artificial insemination (Kurzrok, 1930). In 1933 Goldblat reported that human semen contained a substance that both reduced blood pressure and stimulated smooth muscle (Goldblatt, 1933; Goldblatt, 1935). At about the same time von Euler and colleagues showed that a lipid soluble factor from semen could decrease blood pressure and stimulate smooth muscle contractions as well (Euler, 1935). He named this substance "prostaglandins" assuming that it came from the prostate gland. Later, others were to find that this is not the case.

In the 1950s, Bergström, *et al.*, made a breakthrough in the field by purifying the chemical compound of two major prostaglandins, PGE and PGF. He subsequently demonstrated that prostaglandins are formed from unsaturated fatty acids, primarily from arachidonic acid (Bergstrom, 1967). In the 1960s, work by Samuelsson revealed the processes by which prostaglandins are metabolized and demonstrated the existence of two distinct pathways originating from arachidonic acid, one pathway leading to

prostanoid formation, the other to the formation of leukotrienes (Bergstroem and Samuelsson, 1965).

Not only did Vane's discovery lead to an improved understanding of how prostaglandins are synthesized, he provided the mechanistic insight that the NSAIDs bind directly to cyclooxygenase to inhibit prostaglandin production (Vane, 1971). The mechanism of NSAID action was to either bind covalently to the active sites (aspirin) or to compete with substrate for the binding to the active site (most of the other NSAIDs) of the cyclooxygenase enzyme. In 1982, "for their discoveries concerning prostaglandins and related biologically active substances," Bergström, Samuelsson, and Vane shared the Nobel prize in Medicine (Raju, 1999).

### *Discovery of cyclooxygenase*

Subsequently, extensive advances in molecular biology led to the isolation of *Cox-1* cDNA, corresponding to a genetic locus that spanned 11 exons, covered 25 kb, and encoded a 2.8–3.0 kb mRNA and 68-kDa protein (Yokoyama et al., 1988). Interestingly, the levels of *Cox-1* mRNA did not vary greatly in cell stimulation experiments. In fact, COX-1 was expressed constitutively in many tissues, including the kidney, lung, stomach, duodenum, jejunum, ileum, colon, and cecum of the rat, dog, Rhesus monkey, and human (Kargman et al., 1996). These findings led to the hypothesis that COX-1 is the "housekeeping" enzyme responsible for basal, constitutive prostaglandin synthesis. For example, prostaglandins such as $PGE_2$ and $PGI_2$ appear critical for maintaining physiologic homeostasis.

The existence of a second cyclooxygenase was proposed when sheep epithelial cells were found to exhibit increased prostaglandin production following stimulation with growth factors (Rosen et al., 1989). This also correlated with an increase in the amount of 4.0 kb mRNA that hybridized with *Cox-1* cDNA. A fervent search commenced to identify a second cyclooxygenase enzyme, which was reported by at least three groups. In 1991, Simmons and colleagues identified COX-2 from *src*-transformed chicken fibroblasts, and determined that its sequence was 60-65% similar to that of sheep, mouse, and human COX-1 protein (Xie et al., 1991). *Cox-2* expression was also detected in *src*-transformed fibroblasts and in mouse fibroblasts treated with the tumor promoter tetradecanoylphorbolacetate (TPA) (Kujubu et al., 1991) and human endothelial cells or monocytes treated with TPA or lipopolysaccharide (LPS) (Hla and Neilson, 1992b; O'Banion et al., 1991). Most tissues did not express *Cox-2* constitutively, but various intracellular and extracellular stimuli including cytokines, mitogens, growth factors, and tumor promoters could rapidly induce *Cox-2* expression in many different cell lines. Researchers then began to postulate that COX-2 was an "inducible" enzyme that produces prostaglandins in a wide variety of inflammatory and carcinogenic settings. Hence, COX-2-selective inhibitors, such as Celebrex® and Vioxx®, were developed to treat various inflammatory diseases and later evaluated for their potential as chemopreventive agents (Marnett and DuBois, 2002).

## Eicosanoid Metabolism

Synthesis of prostanoids is initiated by the rate-limiting release of arachidonic acid from the plasma membrane by phospholipase A2. Arachidonic acid is then converted to $PGH_2$ by the enzymatic, two-step action of prostaglandin G/H synthase-1 or -2 (PGHS), commonly referred to as cyclooxygenase-1 and -2 (Fig 1). COX enzymes reside in the endoplasmic reticulum (ER) and nuclear membrane with the substrate-binding pocket precisely orientated to take up the released arachidonic acid. $PGH_2$ is isomerized by various tissue specific prostaglandin synthases to form active lipid molecules. Aside from the prostanoid pathway, arachidonic acid can also serve as a substrate for the formation of hydroxyeicosatatraenoic acids (HETEs), leukotrienes, and lipoxins by the action of lipooxygenase enzymes. A third pathway leads to production of epoxyarachidonic acid via the enzymatic activity of cytochrome P450 monooxygenase enzymes. The metabolic products from the three pathways are collectively known as eicosanoids from the Greek root of *eikosi*, meaning "twenty" for the 20-carbon fatty acid of arachidonate.

The crystal structures of COX-1 and COX-2 are remarkably similar, with one notable amino acid difference that allows for a larger "side-pocket" for substrate access in COX-2 (Smith et al., 2000). $PGH_2$ is subsequently converted to one of several structurally related prostaglandins, including $PGE_2$, $PGD_2$, $PGF_{2\alpha}$, $PGI_2$ (also known as prostacyclin), and $TxA_2$ (thromboxane $A_2$), by specific PG synthases (Funk, 2001). The coupling of $PGH_2$ synthesis to metabolism by downstream enzymes is intricately orchestrated in a cell-specific fashion. For example, thromboxane synthase is found mainly in platelets and macrophages, and $TxA_2$ stimulates platelet aggregation and vessel constriction. Whereas prostacyclin synthase is expressed in endothelial cells, its product,

$PGI_2$, is known for its cardioprotective properties that cause platelet de-aggregation and vessel dilation. PGF synthase is found in the uterus, and two types of PGD synthase are in brain and mast cells. Two PGE synthases have been described: microsomal PGE synthase (mPGES), a member of the MAPEG (Membrane-Associated Proteins in Eicosanoid and Glutathione metabolism) family (Schneider et al., 2004), and cytosolic PGE synthase (cPGES). These two PGESs largely account for $PGE_2$ synthesis (Fig. 1) in mammals.

## Signaling Downstream of Cyclooxygenase in Mammals

There are at least 9 known prostaglandin receptors in mammals, as well as several additional splice variants with divergent carboxy termini (Narumiya et al., 1999). Four of the receptor subtypes bind $PGE_2$ (EP1–EP4); two bind $PGD_2$ (DP1 and DP2) and the FP, IP, and TP receptors bind $PGF_{2\alpha}$, $PGI_2$, and $TxA_2$, respectively. The prostaglandin receptors are categorized as three clusters of a distinct subfamily in the G protein–coupled receptor (GPCR) superfamily of seven transmembrane-spanning proteins. The lone exception is DP2, a member of the chemoattractant receptor subgroup. The "stimulatory" receptors including IP, DP1, EP2, and EP4 that signal through Gs-mediated increases in intracellular cyclic adenosine monophosphate (cAMP) form one cluster (Hirata et al., 1994a; Honda et al., 1993; Namba et al., 1994; Regan et al., 1994); the receptors EP1, FP, and TP form a second group that signals through Gq-mediated increases in intracellular calcium (Graves et al., 1995; Hirata et al., 1991; Namba et al.,

1993). Lastly, the EP3 receptor is regarded as an "inhibitory" receptor as it couples to Gi to decrease cAMP formation (Sugimoto et al., 1992).

Other mechanisms, besides ligand-receptor binding on the plasma membrane, have been implicated in prostaglandin signaling. For example, there is evidence that EP receptors can be internalized into the nucleus (Bhattacharya et al., 1999; Bhattacharya et al., 1998). EP3 and EP4 receptors have been shown to regulate gene transcription by modulating the release of calcium from nuclear calcium pools or by activation of calcium channels (Desai et al., 2000). In addition, prostaglandin metabolites have also been shown to activate transcriptional targets directly by binding to peroxisome proliferator-activated receptors (PPARs), which are members of the orphan nuclear receptor superfamily that constitutively bind to the promoter elements of their target genes (Forman et al., 1997). $PGI_2$ and $PGJ_2$ (a metabolite product of $PGD_2$) have been shown to bind $PPAR_\delta$ and PPARγ, respectively, for direct activation of transcriptional targets. In view of its physiologic significance, gaining a detailed understanding of prostaglandin signaling becomes an especially important area of research.

## Cyclooxygenases and Embryonic Development

Even with our advanced understanding of the prostaglandin signaling we have not elucidated the precise role of these bioactive lipids during embryogenesis. So far most of the work in non-mammalian systems has been focused on identification of functional COX homologues. PGs and other eicosanoids have been identified in both invertebrates and vertebrates. In invertebrates, eicosanoids mediate cellular immunity, play a role in fertility and regulate febrile response (Stanley, 2005). Although COX-like enzymes have

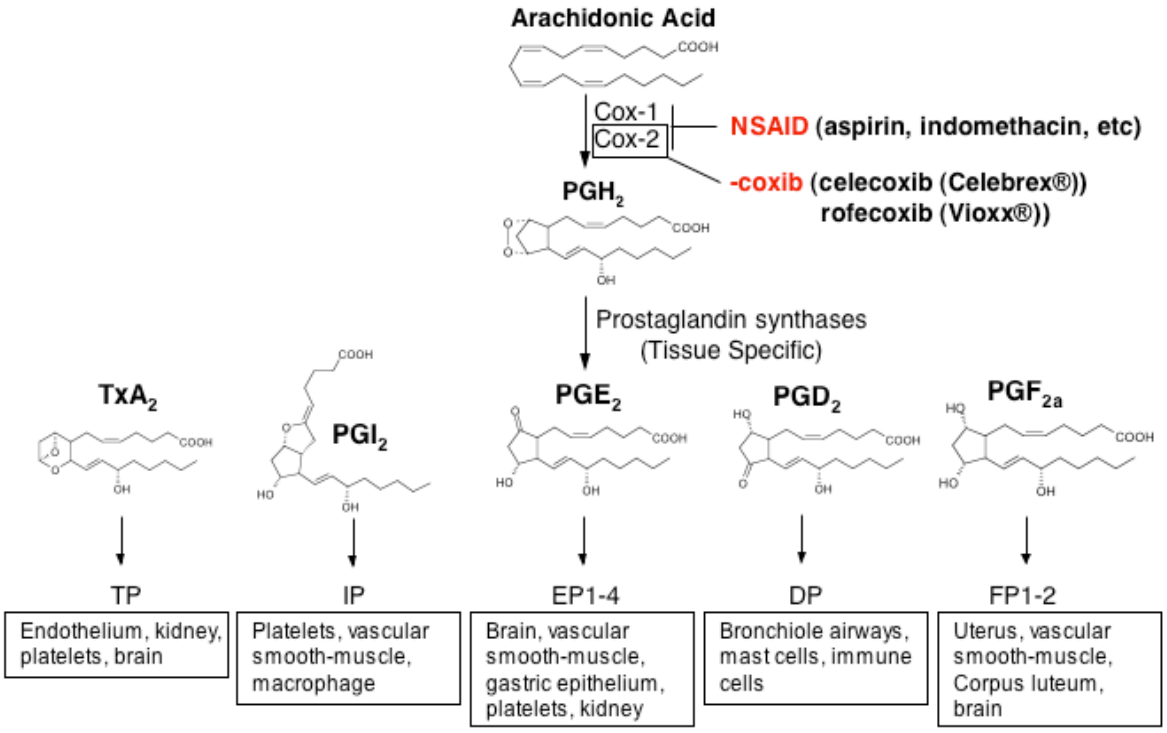


**Figure 1. Prostanoid biosynthetic pathway.** NSAIDs are non-selective inhibitors of cyclooxygenase enzymes, while -coxibs are selective inhibitors of COX-2. Prostaglandin synthases are distributed specifically in various tissues, and boxed inset reveals the spatial expression of prostaglandin receptors in various tissue types.

been identified in invertebrates the exact mechanism of prostanoid biosynthesis is still under debate (Rowley et al., 2005). In vertebrates, a COX-2 homologue has been cloned from rainbow trout (Zou et al., 1999), and COX-1 and COX-2 homologues were identified from brook trout (Roberts et al., 2000) and dog fish shark (Yang et al., 2002). In addition, the zebrafish COXs were shown to be genetically and functionally similar to the mammalian counterparts (Grosser et al., 2002), indicating that the duplication of COX gene likely occurred before the emergence of cartilaginous fish.

In mice, inactivation of genes encoding COX enzymes via homologous recombination didn't reveal much insight into the developmental role for prostaglandins. In heterozygous matings, *Cox-1* null mice were born in the expected ratios and lived to a normal lifespan (Langenbach et al., 1995). Surprisingly, these mutant mice were quite healthy given the putative "housekeeping" function of the COX-1 enzyme. However, *Cox-1* null female mice demonstrated prolonged gestation periods and delayed parturition that ultimately resulted in a reduction in the number of viable progeny. The defective reproduction due to the loss of COX activity could be suppressed by the administration of $PGF_{2\alpha}$ (Gross et al., 1998). This line of investigation suggested that COX-1 function was dispensable for ovulation, fertilization, and implantation of mouse embryos, but indispensable for fulfilling the normal labor term.

*Cox-2* null mice exhibited numerous developmental defects (Dinchuk et al., 1995). Only 60% of the pups survived to weaning, and of those, only 75% survived to one year of age. Death after three weeks of age was mainly due to gastric peritonitis or kidney dysfunction, which suggested that certain somatic tissues require COX-2 derived prostaglandins for normal homeostasis. In addition, *Cox-2* null females also displayed

multiple reproductive defects, including aberrant ovulation, fertilization, implantation, and decidualization, indicating that COX-2 derived prostaglandins are also critical for multiples stages of reproduction (Lim et al., 1997). Indeed, exogenous $PGE_2$ treatment can rescue the defects in ovulation in COX-2 deficient mice, demonstrating that proper follicle expansion and ovulation requires the synthesis of $PGE_2$. Recently, the reproductive defects associated with *Cox-2* inactivation in C57BL/6J/129 mice was suppressed when the same mutation was introduced onto CD1 genetic background that had high endogenous expression of COX-1 in the reproductive organs (Wang et al., 2004b). This showed functional redundancy between COX enzymes and revealed that the levels of prostaglandins ultimately determine the success of reproduction cycle.

## Prostaglandin Receptors and Development

### *Reproduction*

The inactivation of genes encoding prostaglandin receptors by homologous recombination in mice revealed that IP-, EP1-, EP3-, EP4-, and TP-deficient females were essentially fertile, birthing embryos that develop normally. However, EP2-null female mice exhibited multiple reproductive defects (Hizaki et al., 1999; Kennedy et al., 1999; Tilley et al., 1999), delivering fewer pups than their wild-type counterparts, irrespective of the genotypes of mating males. Ovulation was slightly impaired and fertilization dramatically reduced in EP2-deficient mice, leading to the hypothesis that the reproductive failure during early pregnancy in COX-2-deficient mice was due to lack of $PGE_2$ ligands available to bind to the EP2 receptor (Hizaki et al., 1999; Matsumoto et al.,

2001). Gonadotropins induced the EP2 receptor and COX-2 expression in the cumulus and $PGE_2$ stimulated cumulus expansion by elevating cAMP (Hizaki et al., 1999). These studies demonstrated that the $PGE_2$ and EP2 receptor system work as a positive-feedback loop to induce the oophorus maturation required for fertilization, during and after ovulation.

$PGF_{2\alpha}$ has a well-recognized function for stimulating leuteolysis and uterine contraction, important for inducing labor and delivering pups during parturition, respectively. Consistently generation of FP-deficient female mice demonstrated that they do not undergo parturition (Sugimoto et al., 1997). Normally the luteolytic action of $PGF_{2a}$ is required in mice to diminish progesterone levels and thus permit the initiation of labor. In FP null females, production of $PGF_{2a}$ in intrauterine tissues during late pregnancy is significantly reduced and the administration of $PGF_{2a}$ on day 19 is able to restore normal parturition (Sugimoto et al., 1997). The important role of $PGF_{2\alpha}$ in uterine contraction is supported by studies of COX-1 deficient female mice, which exhibit a similar defect in parturition (Gross et al., 1998). In wild-type mice, uterine expression of *Cox-1* mRNA gradually increased from day 15 of pregnancy, reaching maximal levels on day 17 and rapidly decreasing after day 20, the day when parturition normally occurs. Ichikawa and colleagues found that these cycles are altered in FP-deficient mice and the uterine expression of *Cox-1* mRNA was still elevated on day 20 of pregnancy (Tsuboi et al., 2000). This observation suggested that progesterone withdrawal could serve as a negative feedback system for uterine COX-1 expression.

### *Ductus Arteriosus*

The ductus arteriosus is a normal fetal structure, allowing blood to bypass circulation through the lungs, since the fetus does not require its lungs for oxygenation (oxygen is provided through the mother's placenta), and blood flow is shunted from the left pulmonary artery to the aorta. In most cases, the ductus closes soon after birth, and its closure is stimulated by withdrawal of prostaglandins (which act as a dilator) as well as active contraction exerted by increased oxygen tension (Smith, 1998). Administration of NSAIDs leads to closure of the ductus, emphasizing the role of prostaglandins in this process, and indeed, NSAIDs are now used to treat patent ductus arteriosus in neonates. Conversely, vasodilator PGs such as $PGE_1$ maintain the patency of the ductus in neonates with ductus arteriosus (Smith and McGrath, 1994). A study using various synthetic PG analogs suggested that both IP and EP4 receptors are present in the ductus and are involved in maintaining the dilation of this vessel (Smith and McGrath, 1994). The EP4 receptor expression was confirmed by in situ hybridization of its mRNA in the mouse (Nguyen et al., 1997). Although disruption of the mouse IP gene did not appear to cause any abnormality of the ductus (Murata et al., 1997), mice without EP4 receptors died within 3 days after birth, due to marked pulmonary congestion and heart failure (Nguyen et al., 1997), likely resulting from a patent ductus. Administration of indomethacin to mice during late pregnancy elicited premature closure of the ductus in wild-type fetuses, but not in the ductus of EP4-deficient fetuses, indicating that the dilatory effect of $PGE_2$ on this vessel is mediated by the EP4 receptor (Nguyen et al., 1997). This finding is also supported by the phenotype of COX-1, COX-2 double knockout mice, which died shortly after birth due to a patent ductus arteriosus (Loftin et al., 2001).

*Cardiovascular Homeostasis*

Perhaps the most clinically recognized aspect of prostaglandin biology is its role in maintaining cardiovascular homeostasis. $PGI_2$ (prostacyclin) has well-known vasodilating and anti-thrombogenic properties that confer cardioprotective effects. $PGI_2$ acts on platelets to inhibit platelet aggregation and on smooth muscle cells of blood vessels to cause vasodilation. Analyses of mice deficient in the IP receptor revealed that their basal blood pressure and heart rate were not different from those of wild-type siblings (Murata et al., 1997). This result indicated that the $PGI_2$ and IP receptors do not work constitutively in regulating the systemic circulation but more likely work on demand, in response to local stimuli. Furthermore, IP-deficient mice developed and aged normally, without an increased occurrence of vascular incidents. However, an enhanced thrombotic activity was observed in IP-deficient mice in response to endothelial damage (Murata et al., 1997). These findings confirmed the proposed role of $PGI_2$ as an endogenous antithrombotic agent and suggest that this antithrombotic system is activated in response to vascular injury.

On the other hand, $TxA_2$ also acts on platelets to induce platelet aggregation and on smooth muscle cells of blood vessel to cause vasoconstriction. Because of their opposing effects on blood vessels and platelets, a precise balance between the $PGI_2$ and $TXA_2$ is probably important for maintaining vascular homeostasis (i.e., to prevent thrombosis and vasospasm while performing efficient hemostasis). TP-deficient mice showed increased bleeding tendencies and were resistant to cardiovascular shock induced by intravenous infusion of a TP agonist, U-46619, and arachidonic acid (Thomas et al., 1998). The increased bleeding tendency had also been noted in mice harboring in the TP

receptor an arginine to leucine missense mutation, which impairs coupling of the mutant receptor to Gq (Hirata et al., 1994b). Platelets from patients homozygous for this mutation showed no aggregation in response to $TxA_2$. These results supported the notion that the $TxA_2$ and TP receptor signaling do indeed play a physiological role in regulating hemostasis.

$PGE_2$ signaling also acts on smooth muscle cells of the vasculature to regulate blood pressure. To understand which EP receptor is responsible for vessel contraction, Breyer and colleagues administered $PGE_2$ and PGE analogs intravenously into wild-type and EP2-deficient mice and examined the *in vivo* response (Kennedy et al., 1999). They observed that infusion of $PGE_2$ or an EP2 agonist, butaprost, induced a transient hypotension in wild-type mice, whereas injection of the mixed EP1/3 agonist, sulprostone, resulted in an increase in arterial blood pressure. The hypotensive response to butaprost was not observed in EP2-deficient mice, although the hypertensive effects of sulprostone persisted. Surprisingly, $PGE_2$ also evoked considerable hypertension in EP2-deficient mice. The authors suggested that the absence of the EP2 receptor abolishes the ability of the mouse vasculature to vasodilate in response to $PGE_2$ and unmasked a contractile response mediated via interaction with the vasoconstrictor EP receptor(s). Interestingly, when fed a high-salt diet, the EP2-deficient mice developed significant hypertension with a concurrent increase in urinary excretion of $PGE_2$. These results indicated that $PGE_2$ is produced in the body in response to a high-salt diet to decrease blood pressure via the relaxant EP2 receptor and that perturbation of this pathway may be involved in the development of salt-sensitive hypertension. Coffman and colleagues compared the roles of individual EP receptors in males and females (Audoly et al., 1999)

and found that the relative contributions of each EP receptor subtype were strikingly different. In females, the EP2 and EP4 receptors mediated the major portion of the vasodepressor response to $PGE_2$. However in males, the EP2 receptor had a modest role and most of the vasodepressor effect was mediated by the EP1 receptor coupled to phospholipase C. In addition, the EP3 receptor in male mice actively opposed the vasodepressor actions of $PGE_2$. Thus the genetic evidence points to a complex interplay of EP, TP, and IP receptors in regulating cardiovascular homeostasis in mammals.

Given this knowledge about prostaglandin biology in the murine model, can we address a role for COX signaling in embryonic development? The answer is decidedly negative. In the mouse, the maternal contribution of prostaglandins to COX-mutant embryos and the severe defects of the reproductive capacity of COX-deficient female mice, severely hindered the studies of the developmental role for prostaglandins free from maternal interference. It has been hypothesized that the maternal contribution of prostaglandins in the placenta allows the prostaglandin-deficient embryos to develop normally (Reese et al., 2000; Reese et al., 2001). Mammalian blastocysts produce high levels of $PGE_2$ and $PGF_{2\alpha}$, supporting this idea and suggesting a critical requirement for these bioactive lipids during blastocyst development (Davis et al., 1983; Dey et al., 1980). In agreement, murine blastocysts cultured outside of the mother do not survive in the presence of COX inhibitors (personal communication, Wang H, Dey SK, Vanderbilt University Medical Center). Therefore, understanding the developmental function of prostaglandins has been hampered by the lack of a suitable model system.

**Table** Loss-of-Function (LOF) phenotypes in mouse and zebrafish

| Molecules | Mouse Knockout Phenotypes | Species | Reference |
|---|---|---|---|
| COX-1$^{(-/-)}$ | Minimal | Mouse | Langenbach *et al*., |
| COX-1$^{(-/-)}$ female | Prolonged Parturition | Mouse | Gross *et al*., 1998 |
| COX-2$^{(-/-)}$ | Renal Pathology + Gastritis | Mouse | Dinchuk *et al*., 1995 |
| COX-2$^{(-/-)}$ female | Multiple Reproductive Defects | Mouse | Lim *et al*., 1997 |
| COX-1/ -2$^{(-/-)}$ | Patent Ductus Arteriosus | Mouse | Loftin *et al*., 2001 |
| EP2$^{(-/-)}$ | Ovulation and Fertilization Failure<br>⇓ Vasodilatory Response to $PGE_2$ | Mouse<br>Mouse | Kennedy *et al*., 1999,<br>Kennedy *et al*., 1999 |
| EP3$^{(-/-)}$ | ⇑ Vasodilatory Response to $PGE_2$ | Mouse | Audoly *et al*., 1999 |
| EP4$^{(-/-)}$ | Patent Ductus Arteriosus<br>⇓ Vasodilatory Response to $PGE_2$ | Mouse<br>Mouse | Nguyen *et al*., 1997<br>Audoly *et al*., 1999 |
| IP$^{(-/-)}$ | ⇑ Thrombotic Tendency | Mouse | Murata *et al*., 1997 |
| TP$^{(-/-)}$ | ⇓ Thromboembolism and Increased Bleeding Tendency | Mouse | Thomas *et al*., 1998 |
| FP$^{(-/-)}$ | Loss of Parturition | Mouse | Sugimoto *et al*., 1997 |
| | **Embryonic Phenotypes** | | |
| COX-1 LOF | Gastrulation Arrest<br>Vascular Tube Formation | Zebrafish*<br>Zebrafish* | Grosser *et al*., 2002<br>Cha *et al*., 2005 |
| COX-2 LOF | None | Zebrafish* | Cha *et al*., 2005 |
| EP4 LOF | Decreased Cell Motility | Zebrafish*, Cancer | Tsujii *et al*., |

*The phenotypes in zebrafish were assessed before 24 hours post fertilization**1**

## Zebrafish as a Model for Dissecting Prostaglandin Pathway

Recently, FitzGerald and colleagues identified the zebrafish orthologues of genes encoding COX-1 and COX-2 (Grosser et al., 2002). Zebrafish cyclooxygenases have also been shown to be both genetically and functionally equivalent to their mammalian counterparts. These initial studies extended by our work have shown that antisense morpholino oligonucleotides (MOs) translation interference or pharmacologic inhibitors of COX-1 result in gastrulation arrest, while knockdown of COX-2 fails to produce any discernable phenotype (Cha et al., 2005; Grosser et al., 2002). This finding also supports the role of COX-1 as a "housekeeping" enzyme in which a basal level of prostaglandin is required for normal homeostasis.

Zebrafish are an attractive model system in which to address the roles of prostaglandins in embryogenesis for many reasons (Amatruda et al., 2002). Each pair-wise mating gives rise to hundreds of embryos for study on a weekly basis. Most importantly for prostaglandin biology, embryogenesis occurs outside of mother's body, thus not influenced by maternal prostaglandins. Moreover embryogenesis can be visualized at tissue and cellular levels due to the transparency of the developing embryos; molecular markers are available for virtually all the tissues. Zebrafish embryos and their chorions are highly permeable to many drugs, facilitating a temporal control of inhibition of different enzymes involved in prostaglandin signaling. In addition, forward genetic screens have identified mutations in many developmental pathways, allowing one to study functional interactions between prostaglandin signaling and other developmental pathways.

# CHAPTER II

## CYCLOOXYGENASE-1 DERIVED $PGE_2$ PROMOTES CELL MOTILITY VIA THE G-PROTEIN COUPLED EP4 RECEPTOR DURING VERTEBRATE GASTRULATION


Abstract

Gastrulation is a fundamental process during embryogenesis that shapes proper body architecture and establishes three germ layers through coordinated cellular actions of proliferation, fate specification, and movement (Stern 2004). Although many molecular pathways involved in the specification of cell fate and polarity during vertebrate gastrulation have been identified, little is known of the signaling that imparts cell motility. Here we show that prostaglandin $E_2$ ($PGE_2$) production by microsomal $PGE_2$ synthase (Ptges) is essential for gastrulation movements in zebrafish. Furthermore, $PGE_2$ signaling regulates morphogenetic movements of convergence and extension as well as epiboly through the G-protein coupled $PGE_2$ receptor (EP4) via PI3K/Akt. EP4 signaling is not required for proper cell shape or persistence of migration, but rather it promotes optimal cell migration speed during gastrulation. This work demonstrates a critical requirement of $PGE_2$ signaling in promoting cell motility through the COX-1-Ptges-EP4 pathway, a previously unrecognized role for this biologically active lipid in early animal development.

# Introduction

Gastrulation is a fundamental process during embryogenesis that shapes proper body architecture and establishes three germ layers during coordinated cellular actions of proliferation, fate specification, and morphogenetic movements. During zebrafish gastrulation, the morphogenetic movements are comprised of internalization, convergence, extension, and epiboly that ultimately result in formation of embryonic axes at the end of gastrulation. Mounting evidence suggest that distinct molecular signaling networks have both overlapping and non-overlapping functions to regulate these morphogenetic movements, and the molecular pathways that underlie these movements are conserved in various other physiologic and pathologic processes that require cell migration and movement.

Previously it has been shown that phosphoinositide-3 kinase (PI3K) is involved in patterning and morphogenetic movement during vertebrate gastrulation. PI3Ks are key transducers of a variety of upstream signaling that regulate such complex processes as cell proliferation and growth, invasion and metastasis, and chemotaxis. In zebrafish, loss of PI3K function results in loss of polarization and in few cell protrusions in migration mesendodermal cells, but the cells still maintained their directional movement. In addition, loss of Platelet-derived growth factor (PDGF) function also results in similar defects, suggesting that PDGF lies upstream of PI3K to regulate mesendoderm migration. Although it is known that a variety of signaling pathways converge on PI3K, various distinct upstream factors that can regulate specific aspects of PI3K signaling has not yet been identified.

Previous studies in colorectal cancer cells demonstrated that $PGE_2$ signaling lies upstream of PI3K/Akt to regulate colorectal cancer migration and invasive behavior. In addition, studies in zebrafish demonstrated that the injection of antisense morpholino oligonucleotides designed to block the translation of *cox1* transcripts leads to early gastrulation arrest, which is suppressed by co-injection of synthetic *cox1* RNA (Cha et al., 2005; Grosser et al., 2002). The gastrulation arrest phenotype was also recapitulated following treatment with pharmacologic inhibitors of cyclooxygenase, such as indomethacin or sulindac (Cha et al., 2005). These observations prompted us to explore whether $PGE_2$ signaling downstream of COX-1 acts to regulate cell movement through PI3K/Akt pathway during the early developmental process of gastrulation.

## Methods

**Zebrafish Maintenance, Embryo Generation, and Staging.** AB* and TL WT zebrafish strains were maintained as described(Solnica-Krezel et al., 1996). Embryos were obtained from natural spawnings and staged according to morphology as described(Kimmel et al., 1995).

**Cloning of Prostaglandin $E_2$ receptors and synthases** An expressed sequence tag (EST) with sequence similarity to human EP2 and EP4 receptors were isolated by PCR (*Advantage2*, Clontech), and cloned into the pCR2.1 vector (Invitrogen) and used for antisense probe synthesis with SP6 RNA polymerase. For misexpression, the full-length cDNA was cloned into the pCS2+ vector and used for capped RNA synthesis with SP6 RNA polymerase after *Not*I linearization (mMESSAGE mMACHINE, Ambion). The NCBI accession numbers for EP2 and EP4 are DQ286580 and DQ202321, respectively. To obtain full-length prostaglandin $E_2$ synthases, we designed primers against previously isolated sequences of *ptges* and *cpges* (Pini et al., 2005) and isolated them by PCR (*Advantage2*, Clontech). Full-length PCR products were cloned into pCR2.1 vector (Invitrogen) and used for antisense probe synthesis with SP6 RNA polymerase.

**In Situ Hybridization.** Antisense probes for zebrafish *cox1* and *cox2* were synthesized as described (Grosser et al., 2002). Antisense RNA probes were synthesized from cDNAs encoding *six3b* (Kobayashi et al., 1998), *ntl* (Schulte-Merker et al., 1992) and *pax2.1* (Krauss et al., 1991b). Whole mount *in situ* hybridization was performed according to Marlow *et al*. (Marlow et al., 2002). Embryos were analyzed on Zeiss Axioplot and

images were captured with Nikon Coolpix 4500. Each *in situ* experiment was done at least twice, using approximately 20 embryos.

**MO Design and MO/RNA injections** were performed at the one-cell stage as described (Marlow et al., 2002). MOs was designed to the predicted start codon (MO1) and 1st exon-intron boundary(MO2) of *ptges* (underline indicates the predicted start codon)*: ptges*-MO1 (5'-TCAGCAAAAAGTTACACTCTCTCAT-3') and

*ptges*-MO2 (5'-GTTTTGTGCTCTTACCTCCTACAGC).

For EP receptors, we designed two MOs against 5'UTR (MO1) and predicted start codon (MO2): *ep2*-MO1 (5'-GATGTTGGCATGTTTGAGAGCATGC),

*ep2*-MO2 (5'-ACTGTCAATACAGGTCCCATTTTC),

*ep4*-MO1 (5'CGCGCTGGAGGTCTGGAGATCGCGC),

*ep4*-MO2 (5'-CACGGTGGGCTCCATGCTGCTGCTG),

ep4-MOc (5'-CAtGGTGGcgTgCATGCTaCTGCTG, small letters denoting mutated sites). All MOs were obtained from Gene Tools, LLC (Philomath, OR). Zebrafish *ep4* sense-capped RNA was synthesized using mMessage Machine (Ambion, Austin, TX) after template linearization with Enzyme. For phenotype rescue and phenocopy experiments, 80 pg *ep4* RNA and 2 ng *EP4*-MO2 per embryo were used.

**Prostaglandin Rescue Experiments.** For prostaglandin supplementation experiments, we used commercially available prostaglandins (Cayman), $PGE_2$, $PGF_{2\alpha}$, and $PGI_2$. We supplemented Ptges-deficient embryos with 5 μM of PGs in 1% DMSO egg media at the beginning of epiboly process as previously described(Cha et al., 2005).

**Nomarski Time-Lapse Analysis.** Images of lateral gastrula mesodermal cells at midgastrulation (80% epiboly) were carried out as described previously (Myers et al., 2002). Cell movement measurement data obtained in Object-Image (NIH image) was exported to Excel (Microsoft) where cell migration speed, path, direction, turning angle, and LWRs were determined. The movement direction of lateral mesodermal cells was determined at 1-minute intervals.

**Inhibitor Treatment** To block PI3K and PDGF activity, we used LY294002 (Calbiochem), a specific inhibitor of PI3K activity (Vlahos et al., 1994). Embryos were usually treated from 30% epiboly to YPC stage in 10 or 30 μM LY294002 to monitor the phenotypes

**Western Blot Analysis** Embryos were injected with *ptges-MO*, EP2-*MO2*, EP4-*MO*, or treated with LY. Endogenous levels of Akt and ERK activation were measured. Embryos were injected with 2ng dose of *ptges-MO*, EP2-*MO2*, EP4-*MO* WT at 1 cell stage, or treated with $PGE_2$ starting at 30% epiboly stage. Embryos were then collected between 80%-90% epiboly stage, dechorionated manually and homogenized in 50 mM HEPES, pH 7.5, 150 mM NaCl, 1% Triton X-100. Equal amounts of samples were separated by SDS-PAGE and transferred to polyvinylidene difluoride membranes. The membranes were blocked in TTBS (Tris-buffered saline with 0.1% Tween 20) containing either 5% dry milk or BSA. Primary antibody incubations were performed in TTBS with either 5% dry milk or BSA overnight at 4 °C. After washing, the membranes were incubated with the appropriate secondary peroxidase-conjugated antibody for 1 h in TTBS with either 5% dry milk or BSA. Immunoreactive proteins were visualized using the enhanced

chemiluminescence system from Amersham Biosciences. Antibodies to Akt, Erk, phospho-Akt, and phospho-Erk were obtained from Cell Signaling (Beverly, MA).

## Results

### Expression of $PGE_2$ Signaling Molecules during Vertebrate Gastrulation

To understand the downstream effectors of COX-1 signaling during gastrulation, we initially analyzed the highly conserved zebrafish orthologue of $PGE_2$ synthase, Ptges (Pini et al., 2005). The analysis of spatiotemporal expression revealed that *ptges* transcripts are maternally deposited and ubiquitously distributed during gastrulation, revealing that *ptges* and *cox1* transcripts co-localize during gastrulation (Fig. 2A, C). On the other hand, *cox2* transcripts are not detected until after the end of gastrulation specifically in the anterior neuroectoderm (Fig. 2B). Analogous to other systems, these findings suggest that the zebrafish Ptges functions downstream of COX-1 during gastrulation to generate $PGE_2$. Accordingly measurements of prostaglandin levels using mass spectrometry revealed the production of $PGE_2$ during gastrulation, along with $PGF_{2\alpha}$ and $PGI_2$ (Cha et al., 2005), that parallels studies demonstrating the presence of $PGE_2$ and $PGF_{2\alpha}$ in developing mammalian blastocysts (Davis et al., 1983).

### Specific Knockdown of mPGES results in Gastrulation Movement Defects

To address the function of $PGE_2$ signaling during zebrafish gastrulation, we designed two *MOs* to target *ptges* transcripts: one *ptges-MO1* complementary to 5' untranslated region (UTR), expected to block translation, and the second *ptges-MO2* complementary to the first intron-exon boundary, expected to cause a splicing defect. Following *ptges-MO1* or *MO2* injections, we observed severe morphogenetic defects during gastrulation at a dose of 2ng. Ptges-deficient embryos displayed a mispositioned

head and shortened anteroposterior (AP) axis with delayed epiboly that completed only at tailbud or segmentation stage (Fig. 3B). After injection of a 4ng dose of *ptges-MO2*, the embryos underwent complete gastrulation arrest similar to what we observed in COX-1 deficient embryos (Fig. 3C, E). To further characterize the morphological abnormalities associated with the loss of Ptges function, we analyzed the expression of several cell-type specific markers at late gastrulation, anterior neuroectoderm (*six3b*) (Seo et al., 1999), mid-hindbrain boundary (*pax2.1*) (Krauss et al., 1991a), axial and prospective posterior mesoderm (*no tail/brachyury*) (Schulte-Merker et al., 1994). While the transcripts of these markers were detected in Ptges-deficient embryos, their expression domains were shortened anteroposteriorly and widened mediolaterally (Fig. 3J, M). In addition, *no tail* expression in the nascent mesoderm revealed an enlarged blastopore. Marker analyses further suggested Ptges-deficient embryos have defects in several gastrulation movements, including convergence, extension and epiboly. Therefore, we hypothesized that Ptges-derived $PGE_2$ plays an essential role in regulating cell movement during gastrulation.

To address whether lack of $PGE_2$ production is solely responsible for these gastrulation defects, we tested whether the addition of exogenous $PGE_2$, a downstream prostaglandin product of $PGE_2$ synthase, could rescue the gastrulation phenotype in Ptges deficient embryos. Ptges morphants were incubated in control media or media supplemented with $PGE_2$, $PGF_{2a}$, or $PGI_2$, the predominant prostaglandins during zebrafish gastrulation. All morphants treated with $PGI_2$ (n=55) (Fig. 3F) or $PGF_{2a}$ (n=60) (Fig. 3G) exhibited gastrulation defects similar to untreated Ptges-deficient embryos. By contrast, 92% of morphants (n=84) incubated in $PGE_2$ (Fig. 3H) completed epiboly and

survived past tailbud stage (Fig. 8), suggesting that Ptges regulates cell movement through secreted $PGE_2$. Furthermore, we observed that the shapes of expression domains of *pax2.1, six3b,* and *no tail* were partially restored in Ptges morphants treated with $PGE_2$ to those of uninjected control embryos (Fig. 3K, N, T, U). We also found that co-injecting mouse *Ptges* RNA (50 pg) suppressed the gastrulation defects in Ptges morphants in 75% of embryos (n=120, Fig. 3H and Fig. 8), further demonstrating the specificity of the *ptges-MOs*. In addition, the gastrulation arrest in embryos injected with 4ng dose of *ptges-MO2* was also partially suppressed by $PGE_2$ treatment (Fig. 3P, Q). We also tested the effectiveness of the *ptges-MO*. We observed that injection of 2 or 4ng of *ptges-MO2*, targeting the first intron-exon boundary, results in truncation of the transcript size by approximately 82 bp due to deletion of exon 2 (Fig. 9A). Since splicing MO targets beginning of exon2, the splicing machinery targets splicing donor site of intron1 and acceptor site of exon3 to cause deletion of exon2. In addition, some wild-type transcripts remain in embryos injected with 2ng, but not in embryos injected with 4ng of *ptges-MO2*, further demonstrating that injection of 2ng *ptges-MO2* represents a hypomorphic situation (Fig. 9A). Interestingly, overexpression of *ptges* RNA did not lead to any observable gastrulation abnormalities. This is also consistent with our previous finding that incubation of WT embryos with high doses of $PGE_2$ or overexpression of COX-1 do not result in any noticeable defects during development (Cha et al., 2005). The remarkable ability of $PGE_2$ to overcome the gastrulation defect in Ptges morphants and normal gastrulation in embryos with excess $PGE_2$ indicate that this lipid messenger plays an essential but permissive role in gastrulation movements of convergence and extension as well as epiboly.

Specific Knockdown of EP4 results in Gastrulation Movement Defects

Next we sought to identify downstream effectors of $PGE_2$ during gastrulation. In mammals, EP receptors (EP1-4), members of the G-protein coupled receptor (GPCR) family, bind to $PGE_2$ with high affinity to activate downstream signals (Breyer et al., 2001). We searched the NCBI-database and identified zebrafish ESTs with highest sequence similarities to mammalian EP receptors, designated as Ep2 and Ep4 (Fig. 10). Spatiotemporal analysis of *ep2* and *ep4* expression demonstrated ubiquitous distribution of *ep4* transcripts in early embryos before the onset of the zygotic transcription and also later throughout gastrulation, while *ep2* RNA was not detected until the end of gastrulation. RT-PCR analysis confirmed the presence of *ptges* and *ep4*, but not *ep2*, transcripts during gastrulation (Fig. 2D and data not shown). To address the role of Ep2 and Ep4 receptors in early embryonic development, we designed two *MO*s targeting *ep2* and *ep4* receptor transcripts (*MO1* targets 5'UTR, while *MO2* targets start codon), to block translation of the transcripts. Embryos injected with up to 15 ng of *ep2-MO1* or *ep2-MO2* failed to demonstrate any effect on gastrulation, consistent with the lack of expression during early development. However, injection of 2ng of *ep4-MO1* or *ep4-MO2* produced embryos with vegetally shifted heads and shortened AP axes at the end of the gastrula period (95% embryos displayed this phenotype, n=130) (Fig. 4D, E). The shortening of the AP axis was observed throughout the somitogenesis stages, and at 24 hpf EP4-deficient embryos exhibited a shortened AP axis, laterally elongated somites, and wavy notochord (Fig. 4F). This phenotype resembles that of mutants in the non-canonical Wnt/planar cell polarity (PCP) pathway defective in convergence and extension gastrulation movements, such as *knypek* (Topczewski et al., 2001), *trilobite* (Jessen et al.,

2002), and *pipetail* (wnt5) (Rauch et al., 1997). Next, we determined whether *ptges-* and *ep4-MO* demonstrate synergism. Co-injection of sub-optimal dose of *ep4-MO2* (1ng) with *ptges-MO2* (1ng) produced embryos with more severely shortened body axis at 24 hpf as compared those embryos injected with 1ng dose of either *ep4-MO* or *ptges-MO2* (Fig. 9C). This result is consistent with Ptges and Ep4 functioning in the same genetic pathway.

To understand better the morphological differences in EP4-deficient embryos, we examined the expression of several cell type specific genes. The expression of *goosecoid* (*gsc)*, a presumptive prechordal mesoendoderm marker, and *bmp4*, a marker of ventroposterior tissues, were similar to those of uninjected sibling embryos at the shield stage, indicating normal patterning during early gastrulation (Fig. 4K, L). Next we found that dorsal midline expression of *no tail* was widened and its blastopore expression domain animally shifted (Fig. 4O), while the expression domains of neural markers were shortened in the anterior-posterior axis (Fig. 4P), reminiscent of the convergence, extension and epiboly defects in Ptges-deficient embryos. However, in contrast to Ptges and COX-1 deficient embryos, and consistent with the function of EP4 downstream of $PGE_2$, gastrulation defects of EP4 morphants were not suppressed by exogenously supplied $PGE_2$ (0%, n=55). To test the efficiency by which *ep4-MO* inhibit Ep4 function, we designed an EP4-GFP construct. We observed that co-injection of synthetic *ep4-GFP* RNA with *ep4-MO2* resulted in a loss of GFP expression compared to embryos injected with *ep4-GFP* RNA alone (Fig. 9B). These experiments support the argument that the *ep4-MO* leads to a strong EP4 loss of function, consistent with the inability of $PGE_2$ to suppress gastrulation defects. Injection of synthetic *ep4 RNA* at 80pg led to a mild

epiboly, convergence and extension defects and ultimately resulted in a shortened tail at 24 hpf (Fig. 4G). In addition, co-injection of this *ep4 RNA* with *ep4-MO2* suppressed the strong convergence and extension phenotype of EP4 deficient embryos (52% of embryos had partially restored wild-type phenotype at 24hpf; n=72), demonstrating the specificity of the *ep4-MO2*. However, injection of *ep4 RNA* at higher levels led to a dose-dependent defect in convergence and extension (data not shown) and did not suppress the morphant phenotype in rescue experiments. Previously it has been shown that genes which regulate cell movement often have similar phenotypes in loss-of-function (LOF) or gain-of-function (GOF) experiments (Carreira-Barbosa et al., 2003; Jessen et al., 2002; Lin et al., 2005), suggesting that the levels of these gene products must be tightly regulated. These results show that EP4 receptors, similar to Ptges, regulate cell movements without altering patterning and fate specification. In addition, EP4 specifically acts as a receptor for the Ptges-derived $PGE_2$ to regulate cell movement during gastrulation.

EP4 Signaling is Required for Optimal Cell Migration Speed during Dorsal Convergence

The morphological abnormalities observed in Ptges and EP4 deficient embryos are consistent with convergence, extension and epiboly defects. To identify cell behaviors that depend on EP4 receptor function, we performed Nomarski time-lapse analyses of lateral mesoderm cells in WT (2 embryos, 84 cells), control *ep4-MO* injected (*EP4-MOc*) (2 embryos, 80 cells), and *ep4-MO2* injected embryos (6 embryos, 224 cells) at midgastrulation. These moderately elongated cells undergo directed migration towards the dorsal midline along complex trajectories (Carreira-Barbosa et al., 2003; Jessen et al., 2002; Lin et al., 2005). Analysis of total movement speed, accounting for the cell

movement in all directions, showed that cells in EP4-deficient embryos had reduced total speed (75% of the control level). In addition, the net dorsal speed was also attenuated to a similar degree, which was 67% of the control level (Fig. 5D). By comparing the meandering index, a ratio of total to the net path, which measures the tendency of cells to deviate from their directional path, we found that the lateral mesodermal cells in EP4-deficient embryos largely migrate toward the dorsal midline along trajectories similar to the control cells (WT=1.29, n=84; EP4-deficient embryos=1.19, n=224; $p < 0.57$) (Fig. 5E, 6), indicating that the directional cell movement in EP4 deficient embryos is not significantly compromised. In addition, we found that the shape of cells in this population, as determined by length to width ratio (LWR), remains relatively unchanged in EP4 morphants, as compared to EP4-MOc injected or uninjected WT (Fig. 5F) at midgastrulation. The normal cell shape and meandering index associated with defective convergence movements of lateral mesodermal cells in EP4-deficient embryos contrast cellular defects reported for embryos with defective Gα12/13 signaling (Lin et al., 2005). In this situation impaired convergence of lateral mesodermal cells has been linked to rounder cell shapes and less persistent dorsal movement. Therefore, given the above observations and impaired epiboly in COX-1, Ptges and EP4-deficient embryos these studies provide evidence that $PGE_2$ signaling primarily regulates motility of mesodermal cells during gastrulation, without significantly altering cell shape or persistence of migration.

## PI3K/Akt Mediates Gastrulation Movement Downstream of EP4 Receptor

We have previously shown that $PGE_2$ can affect cancer metastasis by increasing proliferation, migration, and invasiveness of cancer cells and the stimulatory effects of $PGE_2$ are dependent upon the activation of the PI3K/Akt pathway (Buchanan et al., 2003a; Sheng et al., 2001). The serine/threonine kinase Akt, or protein kinase B (Akt/PKB) is a direct downstream effector of phosphatidylinositol 3-kinase (PI3K). Akt/PKB lies at the crossroad of multiple cellular signaling pathways and acts as a transducer of inputs initiated by receptor signaling cascades that activate PI3K (Katso et al., 2001). Embryos treated with the PI3K inhibitor, LY294002, exhibited abnormal morphology consistent with defects in convergence and extension, as well as epiboly during gastrulation (Fig. 7B), mimicking phenotypes of Ptges and EP4 deficient embryos. To test whether PI3K/Akt activation is mediated by the EP4 receptor, we determined the phosphorylation status of Akt in embryos injected with *MOs* against *ep2*, *ep4*, and *ptges* transcripts. The zebrafish homolog of Akt has been cloned previously, and was shown to exhibit the phosphorylation sites identified in human Akt, Thr308 and Ser473 (Chan et al., 2002). In both EP4- and Ptges-deficient embryos, we observed a significant decrease in levels of the phosphorylated form of Akt (Fig. 7D, E), while the total Akt levels were constant. Consistent with permissive role of PGE2, the embryos with high levels of $PGE_2$ did not result in increased phosphorylation of Akt (Fig. 7F). We also tested the possibility that the ERK pathway as shown previously (Fujino et al., 2003) can mediate the EP4 signaling and found that ERK signaling is not affected in EP4-deficient embryos (Fig. 6G), showing the restriction of EP4 signaling to PI3K/Akt pathway. Taken together, our data suggest that the EP4 receptor acts upstream of PI3K/Akt pathway to regulate cell movement during gastrulation (Fig. 7H).

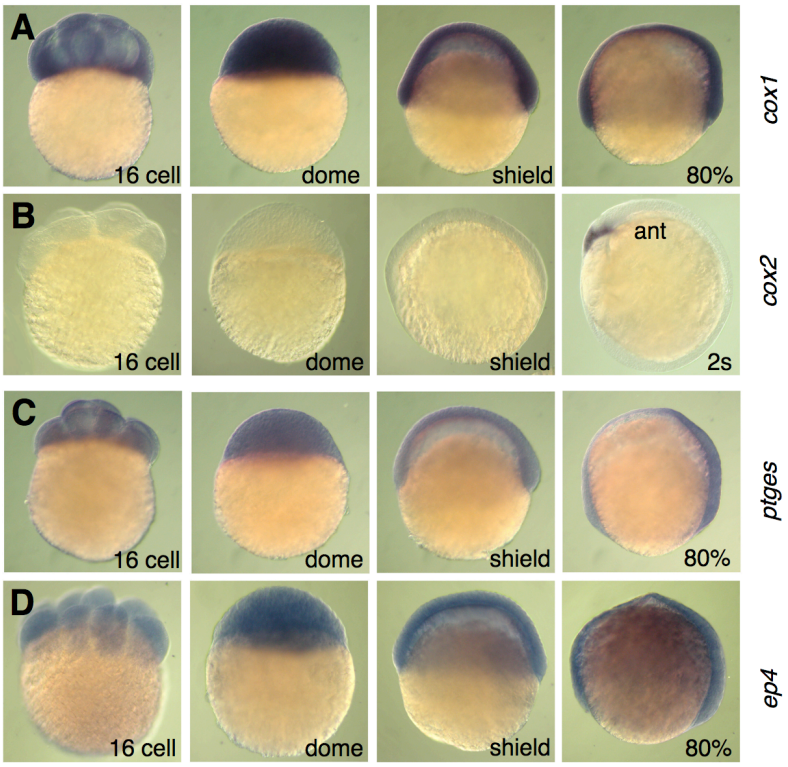


**Figure 2. *cox1, ptges,* and *ep4* transcripts are expressed ubiquitously during gastrulation.** (A-D) Lateral view, dorsal to the right. Expression of *cox1* (A), *cox2* (B), *ptges* (C), and *ep4* (D) transcripts visualized by whole mount *in situ* hybridization. ant, anterior.

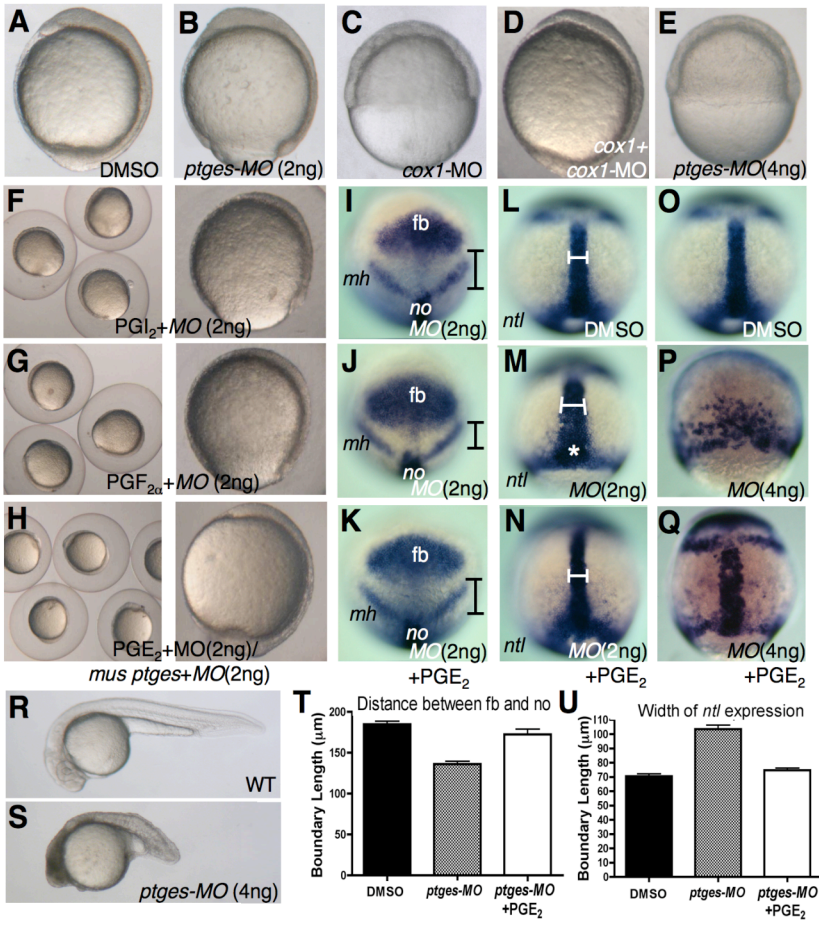


**Figure 3. Ptges-deficient embryos exhibit gastrulation defects that are suppressed by co-incubation with $PGE_2$.** (A-H) Lateral view, dorsal to the right. (I-K) Animal view, dorsal to the bottom. (L-Q) Dorsal view, anterior to the top. (R-S) Lateral view, anterior to the left. (I-K) Boundaries indicate the distance from posterior end of the forebrain to anterior end of notochord. (L-Q) Boundaries indicate the width of the notochord. (A, I, L, O) Control embryos treated with DMSO. (B, F-H, J, K, M, N, S) 2ng of *ptges-MO2* injected embryos. (E, P, Q) 4ng of *ptges-MO2* injected embryos (C, D) 10 ng of *cox1-MO* injected embryos. (D) Embryos co-injected with *cox1-MO* (10ng) and *cox1* RNA (50pg). (F) 2ng of *ptges-MO2* + $PGI_2$ (G) 2ng of *ptges-MO2* + $PGF_{2a}$ (H, K, N) 2ng of *ptges-MO2* + $PGE_2$ (Q) 4ng of *ptges-MO2* + $PGE_2$ (H) Embryos co-injected with *ptges-MO2* (2ng) + mouse *ptges* RNA (100pg). (I-K) Expression patterns of *six3b*, *pax2.1*. (L-Q) Expression pattern of *no tail*. (T) Quantitative analyses of the distance between the forebrain (fb) and notochord (no) in DMSO, *ptges-MO2*, and *ptges-MO2* + $PGE_2$ (n=10 per each group) (U) Quantitative analyses of width of *ntl* expression in DMSO, *ptges-MO2*, and *ptges-MO2* + $PGE_2$ (n=10 per each group). fb, forebrain; mh, mid-hindbrain; no, notochord. White star marks the blastopore to illustrate delayed epiboly. For details see text.

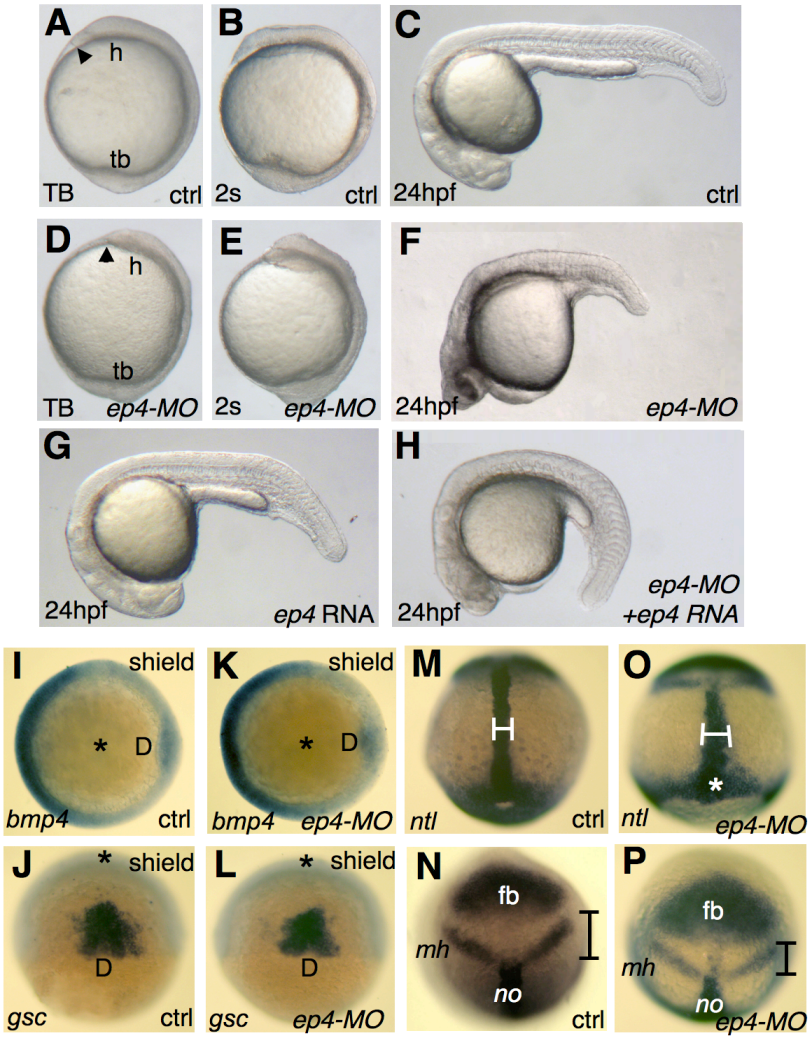


**Figure 4. EP4-deficient embryos exhibit convergence and extension defect during gastrulation.** (A, B, D, E) Lateral view, dorsal to the right. (C, F, G, H) lateral view, anterior to the left. (I, K) Animal view, dorsal to the right. (J, L) Dorsal view, animal pole to the top. (M, O) Dorsal view, anterior to the top. (N, P) Anterior view, dorsal to the bottom. (M, O) Boundaries indicate the width of the notochord. (N, P) Boundaries indicate the distance from the posterior end of the forebrain to the anterior end of the notochord. (A-C, I-J, M-O) Uninjected control. (D-F, H, K-L, O-P) *ep4-MO2* (2ng) injected embryos. (G, H) *ep4 RNA* (80pg) injected embryos. (H) embryos injected with *ep4-MO2* (2ng) + *ep4 RNA* (80pg). (I, K) *bmp4* expressions. (J, L) *gsc* expressions, (M, O) *no tail* expressions. (N, P) *six3*, *pax2*, and *ntl* expressions. h, head; tb, tailbud; D, dorsal; fb, forebrain; mh, mid-hindbrain; no, notochord. Arrowhead points to the location of prechordal plate at tailbud stage. Animal pole is highlighted by black star. White star highlights the blastopore to illustrate delayed epiboly. Black star indiates the animal pole.

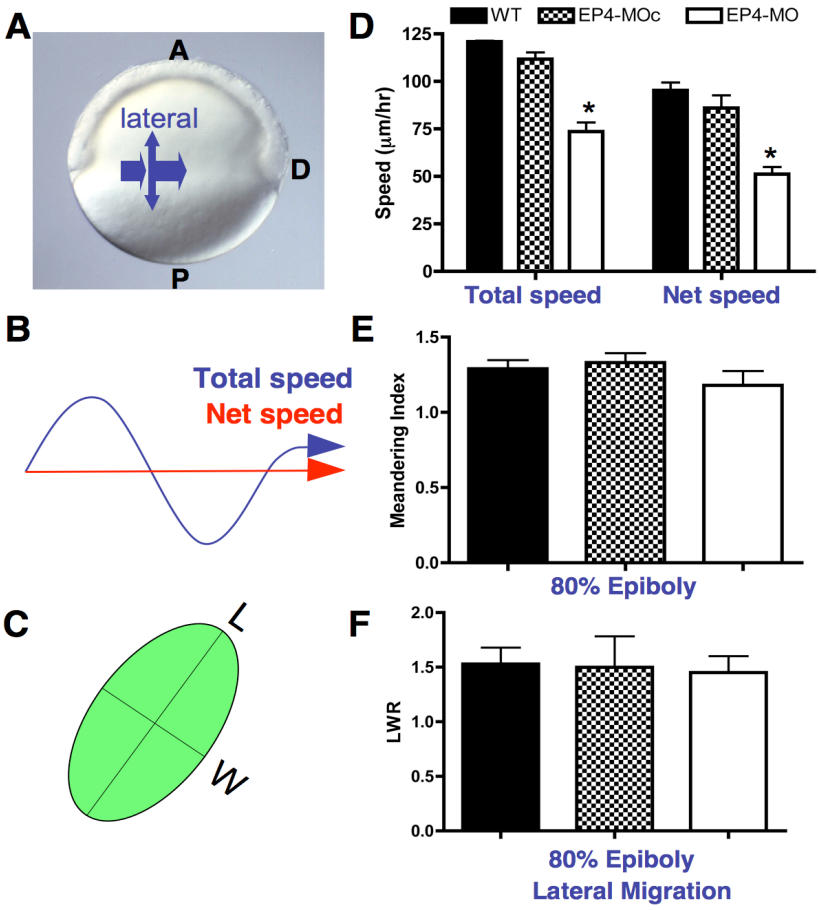


**Figure 5. EP4 deficient embryos exhibit decreased cell motility in dorsal migration, while maintaining normal cell shape.** (A) Domains of convergence and extension movements in zebrafish gastrulae. Orange arrows indicate domains of slow convergence and extension. (B) A diagram to demonstrate the difference between total versus net speed. (C) A schematic representation of the method used to measure cell shape (LWR, length-to-width ratio). (D-F) Cell behavior of mesoderm cells during slow convergence toward dorsal midline. (D) Total and net dorsal speed of lateral mesodermal cells at 80% epiboly. (E) Meandering index of lateral mesodermal cells. The meandering index was determined by dividing total speed by net speed. (F) LWR of lateral mesodermal cells. Lateral mesoderm measurements were made in WT (2 embryos, 84 cells), control EP4-*MO* injected (EP4-MOc) (2 embryos, 80 cells), and EP4-deficient embryos (6 embryos, 224 cells).

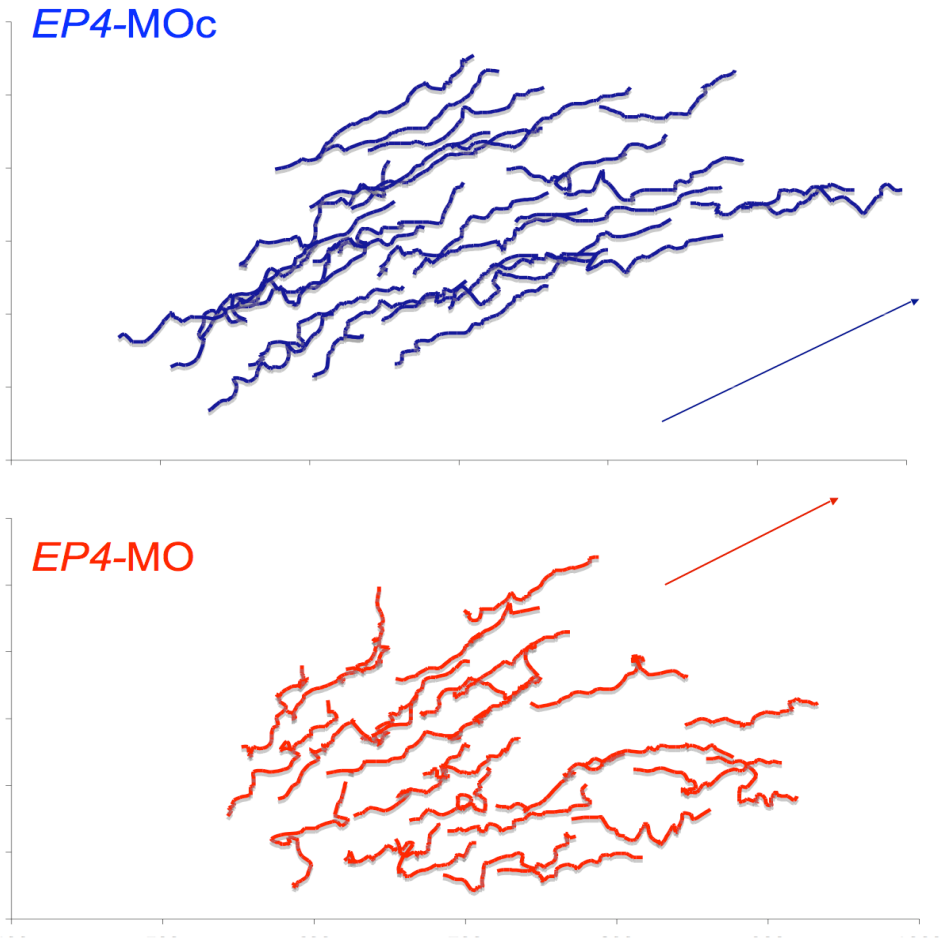


**Figure 6. EP4 deficient embryos exhibit reduced net path, but not persistence of directional movement, during dorsal migration.** Representation of the paths travelled by lateral mesodermal cells at 80% epiboly in embryos injected with control *EP4*-MO (*EP4*-MOc) and *EP4*-MO. The path of each individual cell movement from lateral mesoderm region was plotted over 40 minutes, starting at 80% epiboly. Colored arrows demonstrate the vector representation of the average direction and net length of cells from *EP4*-MOc (blue) and EP4-deficient (red) embryos

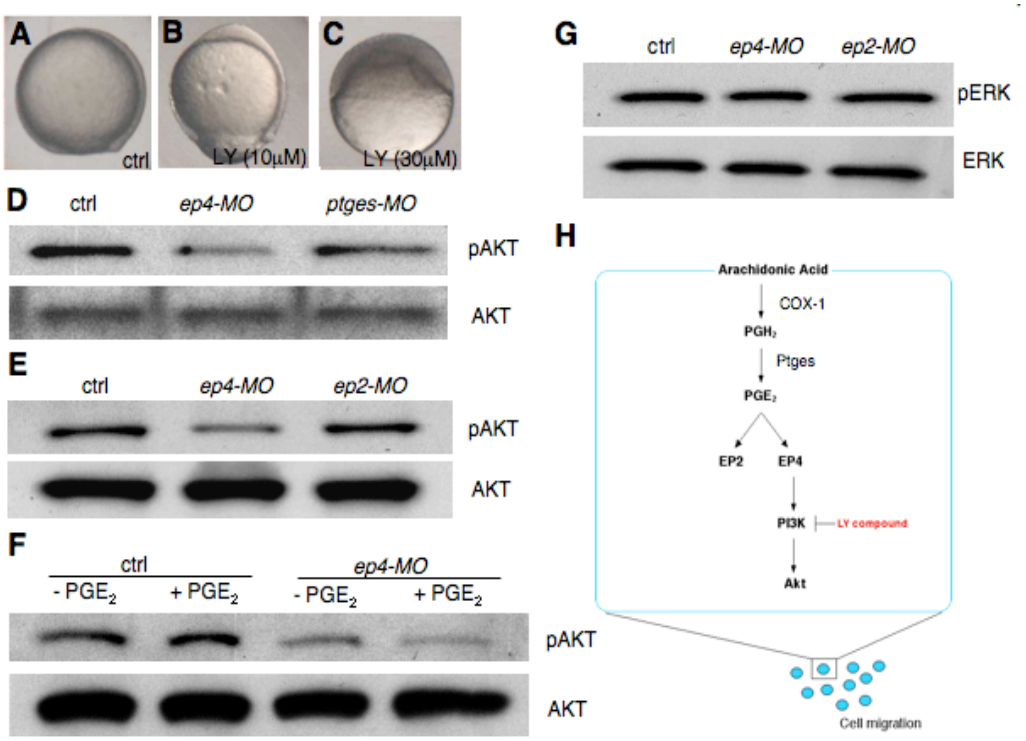


**Figure 7. PI3K/Akt is activated downstream of EP4 signaling.** (A-C) lateral view, dorsal to the right. (B) 10 μM of PI3K/Akt inhibitor, LY294002. (C) 30 μM of PI3K/Akt inhibitor, LY294002. (D) uninjected, *ep4-MO* (2ng), or *ptges-MO* (2ng) injected. (E) uninjected, *ep4-MO* (2ng), or *ep2-MO* (2ng) injected. (F) uninjected or *ep2-MO2* injected (2ng) embryos treated with or without exogenous $PGE_2$ (5 μM). (G) uninjected, *ep4-MO* (2ng), or *ep2-MO* (2ng) injected embryos. (H) Molecular mechanisms for $PGE_2$ signaling in vertebrate gastrulation. Lysates from 2.5 embryos were loaded into each well for Western blot analyses. pAKT, phosphorylated AKT; AKT, total AKT; pERK, phosphorylated ERK; ERK, total ERK. Please see text and method for details.

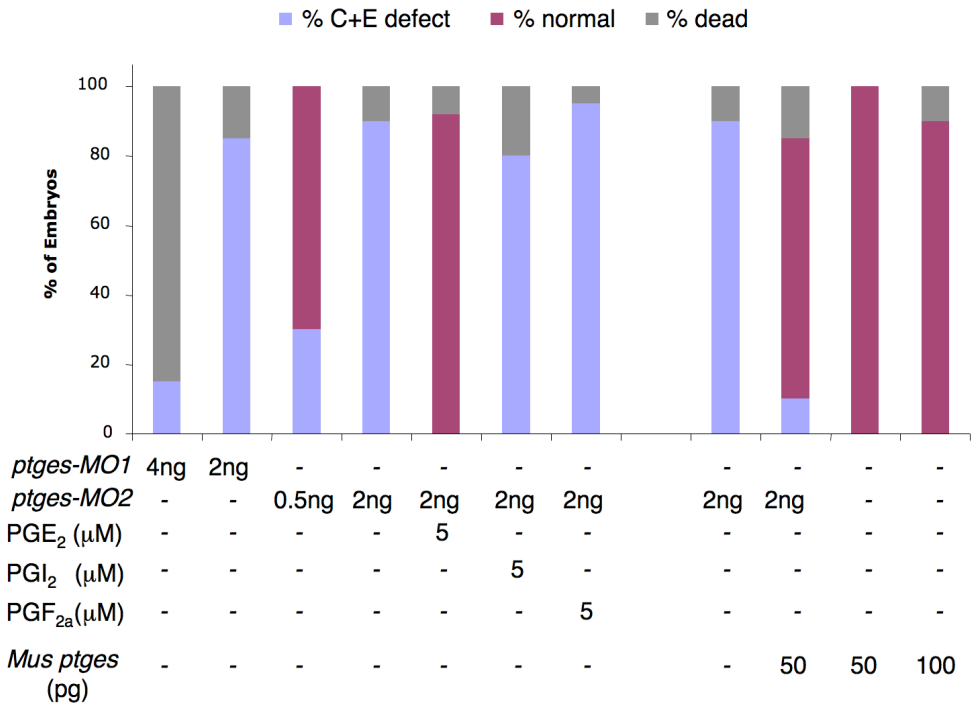


**Figure 8. Specificity of *ptges-MO* in binding endogenous *ptges* transcripts.** *ptges-MO1* was injected at either 4ng (lane 1) or 2 ng (lane 2). *ptges-MO2* was injected at either 0.5ng (lane 3) or 2ng (lanes 4-9). For rescue experiments, each embryo was injected with *ptges-MO2* (2ng) and co-incubated with 5 μM of $PGE_2$ (lane 5), $PGF_{2a}$ (lane 6), or $PGI_2$ (lane 7). Injecting embryos with mouse *ptges RNA* at 50pg (lanes 9-10) or 100pg (lane 11) did not result in gain-of-function phenotype, but co-injection of *ptges RNA* with ptges-*MO2* (2ng) resulted in suppression of the MO-dependent gastrulation defect (lane 9). The percentage is calculated from a total three independent experiments. Gastrulation defects were scored when the embryos display delayed epiboly, mispositioned head, and shortened anterior-posterior axis at the end of gastrulation.

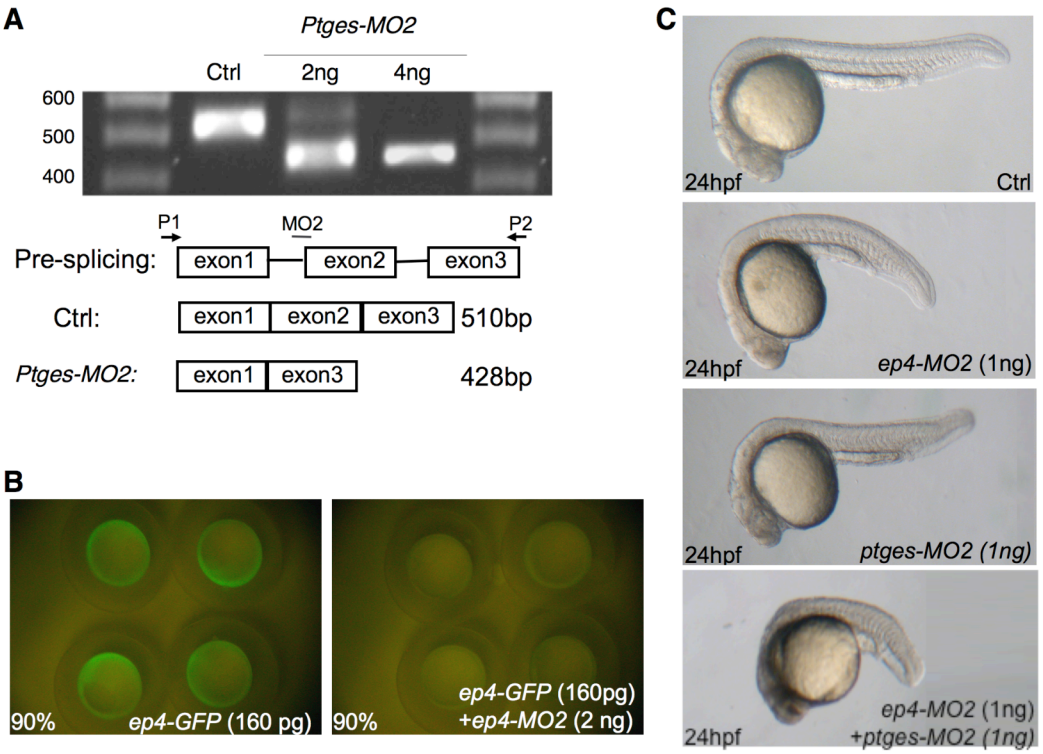


**Figure 9. Morpholinos against *ptges* and *ep4* transcripts are effective and demonstrate synergism.** (A) RT-PCR analyses of *ptges* transcripts from total RNA isolated from 15 embryos in uninjected control (ctrl), 2ng of *ptges-MO2* injected, and 4ng of *ptges-MO2* injected embryos. Diagram demonstrates the loss of exon2 in *ptges-MO2* injected embryos due to blocking of splicing acceptor site. This results in alternative splicing from the splicing donor site at the end of exon1 and acceptor site in the beginning of exon3. This was verified by sequencing of the 428bp band. (B) Images of embryos overexpressing GFP-tagged EP4 receptor injected with *ep4-gfp* RNA alone or co-injected with *ep4-MO2*. (C) *ep4-* and *ptges-MO* reveal synergism at sub-optimal doses. Injection of *ep4-* or *ptges-MO* at 1ng cause mild shortening of zebrafish length at 24 hpf. However co-injection of *ep4-* and *ptges-MO* at 1ng results in synergistic effect on the length of zebrafish embryos at 24 hpf.

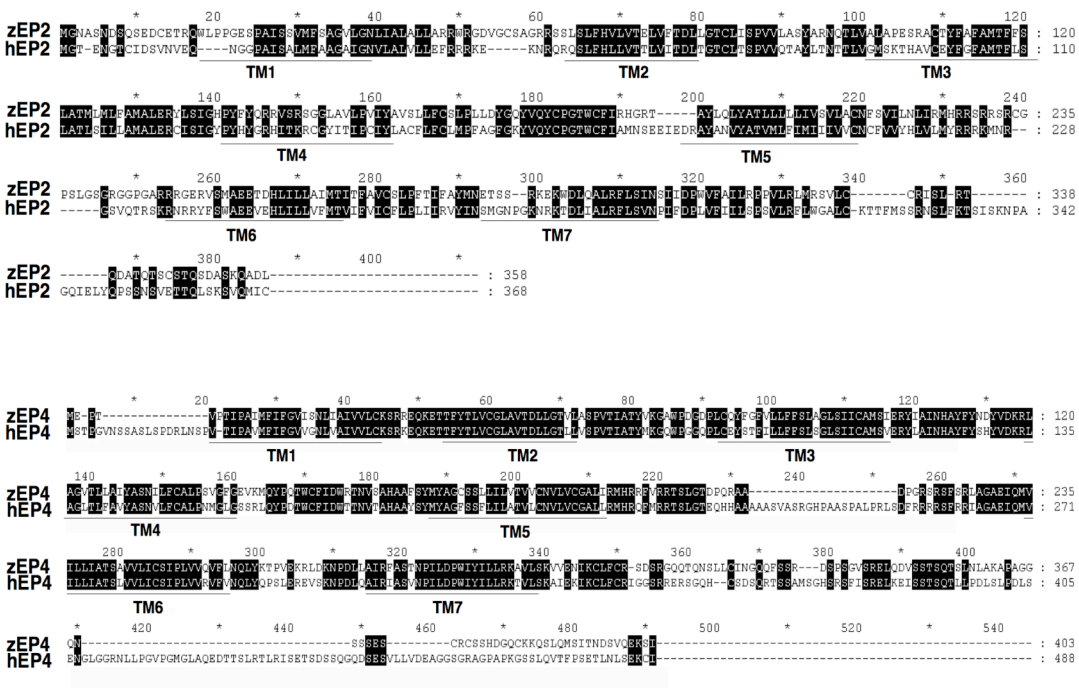


**Figure 10. Sequence analysis of EP4 receptor reveals presence of 7 transmembrane domains.** Consistent with its function as a G-protein coupled receptor (GPCR), EP4 receptor contains 7 TM domains and cytoplasmic tail. In addition the cytoplasmic tail of the human EP4 was longer than the zebrafish form, but there were 30 aa residues that were absolutely conserved between the two species, suggesting that these residues may be critical for EP4 signaling to downstream components.

## Discussion

Previous studies in the mice have not revealed much about the role for prostaglandins in early development. In heterozygous matings, *Cox-1* null mice were born in the expected ratios and lived a normal lifespan (Langenbach et al., 1995). This finding was quite surprising given the putative "housekeeping" function of the COX-1 enzyme. Although *Cox-2* null mice demonstrated numerous developmental defects (Dinchuk et al., 1995), their embryogenesis proceeded normally. Given the vital role of prostaglandins in normal physiologic settings, it has been hypothesized that the maternal contribution of prostaglandins in the placenta allows the prostaglandin-deficient embryos to develop normally (Reese et al., 2000; Reese et al., 2001). Consistent with this notion, mammalian blastocysts produce high levels of $PGE_2$ and $PGF_{2\alpha}$ (Davis et al., 1983; Dey et al., 1980) and murine blastocysts cultured outside of the uterus do not survive in the presence of COX inhibitors (Wang H, Dey SK, personal communication). Therefore the functional role of prostaglandins and the signaling cascades they regulate in early development have been virtually unknown.

By utilizing the externally developing zebrafish embryos as a model system, we circumvented the maternal interference allowing us to study the functional role for endogenous prostaglandins. Our data conclusively demonstrates the conservation of the $PGE_2$ signaling pathway in vertebrates. Previous studies in zebrafish demonstrated that COX-1 derived prostaglandins may be important for early development (Cha et al., 2005; Grosser et al., 2002). Here we show that prostaglandins during gastrulation are produced mainly by COX-1, since COX-2 expression does not take place until after the end of

gastrulation. Furthermore, we demonstrate that expression of Ptges co-localizes with that of COX-1 and the COX-1-Ptges pathway governs the production of $PGE_2$ during gastrulation. Previous work showed that COX-1 is coupled to cytosolic $PGE_2$ synthase (cPGES), while COX-2 is co-localized with microsomal $PGE_2$ synthase (Ptges) (Takeda et al., 2004) in gastrointestinal hamartomas. In zebrafish, Ptges co-localizes with COX-1 during gastrulation, but is co-expressed with both COX isoforms during somitogenesis in the posterior intermediate mesoderm and anterior neuroectoderm (YC, LSK, RND, unpublished data), suggesting that Ptges may be the predominant source of $PGE_2$ during embryogenesis. Consistently, when we injected antisense morpholino oligonucleotides targeting cytosolic $PGE_2$ synthase in zebrafish, we didn't observe any phenotype during gastrulation or thereafter (data not shown). This finding is similar to the situation reported in mice where inactivation of mouse *Ptges* reduces inflammatory responses to carigeenanm, similar to the effects of NSAIDs, while the cPGES-derived $PGE_2$ is relatively unimportant (Trebino et al., 2003), suggesting that Ptges may also be the dominant source of $PGE_2$ during inflammation. Taken together our data suggest that the COX-1-Ptges pathway may solely be responsible for providing basal levels of $PGE_2$ during early development.

During vertebrate gastrulation, changes in the levels of proteins that regulate cell movement can impair gastrulation in either LOF or GOF experiments (Carreira-Barbosa et al., 2003; Jessen et al., 2002; Lin et al., 2005). Similarly, LOF or GOF experiments with EP4 receptor both result in gastrulation defects, while overexpression of *ptges* or treatment with $PGE_2$ do not produce a noticeable change in gastrulation phenotype. In addition, the phosphorylation status of Akt in wild-type embryos treated with $PGE_2$

remains unaltered (Fig. 6F). These data suggest that $PGE_2$ plays an essential, but permissive, role during vertebrate gastrulation. It remains to be determined whether the excess EP4 receptor impairs gastrulation by increased normal signaling or it functions by an antimorphic or neomorphic type of activity.

One of the more interesting questions that arises from this study is why does *ptges* MO cause even stronger phenotype than the one observed in *ep4* morphants? From cell culture experiments, we know that $PGE_2$ can exert effects that are not totally dependent on the EP4 receptor. For example, we have previously reported that $PGE_2$ can indirectly transactivate the PPARd receptor in colorectal adenoma growth in $APC^{min}$ mice (Wang et al., 2004a). Therefore, we hypothesize that during zebrafish gastrulation loss of $PGE_2$ can lead to stronger phenotype than that exhibited by *ep4* morphants due to a disruption of process independent of EP4 pathway. In support of this, there are differences in *ptges* and *ep4* morphant phenotypes at 24 hpf (Fig. 2S and 3F). However, our data convincingly demonstrates the effects of $PGE_2$ on cell movement are dependent specifically on the EP4 receptor.

Another intriguing concept that arises from this study is the conserved role of the EP4-PI3K/Akt pathway in regulation of cell movement. Previous work in zebrafish demonstrated that PI3K/Akt signaling is required downstream of PDGF for proper cell shape, process formation and movement during anterior migration of prechordal mesodermal cells (Montero et al., 2003). However our studies also implicate PI3K/Akt signaling in proper convergence, extension and epiboly processes. We provide two lines of evidence that suggest PI3K/Akt lies downstream of $PGE_2$ during gastrulation: First, we observed decreased activation of Akt in EP4-deficient embryos; Second, loss of function

phenotypes in Ptges and EP4 deficient embryos share similar defects in convergence, extension, and epiboly as those embryos treated with a PI3K inhibitor. With respect to gastrulation cell behaviors that depend on this pathway, our cell tracking data support the notion that EP4 receptor signaling utilizes PI3K/Akt to promote cell motility, rather than cell shape or persistence of migration. Consistently, zebrafish primordial germ cells also depend on PI3K signaling for cell motility during their migration (Dumstrei et al., 2004), while PI3K/Akt signaling has been implicated also in cell movement and directionality during neutrophil migration (Stephens et al., 2002; Wang et al., 2002). These observations suggest that PI3K/Akt is a key regulator of distinct properties of moving cells in various cellular contexts. Therefore we cannot exclude the possibility that EP4 signaling may regulate other aspects of cell behavior during zebrafish gastrulation and development.

So, how does the EP4 receptor activate the PI3K/Akt pathway? As mentioned earlier, the EP4 receptor is a member of GPCR superfamily. Previous studies in mammalian cell culture demonstrated that G-βγ dimers transmit signals from GPCR to a variety of intracellular effectors in distinct cellular contexts (Schwindinger and Robishaw, 2001). One of the targets of G-βγ dimers involves direct activation of PI3K by binding of specific G-βγ dimers to both subunits of PI3K (Leopoldt et al., 1998). Whether G-βγ dimers can transduce EP4 signaling still remains to be seen both in mammalian settings as well as during zebrafish gastrulation. However, our perusal through the zebrafish genome revealed the presence of multiple human Gβ and Gγ homologs and their role during zebrafish gastrulation have not yet been elucidated.

Although $PGE_2$ signaling has been shown to promote migration and invasive behaviors of several cancer cell lines *in vitro* (Buchanan et al., 2003a; Sheng et al., 2001), the cell movement behaviors and molecular pathways regulated by $PGE_2$ signaling *in vivo* are not known. Our studies provide the first evidence that $PGE_2$ signaling regulates cell movement *in vivo* by promoting cell motility during gastrulation. Understanding the detailed mechanism of $PGE_2$ signaling in zebrafish gastrulation may ultimately provide significant insights into how $PGE_2$ regulates various processes that require cell movement, including metastastic spread of cancer.

# CHAPTER III

## CYCLOOXYGENASE-1 SIGNALING IS REQUIRED FOR VASCULAR TUBE FORMATION DURING DEVELOPMENT


Abstract

NSAIDs are utilized widely as an anti-inflammatory, anti-pyretic, and analgesic agent. Since knockdown of COX-1 causes early gastrulation arrest, we investigate the functional role of COX-1 after gastrulation by utilizing NSAIDs as pharmacological inhibitor of COX function. After gastrulation, *cox1* expression becomes restricted to the posterior mesoderm during somitogenesis and to posterior mesoderm organs at pharyngula stage. Inhibition of COX-1 signaling using pharmacological inhibitor as well as after gastrulation results in defective vascular tube formation and shortened intersomitic vessels in the posterior body region. These defects are rescued completely by $PGE_2$ treatment or, to a lesser extent, by $PGF_{2\alpha}$, but not by other prostaglandins, such as $PGI_2$, $TxB_2$, or $PGD_2$. Functional knockdown of COX-1 using antisense morpholino oligonucleotide translation interference also results in posterior vessel defect in addition to enlarged posterior nephric duct, phenocopying the defects caused by inhibition of COX-1 activity. Together we provide the first evidence that COX-1 signaling is required for development of posterior mesoderm organs, specifically in the vascular tube formation and posterior nephric duct development.

## Introduction

Cyclooxygenase inhibitors are one of the oldest and most lucrative drugs to be marketed in the history of mankind. In 2002, more than 41 million prescriptions were filled in the United States for selective COX-2 inhibitors, -coxibs, and total NSAID prescription costs (not including over the counter drugs) totaled $6.5 billion in 2003 (Solomon et al., 2004). Thus any potential relationship between NSAIDs or –coxibs with deleterious side effects poses substantial clinical and public health issues. Recent clinical trials, The Vioxx and Gastrointestinal Outcomes (VIGOR) revealed that selective COX-2 inhibitors increased the relative risk of acute myocardial infarctions (AMIs). A longer term polyp prevention trial (APPROVe) confirmed the deleterious thrombotic side effects of rofecoxib and led to its removal from the market (Bresalier et al., 2005). Others also took a formal experimental approach and found evidence consistent with cardiovascular hazards associated with COX-2 inhibitors. Certain nonselective NSAIDs may also cause cardiovascular deficits similar to those seen in patients treated with COX-2 selective inhibitors (Fitzgerald, 2004). In addition, valdecoxib was taken off the market due to increased complications in patients on this drug following cardiac surgery (Nussmeier et al., 2005). Celecoxib remains available for use but with a very stern warnings about possible cardiovascular side effects.

In addition, one of the notorious side effects associated with high-dose ingestion of NSAIDs use include dyspepsia, gastritis, peptic ulcer, gastrointestinal bleeding, hemorrhagic stroke, and perforaton of gastroduodenal ulcers (Wolfe et al., 1999), all involving various aspects of angiogenesis. NSAIDs also inhibit wound healing, delaying

the healing of ulcers. COX-2-selective NSAIDs do not produce gastroduodenal ulcers, but they also appear to inhibit ulcer healing, similar to non-selective NSAIDs (Halter et al., 2001). Therefore understanding the mechanisms by which NSAIDs cause such deleterious side effects is an important clinical question.

Previously, Grosser *et al*. elegantly demonstrated that the non-selective COX inhibitor, indomethacin, specifically inhibits the formation of PGs in live zebrafish. Indomethacin was a potent inhibitor of COX-1 derived PGs, while both indomethacin and NS-398, COX-2 selective inhibitor, were effective inhibitors for COX-2. Similar $IC_{50}$ were obtained for both inhibitors (Grosser et al., 2002). Since zebrafish is an emerging model for chemical screening, we tested the inhibitors of cyclooxygenase in zebrafish embryos to understand the phenotypic defects associated with inhibition of prostaglandin production after gastrulation.

## Methods

**In Situ Hybridization.** Antisense probes for zebrafish *cox1* and *cox2* were synthesized as described(Grosser et al., 2002). Antisense RNA probes were synthesized from cDNAs encoding *fli1*(Krauss et al., 1993), *flk-1* and *pax2.1*(Krauss et al., 1991a). Whole mount *in situ* hybridization was performed according to Marlow *et al.*(Marlow et al., 2002). All images were captured with Nikon Coolpix 4500. Each *in situ* experiment was done at least twice, and approximately 20 embryos were used in each experiment.

**Morpholino Design and RNA injections** were performed at the one-cell stage as described (Marlow et al., 2002). Morpholino antisense oligonucleotide was designed to the predicted start codon of *cox-1* (underline indicates the predicted start codon)*: cox1-*MO (5'-TCAGCAAAAAGTTACACTCTCTCAT-3') as described (Grosser et al., 2002). Mismatch morpholino against *cox1* was also designed to the predicted start codon of *cox-1* (underline indicated the predicted start codon and italic letters indicates mismatched resides): (5'-TCAcCAtAAAcTTACACTgTCTgAT-3'). Both morpholinos were obtained from Gene Tools, LLC (Philomath, OR). Zebrafish *cox-1* sense-capped RNA was synthesized using mMessage Machine (Ambion, Austin, TX) after template linearization. For phenotype rescue and phenocopy experiments, 40–100 pg *cox-1* RNA and 4-10 ng *cox1-MO* were used per embryo.

**NSAID pharmacology** To inhibit COX activity, embryos were treated with indomethacin, a non-selective cyclooxygenase inhibitor. Embryos were incubated overnight at 28 C in 24-well dishes. Indomethacin (Cayman), celecoxib (Cayman), and NS-398 (Cayman) were incubated in embryo medium (EM) with 1% DMSO. Treatment

of embryos at 2-8 cell stage with 50 μM indomethacin results in gastrulation arrest phenotype. We treated embryos with 40 μM indomethacin at tailbud stage or early somitogenesis stage to observe posterior vessel defect. Celecoxib was used in dose ranging from 1μM to 150 μM and NS-398 was used at dose ranging from 1 μM to 150 μM. Both were treated starting at tailbud stage.

**Prostaglandin Assays** Endogenous levels of prostanoids were measured. Embryos were isolated at 80% epiboly stage and 24 hpf. The PG content ($PGE_2$, $PGF_{2\alpha}$, $PGD_2$, thromboxane $B_2$ ($TXB_2$, a stable metabolite of $TXA_2$) and 6-keto-PGF1α, a stable metabolite of $PGI_2$) of developing embryo was then quantified by utilizing gas chromatography/negative ion chemical ionization mass spectrometric assays. Each bar indicates the mean of three independent measurements, with each group consisting of at least 100 embryos.

**Prostaglandin Rescue Experiment** To rescue indomethacin mediated phenotypic effects, we used commercially available prostaglandins (Cayman), $PGE_2$, $PGF_{2a}$, $PGI_2$, $TxB_2$, and $PGD_2$. All PGs were incubated alone at concentration ranging from 1 μM to 100 μM, except for $PGI_2$, which was tested at 0.1 μM to 100 μM. For rescue experiments we supplemented indomethacin (40μM) treated embryos with 1-10 μM of PGs at 1% DMSO egg media. Both indomethacin and PGs were incubated at tailbud or early somitogenesis stages.

## Results

### Expression of COX-1 during Embryogenesis

*Cox1* maternal transcripts were distributed ubiquitously in zebrafish embryos at early cleavage stages and its expression persists in all cells during cleavage, blastula, and gastrula stages (Fig. 11A). At the end of the gastrula period (10.5 hours post fertilization, hfp), we observed *cox1* expression localizing in the posterior half of the embryo (Fig. 11B, 11C). At the 7 somite stage, higher levels of *cox1* expression were detected in the bilateral stripes in intermediate and lateral plate mesoderm, which give rise to future mesodermal organs, such as blood vessels and nephric ducts. These stripes co-localized with the endothelial marker, *fli1*(Krauss et al., 1993) (Fig. 11G), and the nephrogenic marker, *pax2.1*(Krauss et al., 1991a) (Fig. 1H), indicating that *cox1* is expressed in the posterior region and enriched in both posterior intermediate and lateral plate mesoderm. During the late segmentation stage, intense expression of *cox1* was seen broadly in the developing posterior body (Fig. 11D), and eventually became confined to the distal end of the nephric duct, forming vasculature, hypochord and posterior end of notochord at the 24 hpf (Fig. 11E). *cox1* staining was not significantly altered in *casanova* mutant (Fig. 11F), which lack gut tube and do not express any molecular markers of endoderm differentiation(Alexander et al., 1999; Kikuchi et al., 2001). This is consistent with *cox1* being expressed largely in posterior mesoderm. Therefore we hypothesized that COX-1 may be an important regulator of posterior mesoderm development.

### Pharmacologic Inhibition of COX-1 Results in Posterior Mesoderm Defects

We then tested whether use of cyclooxygenase inhibitors would lead to early developmental defects, specifically in posterior mesoderm. Previously, Grosser *et a*l. showed that indomethacin, a non-selective COX inhibitor, and NS-398, a selective COX-2 inhibitor, could specifically inhibit the respective zebrafish enzymes *in vitro* as well as *in vivo*(Grosser et al., 2002). Indomethacin treatment, starting at the 2-8 cell stage, caused phenotypic defects in a dose-dependent manner from 25 μM to 50 μM. At the dose of 50 μM, we observed gastrulation arrest at 50% epiboly stage (Fig. 12A). At 40 μM, embryos completed epiboly and gastrulation, but, at the end of embryogenesis, they exhibited a gross disorganization of the posterior mesodermal region of the embryo (Fig. 12B, 13, 14). Treatment with 25 μM indomethacin resulted in a milder defect in posterior mesoderm formation. This area of defective development in the embryo parallels the location of *cox1* expression (Fig. 11D,E).

To test whether this phenotypic defect was mediated exclusively by COX-1, we incubated the embryos with a COX-2 selective inhibitor NS-398. However, even at 150 μM NS-398, we failed to see any difference in the morphology of treated embryos compared to the untreated wild-type embryos (Fig. 12G). Also, incubating the embryos with another COX-2 selective inhibitor, celecoxib, failed to elicit any observable phenotypic changes even at doses up to 150 μM (Fig. 12G), while COX-1 selective inhibitor, SC-560, did result in posterior mesoderm defect similar to indomethacin treatment (data not shown). Taken together, these results indicate that pharmacologic inhibition of COX-1, but not of COX-2 results in posterior mesoderm defects.

## Specfic Inhibition of COX-1 by Indomethacin

To examine if the specificity of the phenotypic changes associated with indomethacin treatment resulted directly from inhibition of COX-1 enzymatic activity, we tested whether supplementing treated embryos with prostaglandins could suppress the indomethacin-mediated defects. We co-incubated the indomethacin treated embryos with $PGE_2$, $PGF_{2\alpha}$ or $PGI_2$ in separate experiments, since these PGs are the predominant prostaglandins found during embryogenesis. We found that $PGE_2$ and, to a lesser extent, $PGF_{2\alpha}$ completely suppressed the development of posterior mesodermal defects (Fig. 12D, 12F) observed in embryos treated with indomethacin alone (Fig. 12B). Coincubation of indomethacin and $PGE_2$ (Fig. 2C) or $PGF_{2\alpha}$(Fig. 12E) can also effectively rescue gastrulation arrest phenotype. $PGE_2$ treatment rescued the gastrulation arrest at both 1 μM and 10 μM, while $PGF_{2\alpha}$ could only suppress the phenotype at 1 μM (Fig. 12G). We found that $PGE_2$ and $PGF_{2\alpha}$, in combination, also rescued both posterior mesoderm defects, while other prostaglandins, $PGI_2$, $PGD_2$, or $TxA_2$, had no effect (Fig. 12G). It could be argued that using 40 - 50 μM dose may produce COX-independent effects, but the fact that we can rescue the posterior defects with $PGE_2$ argues against this claim. Moreover we demonstrated the actual levels of indomethacin in embryonic tissues are significantly lower than the level of indomethacin in media in which the embryos were incubated in these experiments (data not shown). Analyses of prostaglandin profiles during zebrafish development showed that $PGE_2$, $PGF_{2\alpha}$, and $PGI_2$ are the major prostanoids present in the embryos during the gastrulation stage (7 hpf), while $TxA_2$ was found at very low levels, and $PGD_2$ was virtually undetectable (Fig. 20). Together these results indicate that COX-1 derived $PGE_2$ and, to a lesser extent, $PGF_{2\alpha}$ play critical roles in the regulation of gastrulation and posterior mesoderm development.

## Inhibition of COX-1 Results in Defective Vascular Tube

To determine what specific posterior mesodermal structures depend on COX-1 derived prostaglandins, we analyzed phenotypic defects in embryos treated with 40 μM indomethacin. We utilized *fli1:*EGFP fish, a transgenic line that expresses green fluorescent protein in all blood vessels under the control of the *fli1* promoter(Lawson and Weinstein, 2002c). Normally at 24 hpf, posterior vasculature consists of dorsal aorta that makes a 180° turn at its caudal end to form the caudal vein. The caudal vein forms a network veinous plexus that continues into the trunk, becoming a single distinct posterior cardinal vein (PCV) (Isogai et al., 2001). Intersegmental vessels sprout and elongate from the dorsal aorta and posterior cardinal vein to form dorsal longitudinal vessels (DLV). To our surprise, indomethacin treatment caused the posterior vasculature to remain as a single distended vessel at 24 hpf (Fig. 13B inset) and 48 hpf (Fig. 13D), compared to the untreated embryo (Fig. 13A inset, Fig. 13C). At day 2 a clear demarcation was seen between arterial and venous vessels in control embryos (Fig. 13C), whereas indomethacin-treated embryos continued to exhibit a single vessel, unable to differentiate into distinct arterial and venous system (Fig. 13D). We found that indomethacin treatment results in distention of the posterior vasculature at 24 hpf (Fig. 13F). Circulation of blood was markedly slowed and blood cells were pooled in the distended vessel. These circulation defects results in decreased hydrostatic blood pressure and led to pericardial edema at 48 hpf, and all treated embryos died within the first five days post fertilization.

## Inhibition of COX-1 Results in Reduction of Posterior Veninous Vessel

We then performed *in situ* hybridization revealing expression of several blood vessel markers to understand the specific defects in the vasculature caused by COX-1 interference. Untreated embryos showed continuous *flk1* ((Liao et al., 1997) staining in the dorsal aorta as well as the posterior cardinal vein (Fig. 3G), whereas treated embryos show a significant reduction of *flk1* staining in the posterior vasculature (Fig. 13H). The marked reduction in *flk1* staining is clear when compared to untreated embryos (Fig. 13G). We then examined arterial and venous specific markers to further understand the defect in vessel formation. *ephB2* , a marker specific artery, showed similar staining between untreated (Fig. 13I) and treated embryos (Fig. 13J). However staining with *flt4* (Lawson et al., 2001), a marker specific for vein, revealed a decrease of posterior cardinal vein as well as venous plexus found in caudal vein (Fig. 13L), compared to untreated embryos (Fig. 13K). Most striking reduction was the in the boundary separating arterial and veinous vessel, which is absent also in the blood vessel of indomethacin-treated *fli1:*GFP transgenic (Fig. 13B inset). Next we sectioned *flk1* stained embryos both treated with indomethacin and untreated. Cross-sections of anterior and posterior trunk portion of *flk1* embryos revealed that proper tube formation did not occur in indomethacin treated embryos. Anterior (Fig. 14C) and posterior (Fig. 14D) sections of the untreated embryo show two distinct tube structures, dorsal aorta (DA) dorsally and posterior cardinal vein (PCV) ventrally, while inhibition of COX-1 results in defective tube formation and strong reduction of PCV staining both in the anterior (Fig. 14G) and posterior (Fig. 14H) sections of the trunk. These experiments strongly suggest that the indomethacin treated embryos are unable to form separate tubes of artery and veinous systems, but, instead, form a single, distended vessel.

To understand the timing requirement of COX-1 function in vasculogenesis, we varied the starting time of treatment from the tailbud stage (10 hpf) to 36 hpf and found that altering the initiation time did not affect the vascular defect analyzed at 38 hpf (Fig. 15). In addition, we initiated treatment at the 5 somite stage and discontinued treatment at various points before 32 hpf. Interestingly we found that stopping treatment anytime before 25 somites resulted in the absence of a posterior vessel defect, indicating that COX-1 derived prostaglandins are required after 25 somite stage for posterior vessel formation (Fig. 15). Angioblasts first coalesce to form a single cord at 22 somite stage. Then arterial and venous angioblasts undergo morphogenesis and eventually form distinct domains and align in the form of rudimentary and distinct tubes beginning at 26 somite stage (Parker et al., 2004). Based on these results we hypothesize that COX-1 may function at this cord-to-tube transition to regulate proper tube formation. Therefore inhibition of COX-1 activity at 24-26 stage mostly likely results in vascular tube formation.

Inhibition of COX-1 activity also causes defect in intersomitic vessel formation. Intersomitic vessels begin to grow out of dorsal aorta at around 20 hpf, while the angioblasts are actively coalescing to form a vascular cord (Isogai et al., 2003). In *Fli1*-GFP transgenic embryos, treatment with indomethacin resulted in shortened or absence of intersomitic vessel at 24 hpf (Fig. 13B), compared to the longer intersomitic vessels seen in untreated embryos. Removing the indomethacin at 24 hpf did not restore the intersegmental vessel formation at 48 hpf, and 30% of the intersomitic vessels were still shortened. To understand whether the shortening of intersomitic vessel is due to a primary defect in the vessel formation, we examined whether indomethacin causes defect

in somite formation. Since intersomitic vessels transverse through the boundary between the myotomes (Isogai et al., 2003), we stained the treated embryos with vinculin antibody and found that the somite boundary is largely normal in treated embryos. Somites are formed correctly in treated embryos, both in the trunk and tail regions (Fig 15B, D). In addition, expression of *vegf* gene, encoding a key regulator of intersomitic vessel angiogenesis, in the somites also appeared unaltered in both control and treated groups (Fig. 15E, F). Therefore we conclude that defect in somite formation or VEGF production is not responsible for shortened intersomitic vessels, suggesting a direct involvement of COX-1 in intersomitic vessel development.

Functional Knockdown of COX-1 by Morpholino

We next asked whether interference with COX-1 translation, using antisense morpholino-modified oligonucleotides (MO), mimics the posterior mesoderm defects of indomethacin treatment. Given that embryos injected with high doses of MOs halt development at early gastrulation (Grosser et al., 2002), we injected the embryos with lower amounts of *cox1-MO* that allowed them to progress past gastrulation. Interestingly, at 4 ng and 8 ng, 80% of embryos completed epiboly and gastrulation, but they eventually developed defects in posterior mesoderm structures similar to those seen following indomethacin treatment. In particular morphant embryos also developed disorganized posterior vasculature similar to those seen in indomethacin treated embryos, whereby blood cells pooled in the posterior vessel region (Fig. 17B arrow) compared to uninjected embryos (Fig. 17A). In addition, staining with *pax2.1*(Majumdar et al., 2000) revealed increased posterior nephric duct size in morphants (Fig. 17G), compared to the

control siblings (Fig. 17D arrow). The increased staining correlated with the enlarged posterior nephric duct that persisted at day 2 (Fig. 17H arrow) and day 3 (Fig. 17I arrow). Enlargement of posterior nephric duct also occurred with lower dose of indomethacin (25 μM), in which milder vessel defect and enlarged posterior nephric duct was seen, another evidence that we were treating the embryos with physiologically relevant dose of indomethacin.

Injection of higher dose of *MOs* at 10 ng/embryo led to gastrulation arrest at around the 40-50% epiboly stage in over 90% of the injected embryos (Fig. 18A), similar to the results seen in embryos treated with 50 μM indomethacin during gastrulation. Co-injecting *cox1-MO* with synthetic *cox1* RNA, harbouring five point mutations in the MO binding region, suppressed the gastrulation arrest and allowed the embryos to progress past the tailbud stage (Fig. 18B), demonstrating that *cox1-MO* specifically binds to endogenous *cox1* mRNA and does not interfere with translation of different transcripts. From these results, we conclude that functional knockdown of COX-1 with *cox1-MO* results in posterior mesoderm defects and gastrulation arrest similar to that seen with pharmacologic COX-1 inhibition.

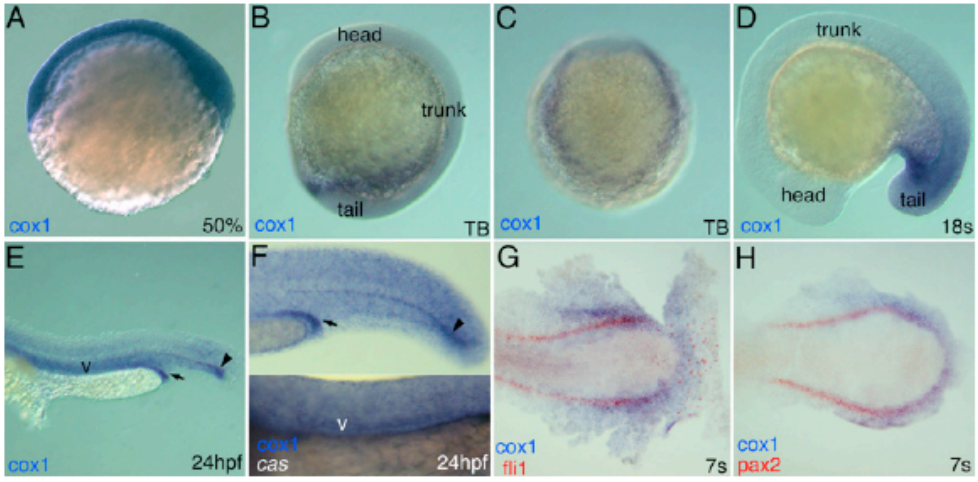


**Figure 11 *cox1* transcripts localize to posterior mesoderm organs.** Whole mount in situ hybridization of *cox1* (A-H) reveals that its transcripts are ubiquitously present at shield stage (A) and localize posterior half of the embryos at tailbud stage (B-C) from side view (B) and from posterior view (C). *cox1* co-localizes with *pax2* (H) and *fli1* (G) at 7 somite stage, indicating its expression in posterior intermediate mesoderm and posterior lateral mesoderm. During late somitogenesis stage *cox1* transcripts strongly stain posterior mesoderm (D). At 24 hpf, *cox1* can be seen in various mesodermal structures (E). Expression of *cox1* is not altered in *casanova* mutant, deficient in endoderm derivatives (F). Arrow indicates the distal end of pronephric duct, Letter v indicates the vasculature and arrowhead points to the distal hypochord. Scale bar: 100μm

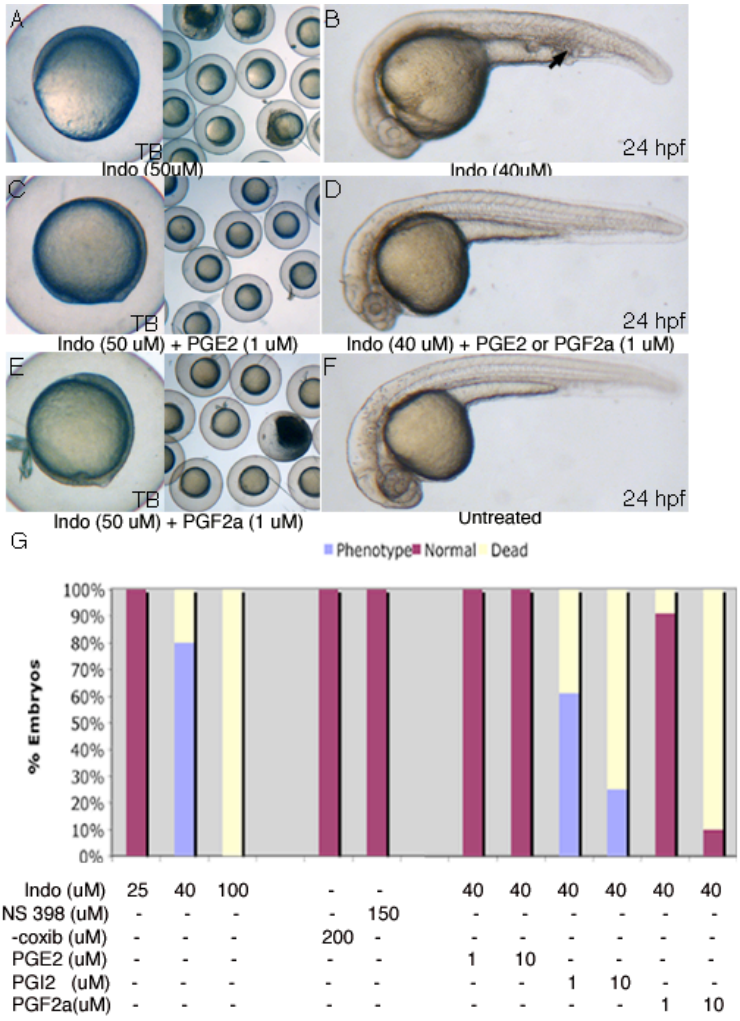


**Figure 12 $PGE_2$ and $PGF_{2a}$ can rescue both gastrulation arrest and posterior mesoderm defects induced by indomethacin.** Indomethacin treatment results in gastrulation arrest at 50 μM (A) and posterior mesoderm defects at 40 μM (B arrow). Supplementing indomethacin with $PGE_2$ or $PGF_{2a}$ can suppress both the gastrulation arrest (C, E) and posterior mesoderm defects (D). COX-2 inhibitors, NS-398 or celecoxib, do not cause any noticeable phenotypic defects up to 150 μM (G). In zebrafish embryos, $PGE_2$, $PGF_{2a}$, and $PGI_2$ are the major prostaglandins present (H). Two independent experiments were performed (n > 80) in indomethacin, celecoxib, and NS-398 treatment groups. In rescue experiments, we performed three independent experiments (n>80) in each of the treatment groups.

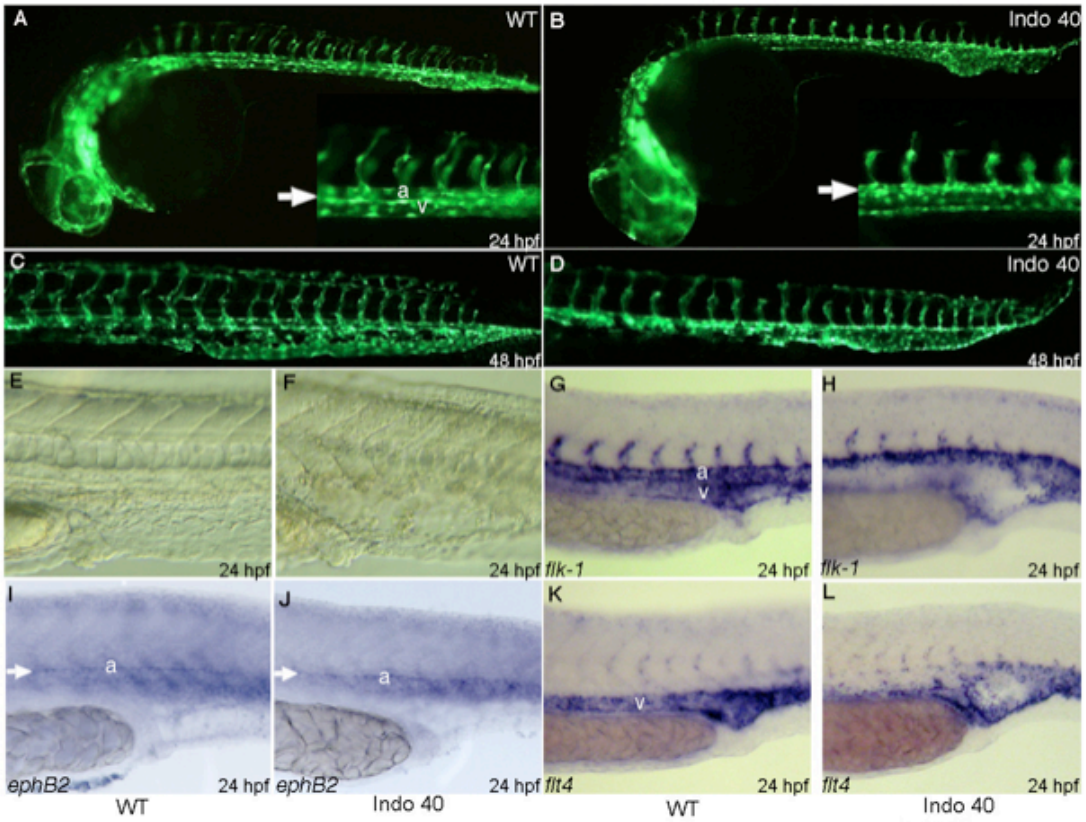


**Figure 13 COX-1 inhibition results in posterior vessel defect.** *Fli1:*EGFP transgenic embryos were treated with 40 μM indomethacin at tailbud stage. Compared to untreated embryos (A, C), indomethacin treatment results in distended posterior vasculature at day 1 (B, arrow). Posterior vasculature cannot differentiate into dorsal aorta and posterior cardinal vein (B, inset arrow). At day 2, clear distinction between dorsal aorta and caudal vein can be seen in untreated embryo (C), but a single distended posterior vessel remains in treated embryo (D). In addition, intersomitic vessels are either shortened or absent at day 1(B, inset) and results in shortened intersomitic vasculature at day 2 (D). Indomethacin treatment results in distention of posterior vasculature at day 1 (F), compared to untreated embryos (E). Staining with *flk1,* an endothelial marker, reveal the significant reduction or absence of venous staining at day 1 in treated embryos (H), compared to the wildtype (G). While *ephB2*, arterial marker, staining shows similar staining between wt (I) and treated embryos (J), *ephB4*, vein marker, staining shows absence of of veinous staining (L).

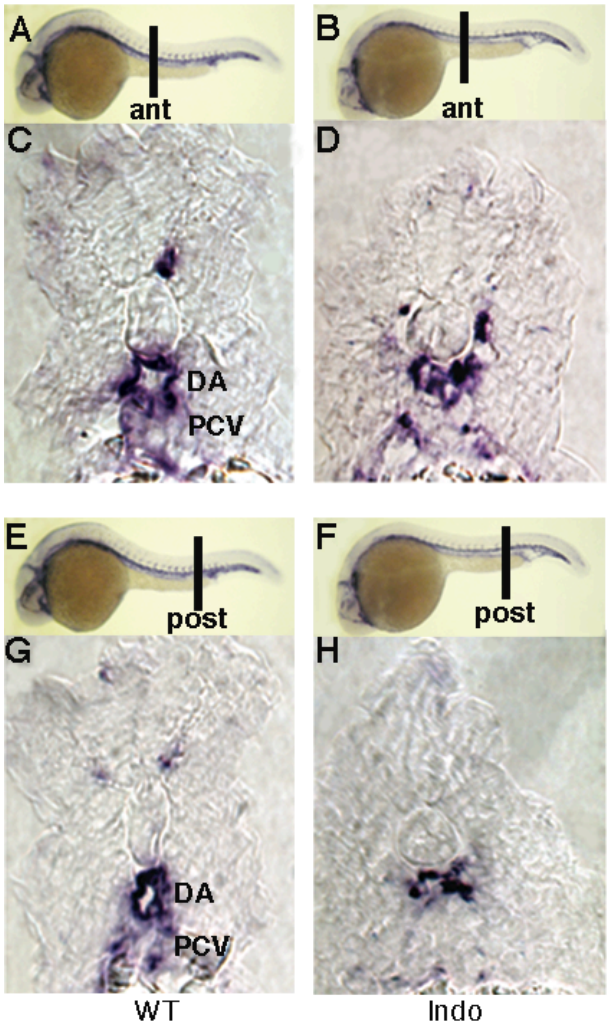


**Figure 14 COX-1 inhibited embryos are unable to form distinct dorsal aorta and posterior cardinal vein.** Cross-section of anterior (A, C) and posterior trunk (E, G) in untreated embryos displays distinct dorsal aorta and posterior cardinal vein. Indomethacin treated embryos do not form two vascular tubes, but display one distended vessel both in anterior (B, D) and posterior sections (F, H). ant, anterior; post, posterior. All embryos were sectioned after staining with *flk1*.

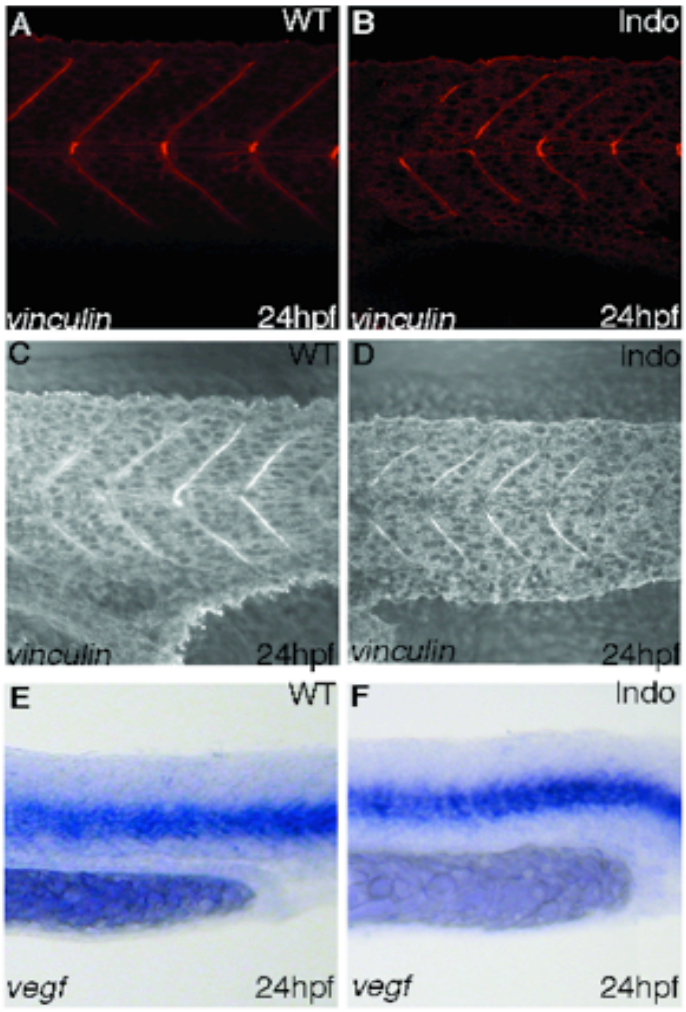


**Figure 15 Inhibition of COX-1 does not cause defect in somite formation or VEGF production.** Vinculin staining, marker for intersomitic boundary, reveals normal somite formation in the trunk both in control group (A) and treated group (B). In the tail region, vinculin staining is overlayed on DIC image of the same area. It also reveals that somite formation of the tail is unchanged in control group (C) vs. treated group (D). Somites in the treated embryos produce normal amounts of VEGF (F), compared to the untreated group (E).

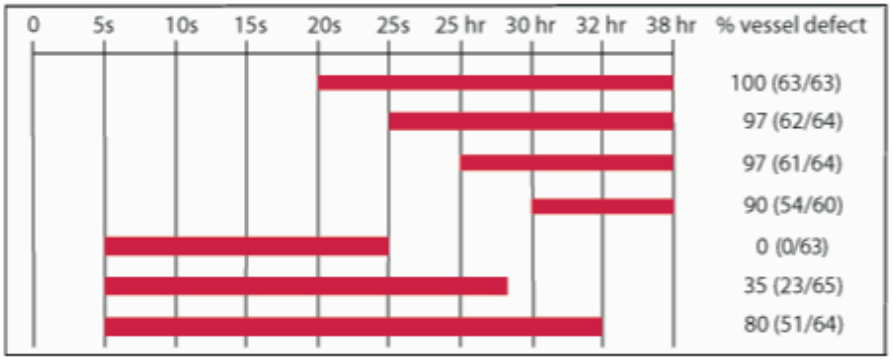


**Figure 16 COX-1 signaling is required after 25 somite stage for posterior vessel formation.** We monitored the presence of posterior vessel defect at 38 hpf after starting indomethacin treatment at various stages. Starting treatment at 20s, 25s, 25hr, or 30 hpf to 38 hpf all resulted in posterior vessel defect. Starting treatment at 5s to any stage before 25 somites resulted in absence of posterior vessel defect, whereas ending the treatment at 28 hpf and 32 hpf resulted in posterior vessel defect in 35% and 80% of the embryos respectively.

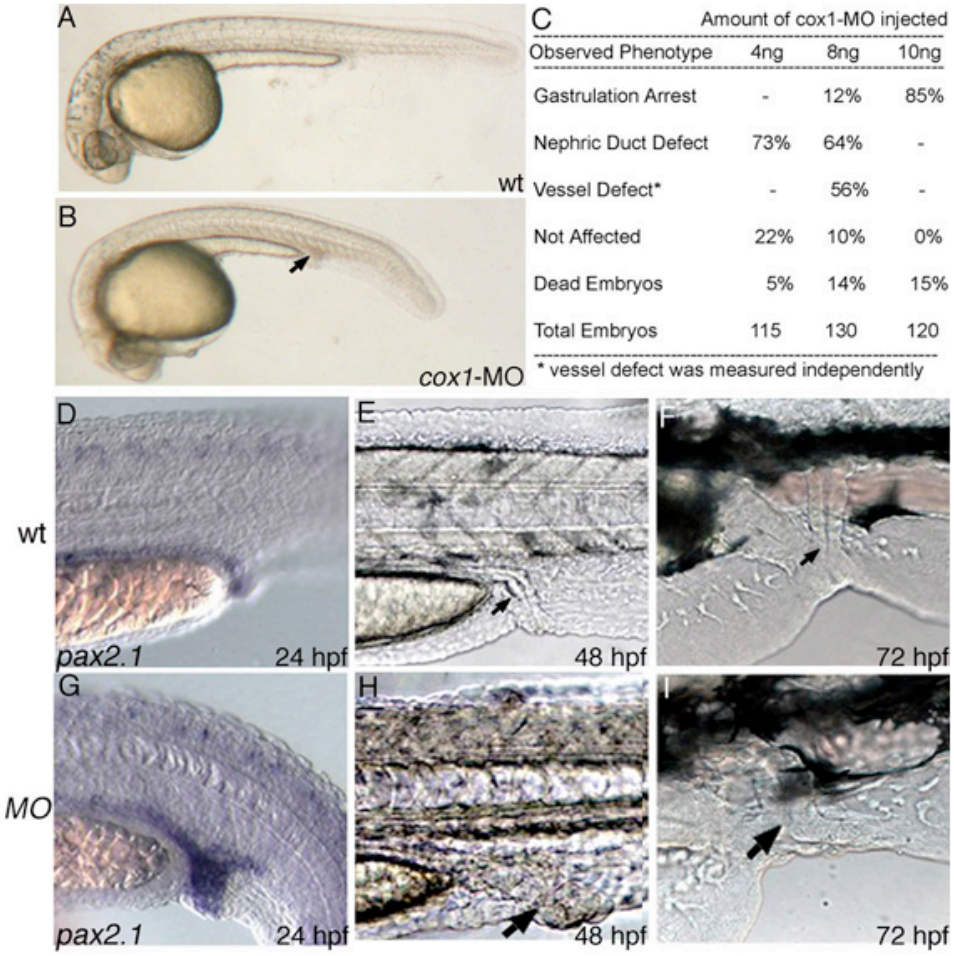


**Figure 17 *cox1-MO* causes posterior mesoderm defect similar to indomethacin induced phenotype.** Compared to uninjected wildtype (A), injection of 8ng *cox1-MO* (B) specifically disrupted posterior mesoderm organs at 1 day, including enlarged nephric duct and distended posterior vasculature (B arrow). Staining with *pax2.1* shows increased pronephric duct size in the posterior region (D). As the embryos develop, the increased staining correlate to increase in posterior nephric duct size at day 2 (D, arrow) and day 3 (F, arrow), compared to wildtype (B,C).

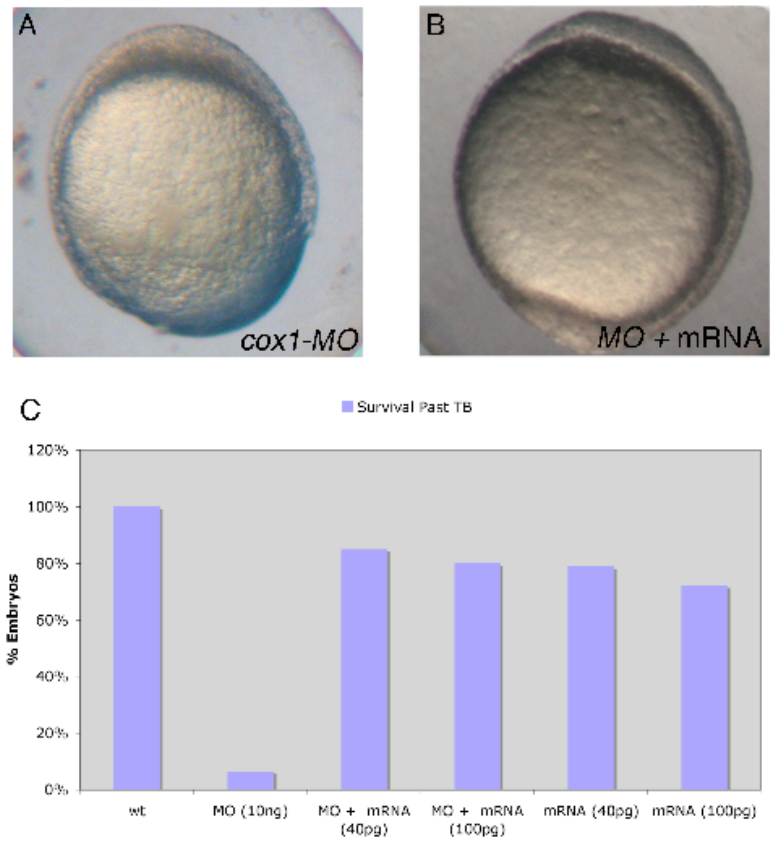


**Figure 18 COX-1 knockdown results in gastrulation defect at high dose.** Injection of 10ng *cox1-MO* results in gastrulation arrest at ~50% epiboly stage (A). Gastrulation arrest defect by *cox1*-MO can be rescued by co-injecting synthetic *cox1* RNA, mutated in MO-binding site (B). Black bar indicates the percentage of embryos that survived to tailbud (C). In the rescue experiments (lane 3, 4), we tested at least 80 embryos from two independent experiments, while in *cox1* RNA injection groups (lane 5, 6), at least 50 embryos from two independent experiments were analyzed.

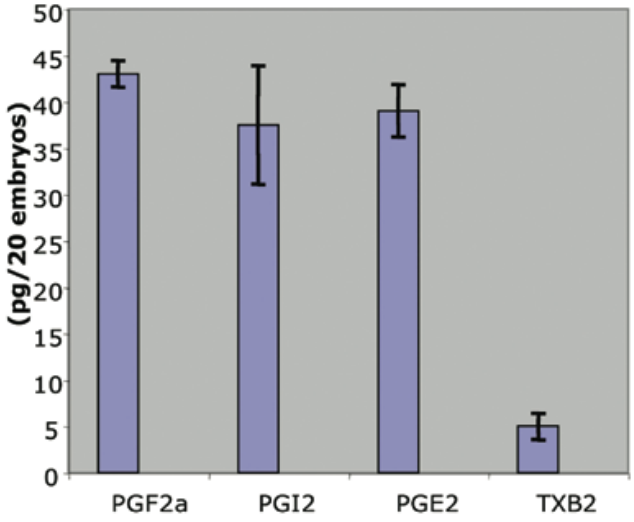


**Figure 19 $PGE_2$, $PGF_{2a}$, and $PGI_2$ are the major prostaglandins present in zebrafish embryos during gastrulation.**

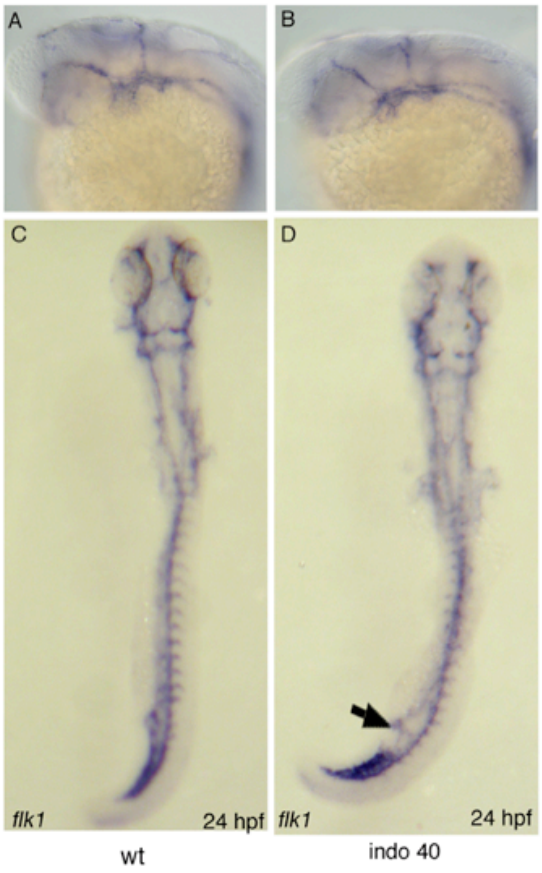


**Figure 20 Vessel defect is specific for posterior vasculature.** Anterior vasculature appears to progress normally since *flk1* staining in indomethacin treated embryos (B) does not differ from untreated embryos (A). However in the posterior vasculature, caudal vein is absent in the indomethacin treatment embryos (D arrow), while untreated embryos (C) show continuous *flk1* throughout posterior vasculature.

## Discussion

Limitations in murine model for elucidating COX biology prompted investigation in zebrafish model system. During embryogenesis COX-1 shows specific spatiotemporal expression in the posterior mesoderm, which localizes to posterior nephric duct, cloaca, hypochord, and blood vessel. In mammals, normal endothelial cells and vascular smooth muscle cells have also been shown to express COX-1 (Hla and Neilson, 1992a). Grosser *et al*. elegantly demonstrated that COX inhibitors, indomethacin, suppressed the formation of PGs in live zebrafish. Indomethacin was a more effective inhibitor of COX-1 derived PGs, while both indomethacin and NS-398 were effective inhibitors for COX-2. Similar $IC_{50}$ were obtained for both inhibitors (Grosser et al., 2002). Treatment of indomethacin in zebrafish resulted in dramatic defect in posterior vasculature, in which single, distended blood vessel remained after 2 days, instead of the blood vessel differentiating into artery and vein. Treatment with NS-398 has did not have any effect on normal vessel formation. We also observed similar defect in vessel formation with COX-1 morpholino treatment, but not to the similar degree of defect as indomethacin treatment. Since high-dose COX-1 morpholino treatment results in gastrulation arrest, we can only utilize lower dose of COX-1 to monitor vessel defect. Therefore, we can achiever greater inhibition of COX-1 activity by indomethacin to observe vasculature defect. Indomethacin treatment specifically inhibits COX activity, since reconstituting $PGE_2$ can suppress vessel defect.

COX-1 is expressed in the developing vasculature and regulates arterio-venous differentiation. It appears that COX-1 function is critical for vascular tube formation by

angioblast at around 25 somite stage. At 24 hpf, we observed the presence of arterial and veinous endothelial cells by both *flk1*, *flt4*, and *ephB2* staining, but the boundary between arterial and veinous structures was completely missing. We hypothesize that COX-1 regulates proper morphogenesis of the angioblast during tubulogenesis. However, it is not clear why there is a strong downregulation of veinous staining, compared to arterial staining. Perhaps there may be a defect in veinous differentiation as well as angioblast morphogenesis. It would be important to carefully analyze the cellular changes in the affected angioblasts to understand how COX-1 functions during tube formation.

In the zebrafish model, COX-1 does not appear to be expressed in the sprouting angiogenic vessels, rather, it is localized to the pre-existing vessel. Therefore, the reduction of angiogenic vessels in indomethacin-treated embryos indicate that disruption of the pre-existing vessel results in reduction of the derivative angiogenic vessel, presumably by disruption of vascular integrity, rendering it unable to support angiogenesis. In agreement with the zebrafish data, Tsujii *et al.* demonstrated that paracrine effects of COX derived prostaglandins is required for HUVEC tube formation induced by colon cancer cells, HCT-116, since aspirin or COX-1 antisense oligonucleotide can inhibit tube formation. Therefore, it is plausible that initial stages of angiogenesis require the function of COX-1 by the pre-existing vessel, where as pathogenic stages of angiogenesis may require higher level of PG produced by COX-2.

With respect to the role of $PGE_2$ during intersomitic vessel angiogenesis, mounting evidence from mammalian systems support the notion that $PGE_2$ is critical for both developmental as well as cancer angiogenesis. During implantation and decidualization, COX-derived prostaglandins (PGs) are important for uterine vascular

permeability and angiogenesis (Matsumoto et al., 2002). Various studies also indicate that NSAIDs inhibited angiogenesis *in vivo* (Majima et al., 1997), primarily by downregulating $PGE_2$ levels. Additionally, $PGE_2$ upregulated the production of angiogenic factors (VEGF, bFGF), which are important for the growth and survival of endothelial cells and stimulates vascular endothelial cell proliferation, migration, and tube formation (Iniguez et al., 2003). This is consistent with our findings in zebrafish. During intersomitic vessel angiogenesis, sprouting of the intersomitic vessels from the dorsal aorta occurs by angiogenesis, whereby angioblasts migrate along the dorsal aorta from the posterior lateral mesoderm and sprout at the intersomitic boundaries (Lawson and Weinstein, 2002b). In embryos in which COX-1 activity was inhibited by indomethacin, approximately 70% of the intersomitic vessels were either shortened or absent at 1 day post-fertilization but somite differentiation occurred normally (Cha et al., 2005), leading us to hypothesize that the lack or inhibition of COX-1 activity by NSAIDs cause a primary defect in the angioblast migration and proliferation either in the dorsal aorta or the posterior cardinal vein, and not by other secondary defects in development such as somite formation.

This study opens an exciting area of investigation focused on dissecting the prostanoid pathway in zebrafish model system. Prostaglandin biosynthetic pathway is conserved in zebrafish, therefore the next step would be to perform functional analysis on downstream receptor pathways. Since COX enzymes are conserved, it is reasonable to hypothesize that prostanoid receptor pathways are also conserved. True to this hypothesis, our analysis through zebrafish genome database revealed presence of numerous prostaglandin receptors, including IP and FP receptors (YC, LSK, RND,

unpublished observations). Therefore, it would be important to understand what downstream receptor mediates prostaglandin signaling in formation of vascular tubes. In addition, the increased risk of cardiovascular defects in patients who ingest high doses of COX-2 inhibitors illustrates the importance of understanding prostaglandin pathway in regulating cardiovascular homeostasis.

# CHAPTER IV

## CONSERVED FUNCTION OF CYCLOOXYGENASES AS REPRESSOR OF HEMATOPOIESIS


### Abstract

Microarray gene profiling analyses in zebrafish embryos treated with non-selective COX inhibitor showed upregulated/increased expression of genes specific for blood cells. We confirmed through whole mount *in situ* hybridization experiments that *mpo,* a marker for granulocytic lineage of blood cells, and *e1b*, a marker for red blood cells, are indeed upregulated in embryos treated with indomethacin. Further we showed that the COX-1 activity is required for regulating blood cell development after 22 somite stage, similar to what we observed for its function in vascular tube formation, leading to the hypothesis that COX-1 is required in the intermediate cell mass after 22 somite stage for proper formation of blood vessel and blood cells. We further hypothesize that COX derived prostaglandins may also be important repressor of hematopoiesis in mammals since COX-2 null mice develop extremedullary hematopoiesis in spleen.

## Introduction

Many of the genes involved in blood-vessel formation and blood cell development in mammals also have been shown to function in similar ways in zebrafish. In mammalian cell culture models, human umbilical vein endothelial cells (HUVECs), treated with COX-1 antisense oligonucleotides exhibited defects in endothelial tube formation, while those treated with COX-2 antisense oligonucleotides did not (Tsujii et al., 1998). Consistently previous reports demonstrated the NSAIDs inhibit angiogenesis *in vivo*, primarily by downregulating $PGE_2$ levels. These findings are consistent with our results in zebrafish in which both NSAIDs and *cox1-MO* cause defects in vascular tube formation and inhibit angiogenesis of intersomitic vessels. In addition *cox1*, not *cox2*, expression is observed in endothelial tissue during vertebrate development.

During the early segmentation stage, *cox1* transcripts are distributed broadly along the lateral mesodermal cells and co-localize with *fli1*, an early marker for hematopoietic stem cells (HSC) and endothelial cells (EC), as well as *scl*, another marker for HSC and EC. Despite the well-known function of COX derived prostaglandins in endothelial cell development and maintenance, their roles in hematopoiesis remain poorly understood. Based on these findings, we explored the possibility that the mechanism by which NSAIDs cause defects in blood vessel formation may be due to their essential role in regulating proper endothelial and hematopoietic stem cell interaction during early development.

## Material and Methods

**Microarray analysis** Embryos were treated with NSAIDs starting at tailbud stage to 24 hpf. As a control, another group of embryos were treated with DSMO. As a quality control, a subset of the segregated embryos was kept until later stages, when the morphologic phenotype was apparent. Total RNA from batches of approximately 50 embryos was purified using the RNeasy kit (Qiagen, Woodland Hills, CA). Hybridization and data acquisition were performed at the Microarray Core Facility at Vanderbilt University, Nashville, TN. For each hybridization, amplified sample RNA was labeled with Cy5 and labeled amplified control RNA was labeled with Cy3 in a reverse transcriptase reaction employing 8 g of random hexamer primer, Cy5- or Cy3-labeled dCTP, and Superscript II reverse transcriptase (Invitrogen, Carlsbad, CA). Reaction products were purified using a Qiagen PCR purification kit, desiccated in the speedvac concentrator, and resuspended in 5X SSC hybridization buffer (sodium chloride, sodium citrate buffer, 25% formamide, and 0.1% SDS), and 10 g of carrier polyA RNA. After heating the mixture to 100°C for 2 min, hybridization was performed at 42°C on pretreated Affymetrix zebrafish microarray slides for 14 h in a water bath. After washing and air-drying, slides were scanned with the GenePix scanner 4000B and its software (Axon Instruments, Union City, CA). Scanned intensity data were further analyzed with GeneSpring software (Silicone Genetics, Redwood City, CA).

**Microarray Data Analysis** All datasets were normalized by Lowess intensity-dependent normalization (Yang *et al*., 2002) as implemented in GeneSpring 6.1. We then deleted unreliable features that included apparent artifacts, low signals (sum of medians,

subtracted background, below 500), and nonreproducible features in 2 independent hybridizations for DMSO vs NSAID treated embryos at 24 hpf. We selected the ~30 top-ranked differentially regulated genes as those that exhibited 2-fold or greater differences in expression.

**EST Clones and In situ hybridization** *Mpo* EST clone (GenBank sequence ID AF349034.1), and beta globin e1 EST clone (GenBank sequence ID NM_131759) were obtained from Openbiosystems. *pu.1* plasmid for *in situ* were kindly provided by Dr. Weinstein (NIH). Antisense probes for *in situ* hybridization was synthesized using RNA polymerase kit (Ambion). Whole mount *in situ* hybridization was performed according to Marlow *et al.* (Marlow et al., 2002). Embryos were analyzed on Zeiss Axioplot and images were captured with Nikon Coolpix 4500. Each *in situ* experiment was done at least twice, using approximately 20 embryos.

## Results

### Microarray Analysis Reveal Upregulation of Blood Cell Markers

Based on the broad expression of *cox1* in the ventro-posterior mesoderm that gives rise to both blood vessel and blood cells, we hypothesized that COX-1 function may also be required for hematopoiesis. In order to test this hypothesis, we performed comparative gene expression profiling using Affymetrix microarray on 1-day old zebrafish embryos after treatment with indomethacin and compared the data set from this group with those of untreated control group. We normalized our dataset based on two independent hybridizations and selected the ~30 top-ranked differentially regulated genes as those that exhibited 2-fold or greater differences in expression. We found that only 14 genes were upregulated and 7 genes were downregulated in the indomethacin-treated group compared to untreated control group (Fig. 21). Another surprising result from the microarry data was that out of 14 genes that were upregulated, 5 of the genes were previously shown to be expressed in the blood cells. Myeloperoxidase (mpo), a well known marker for granulocytic lineage of blood cells, and embryonic beta globin e1 (e1b), a marker for red blood cells, were upregulated 9.2 fold and 4.6 fold, respectively.

In order to validate the microarray results, we performed whole mount *in situ* hybridization experiments with antisense probes for these genes in 1-day old zebrafish embryos that were treated with indomethacin and used DMSO treated embryos as control. We found that consistent with the microarray results, *mpo* and *e1b* markers were significantly increased in the zebrafish embryos (Fig. 22A-D). As a control we performed *in situ* for *pu.1*, a marker for myeloid lineage, whose expression level was not

significantly altered in indomethacin treated embryos based on the microarray results. As expected, *pu.1* expression level was unaltered compared to untreated control group (Fig. 22E-F). There are few reasons for the relatively unchanged expression level of *pu.1*: one may be that *pu.1* is not expressed at high enough levels to detect differences since cells that express *pu.1* are less abundant than those of *mpo* and *e1b*; another explanation may be that the indomethacin treatment affects blood stem cells that give rise to granulocytic and RBC lineages, while the myeloid lineage is unaffected.

COX-1 Function is required after 22-somite stage for Proper Blood Cell Development

To understand the timing requirement for COX-1 function in hematopoiesis, we varied the indomethacin treatment period in zebrafish at different time points during embryogenesis. We found that the timing of COX-1 function as a hematopoietic repressor is similar to what we observed for its role during vascular tube formation. We initiated the treatment starting at tailbud and washed out the indomethacin solution at various stages during segmentation. We observed that if the treatment period was terminated before 22 somite stage, the embryos still maintained normal blood cell numbers compared to the control group (Fig. 23). However, if the treatment period persisted in the embryos past 22 somites, the embryos had defects in both in vascular tube formation as well as hematopoiesis. Since the intermediate cell mass (ICM), which give rise to both blood cells and endothelial cell, begin to migrate in the midline at ~20 somite stage, our evidence suggest that COX-1 function is required in the ICM to regulate proper differentiation of endothelial and blood cell precursors (Fig. 24).

COX Function is required to Repress Hematopoiesis in Mammals

To understand whether COX function is also required for proper hematopoiesis in mammals, we analyzed various hematopoietic organs in COX-1 and COX-2 null mice. We observed that COX-2 null mice consistently developed enlarged spleen by the adult stage (Fig. 24). Analyses of Hematoxylin and Eosin Stain (H&E) sections of the spleens showed that COX-2 null mice developed full tri-lineage hematopoiesis with increased germinal centers as well as blood cell counts (Fig. 24B), while the wild-type spleen was clearly dormant shown by a well-demarcated white and red pulp. Although spleen is an organ that primarily functions to filter old red blood cells and foreign organisms, it also has an additional role as an auxiliary hematopoietic site during strenuous conditions. We observed that COX-2 null mice, compared to wild-type littermates, show increased splenic mass (290g vs 145g), increased peripheral granulocytes, and upregulated peripheral G-CSF (Fig. 25A-D). In addition, when we divided the serum G-CSF level by the splenic mass in both COX-2 and wild-type animals, we observed that the ratio of G-CSF to splenic mass was similar in both COX-2 null and wild-type mice, demonstrating that increased G-CSF level in COX-2 null mice may be directly proportional to the increased splenic mass. Taken together, we hypothesize that the COX derived prostaglandins have an important role in regulating hematopoiesis and that this function may be evolutionarily conserved among vertebrates.

| Accession ID | Affymetrix ID | Fold (up) | Up-Regulated Gene Description |
|---|---|---|---|
| AB078927.1 | Dr.9478.1.S1_at | 11.3 | CYP1A mRNA for cytochrome P450 1A* |
| BM082478 | Dr.14719.1.A1_at | 10.5 | Human Adp-Ribosylation Factor 1 Complexed With Gdp |
| AF349034.1 | Dr.9478.2.S1_at | 9.2 | myeloperoxidase mRNA* |
| NM_131759 | DrAffx.1.85.A1_at | 4.6 | Embryonic beta globin e1* |
| AY180110.1 | Dr.3583.1.S1_at | 3.7 | sulfotransferase mRNA* |
| AW019592 | Dr.3029.1.A1_at | 3.7 | S17512 glucuronosyltransferase - human |
| AW595366 | Dr.10183.1.S2_at | 3.5 | early growth response 1 |
| BM957279 | Dr.12986.3.S1_a_at | 2.5 | c-fos (Cellular oncogene fos)* |
| NM_131695.1 | Dr.23469.1.S1_x_at | 2 | MHC class II alpha chain 3 (a3), mRNA* |

| Accession ID | Affymetrix ID | Fold (down) | Down-Regulated Gene Description |
|---|---|---|---|
| BE605271 | Dr.10379.1.A1_at | -3 | prostaglandin-I synthase (EC 5.3.99.4) - human |
| AI396977 | Dr.8153.1.S5_a_at | -2.5 | claudin g |
| AL904095 | DrAffx.3.1.A1_at | -2.5 | pseudouridylate synthase 1 |
| BI984759 | Dr.13557.2.S1_at | -2.5 | polyadenylate binding protein-interacting protein (PBPP-2) |
| NM_130960.1 | Dr.8208.1.S1_at | -2.4 | lefty1 (lft1) |
| U89709.1 | Dr.263.2.S1_at | -2.1 | plasticin |
| BQ419577 | Dr.18531.1.S1_at | -2 | G protein-coupled receptor kinase 5 - human (fragment) |
| CA471246 | Dr.15153.2.S1_at | -2 | S44346 RAD23 protein homolog - human |

*Described in the literature to be expressed in blood cells

**Figure 21 Microarry analyses reveal that blood cell markers are upregulated in embryos treated with NSAIDs**

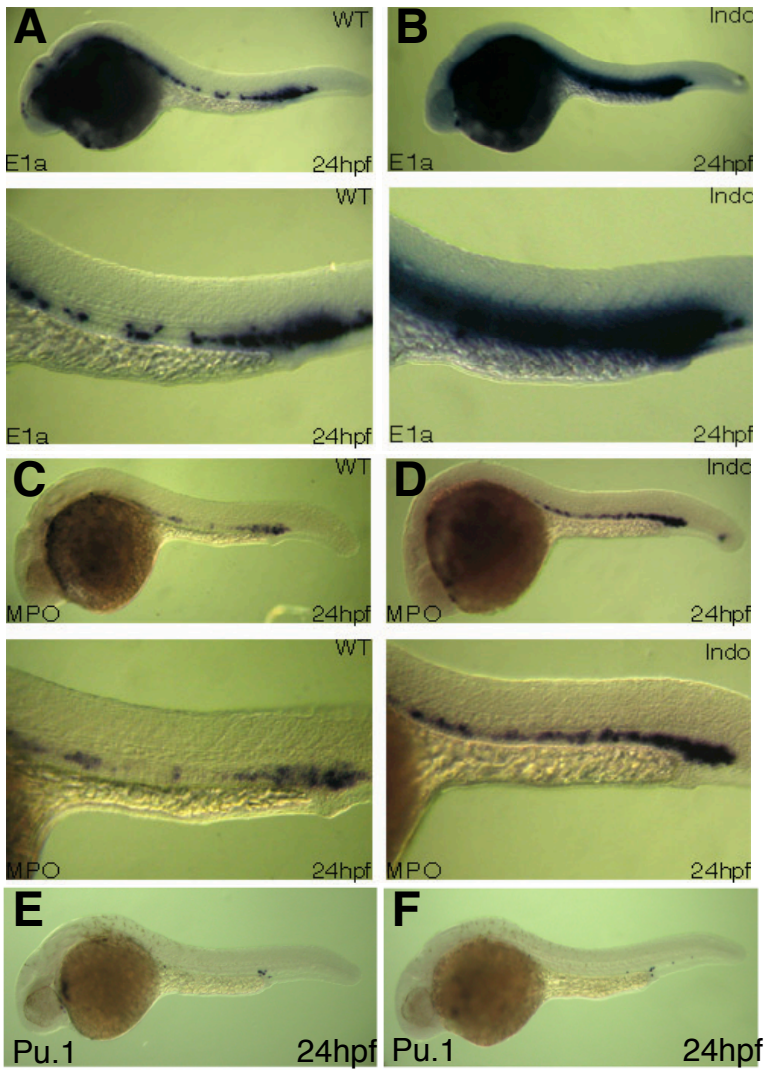


**Figure 22 NSAIDs cause upregulated of blood cell markers, *e1a* and *mpo*.** Lateral view of embryos at 24 hpf. Embryos were either untreated (A,C,E) or treated with indomethacin starting at tailbud stage continuously to 24 hpf (B, D, F). After fixation, embryos were stained with *e1a* (A, B), *mpo* (C, D), or *Pu.1* (E,F)

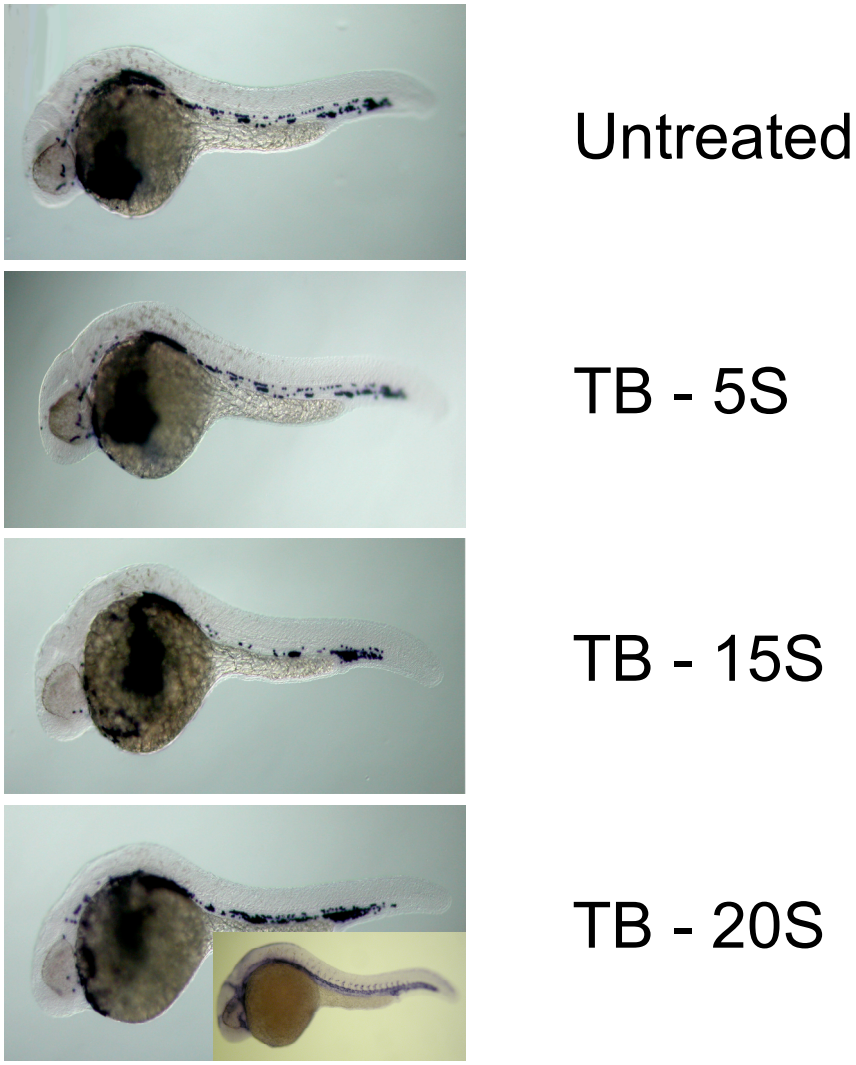


**Figure 23. COX-1 function is required after 20 somite stage to regulate hematopoiesis.** Lateral view of embryos at 24 hpf. Embryos were either untreated or treated with indomethacin starting at tailbud stage continuously to 24 hpf. After fixation, embryos were stained with *e1a*, a marker for red blood cells.

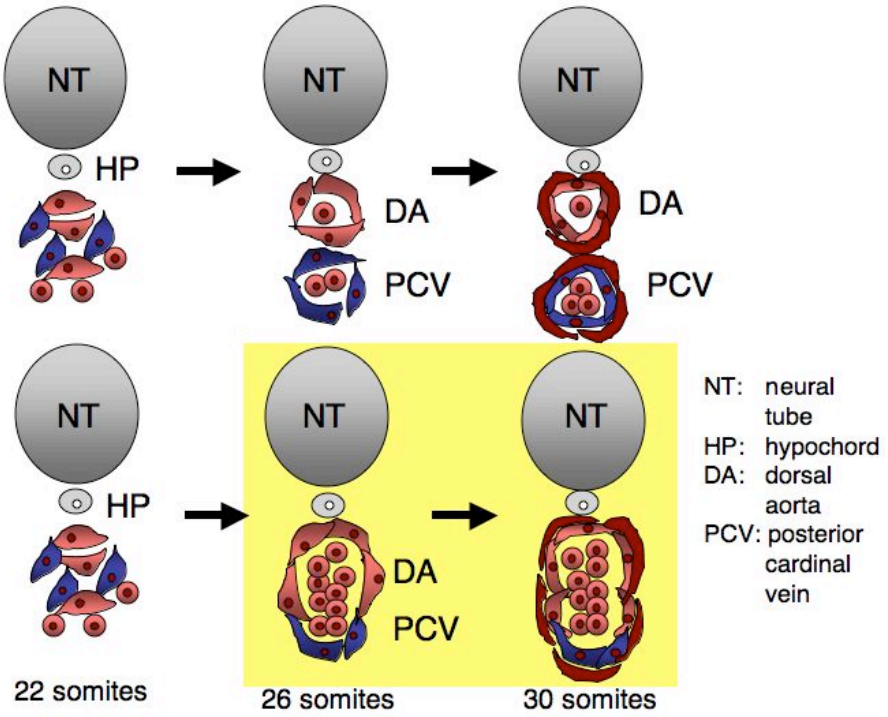


**Figure 24. Model for Vascular Tube Defect.** Intermediate cell mass (ICM) that give rise to both endothelial and hematopoietic stem cells congregate at the midline at 22 somite stage. NSAIDs function at 22 somite stage to allow proper differentiation of intermediate cell mass.

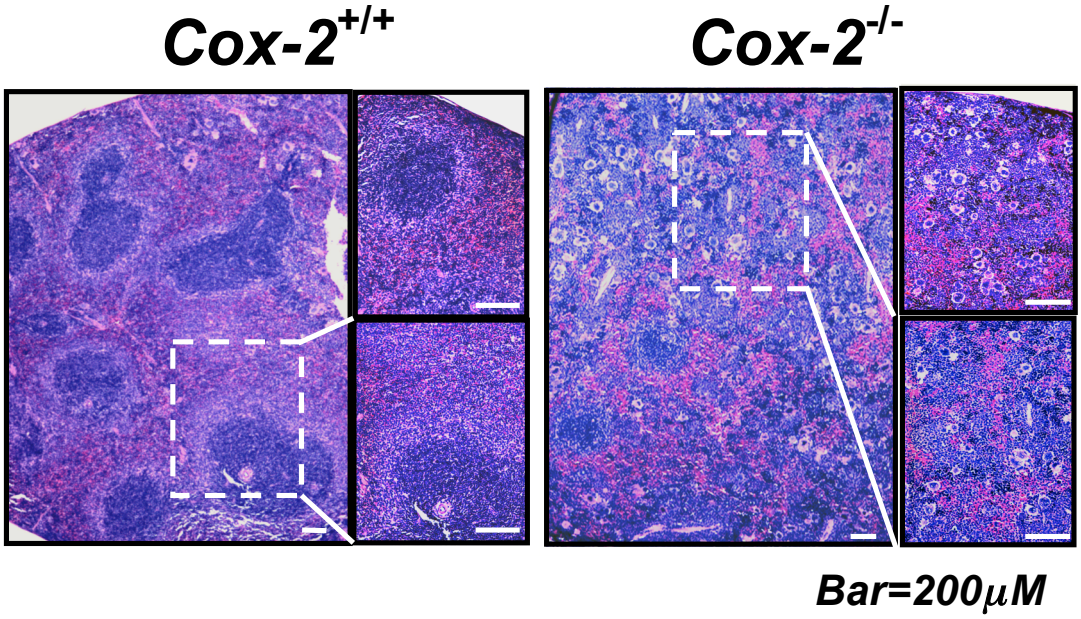


**Figure 25. COX-2 null mice develop extra-medullary hematopoiesis in the spleen.** H&E staining of sections of the spleen from COX-$2^{-/-}$ mice and wiltype littermates. A representative section is shown. Dotted line refers to the magnified area.

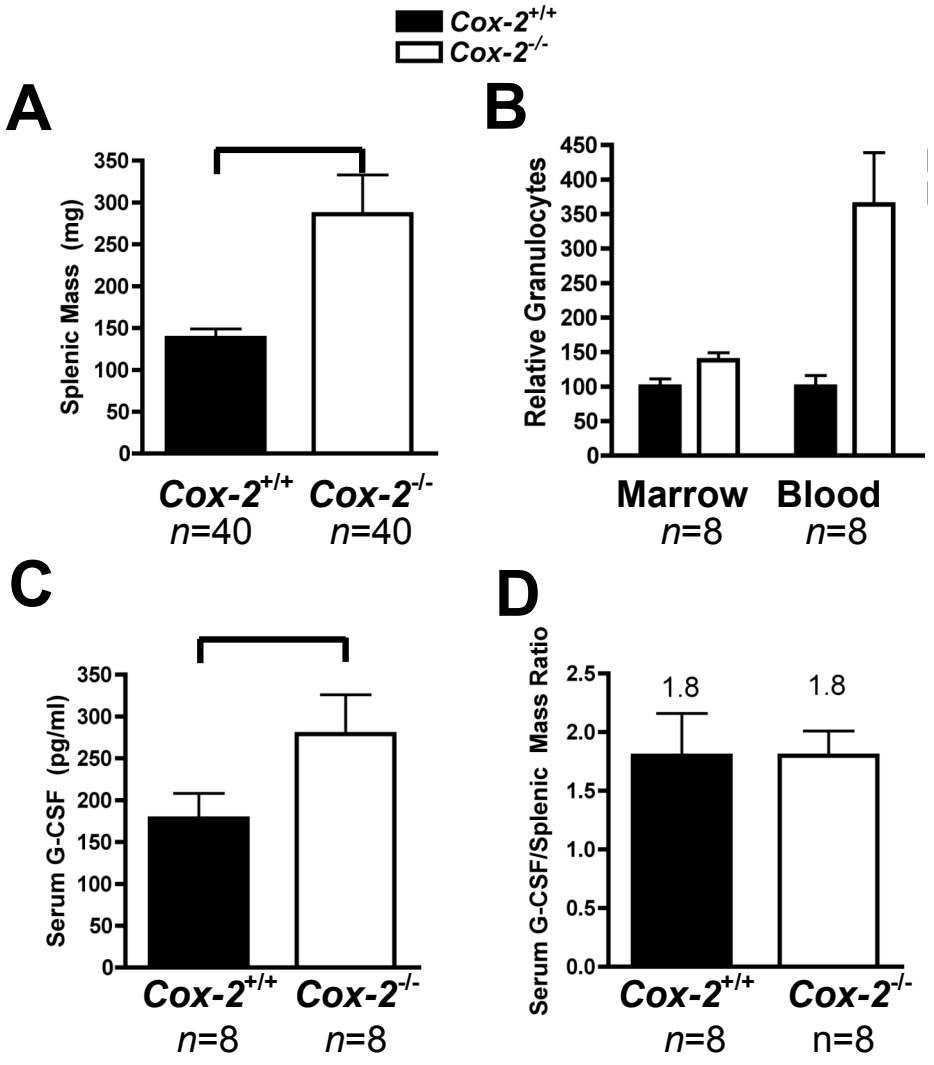


**Figure 26 Increased peripheral granulocytes is due to splenomegaly in COX-2$^{-/-}$mice.** Whole spleen weight was measured from 3-5 months old mice. Relative peripheral granulocytes were measured using FACS analyses. Serum G-CSF was measured from ELISA kit as described in the manufacturer's protocol. Difference in splenic weight between WT and COX-2$^{-/-}$ mice (A)Relative granulocytes number was measured (B). Level of serum G-CSF was measured (C) Ratio between serum G-CSF and spleen mass (D).

## Discussion

In this study, we explored the possibility that COX-1 function is important for proper regulation of hematopoietic stem cells and endothelial precursors. During embryogenesis, expression of *cox1* is observed in the posterior lateral mesodermal cells during early segmentation stage. In zebrafish, the presumptive mesodermal tissues that give rise to hematopoietic stem cells, blood vessel, and pronephric duct arise from posterior lateral mesoderm during segmentation. Co-localization experiments of using *cox1* and *fli1,* endothelial and hematopoietic precursor marker, revealed that *cox1* is expressed broadly in the posterior lateral mesoderm and encompass the expression of *fli1*. After segmentation, expression of *cox1* is observed in the developing blood vessel and posterior nephric duct at 24 hpf. In addition, we have previously demonstrated that inhibition of COX-1 function with NSAIDs leads to defects in proper differentiation of artery and vein that ultimately leads to a failure in vascular tube formation. These studies led to our hypothesis that COX-1 function is necessary for proper development for blood vessel and blood cells.

We observed that NSAID treatment of zebrafish embryos results not only in defective vascular tube formation, but also in augmentation of granulocytic and erythrocytic lineage of blood cells, as revealed by *mpo* and *e1a* expression analysis. Previous studies in murine models revealed that treatment of mice with indomethacin led to increased cellularity both in the spleen and bone marrow (Razin et al., 1981) with concomitant increase in Colony-Stimulating Factor (CSF). This initial observation was subsequently re-affirmed by several other groups. In one study, treatment with low dose

of indomethacin resulted in minor increase in bone marrow cellularity and pluripotent hemopoietic stem cells (CFU-S) and dramatic increase in granulocyte-macrophage progenitors (CFU-GM) and splenic erythropoiesis. Indomethacin exposure caused increased macrophage function as evidenced by enhanced phagocytosis. Lymphoproliferative response of splenocytes to T-cell mitogens was depressed while B-cell mitogenic response was not affected. These results indicated that indomethacin affects selective components of the immune and hemopoietic cells at dose levels that cause no other significant pathological alterations (Boorman et al., 1982). In addition, NSAIDs have also been shown to facilitate hematopoietic recovery following cytotoxic 5-Flurouracil (5-FU) treatment, indicating that inhibition of prostaglandin synthesis may have a role in accelerating hematopoietic recovery mainly by increasing CFU-GM (O'Reilly and Gamelli, 1990). Therefore we hypothesize that prostaglandins' function is essential for proper hematopoieis events and that this function may be evolutionarily conserved.

So how do NSAIDs affect hematopoiesis in zebrafish? In zebrafish hematopoietic stem cells and endothelial cells both derived from similar population of stem cells, termed ICM. During late segmentation, the intermediate mesodermal cells initially reside lateral to the somites. However these cells start to migrate medially to form group of cell ventral to the neural tube and somites to form ICM. These cell types then undergo rapid morphogenesis, proliferation, and differentiation to give rise to various vascular cell types. Treatment of NSAIDs in zebrafish revealed that COX function is required after 22 somite stage, after the ICM is formed. Terminating the treatment of NSAIDs at any stage before 22-somite fail to give rise to either vascular or

hematopoietic defects, indicating that NSAIDs do not affect the initial formation of intermediate mesodermal cells nor do they affect the migration of lateral mesodermal cell. Only when NSAIDs are treated past 22-somite stage do we begin to observe defects in both endothelial and hematopoietic cells, demonstrating that NSAIDs specifically affect ICM. Another evidence that NSAIDs specifically function on ICM comes from our analysis using endothelial specific markers. Previous studies have demonstrated that the initial derivation of red blood cells mainly occurs in the venous endothelial cells (Amatruda and Zon, 1999). In our analyses of blood vessel markers, we observed a strong downregulation of venous endothelial cells, while the arterial markers were relatively unaffected (Cha et al., 2005). Since veinous cells are formed only after the ICM undergoes differentiation, we provide second line of evidence that NSAIDs effect on hematopoiesis occurs by inhibiting proper differentiation of ICM into veinous cells.

Taken together we provide evidence that COX-derived prostaglandins may have an evolutionarily conserved function as an inhibitor of hematopoiesis. Therefore it would be important to address the cellular and molecular mechanisms behind this role of prostaglandin by using zebrafish as a model. Next question is to understand what specific prostaglandin receptor pathway is required for proper hematopoiesis and define the specific cellular mechanism this pathway affects. Based on our data, COX signaling may be required for both morphogenesis as well as differentiation of blood cell and vessel. Therefore it would be important to analyze these defects at a cellular and molecular level to reveal insight into the hematopoietic role for prostaglandins in zebrafish.

# CHAPTER VI

# CONCLUSION

## Zebrafish as a Model for Dissecting Prostaglandin Pathway

Recently, FitzGerald and colleagues identified the zebrafish orthologues of genes encoding COX-1 and COX-2 (Grosser et al., 2002). Zebrafish cyclooxygenases have also been shown to be both genetically and functionally equivalent to their mammalian counterparts. These initial studies extended by our work have shown that antisense morpholino oligonucleotides (MOs) translation interference or pharmacologic inhibitors of COX-1 result in gastrulation arrest, while knockdown of COX-2 fails to produce any discernable phenotype (Cha et al., 2005; Grosser et al., 2002). This is consistent with our finding that *cox1* transcripts were expressed ubiquitously during gastrulation, while *cox2* expression was not detected until the end of the gastrula period (Cha et al., 2005). This finding also supports the role of COX-1 as a "housekeeping" enzyme in which a basal level of prostaglandin is required for normal homeostasis.

Zebrafish are an attractive model system in which to address the roles of prostaglandins in embryogenesis for many reasons (Amatruda et al., 2002). Each pair-wise mating gives rise to hundreds of embryos for study on a weekly basis. Most importantly for prostaglandin biology, embryogenesis occurs outside of mother's body, thus not influenced by maternal prostaglandins. Moreover embryogenesis can be visualized at tissue and cellular levels due to the transparency of the developing embryos; molecular markers are available for virtually all the tissues. Zebrafish embryos and their

chorions are highly permeable to many drugs, facilitating a temporal control of inhibition of different enzymes involved in prostaglandin signaling. In addition, forward genetic screens have identified mutations in many developmental pathways, allowing one to study functional interactions between prostaglandin signaling and other developmental pathways.

## Blood Vessel Development

Taking advantage of the extra-embryonic development of zebrafish embryos, we recently further characterized the functional requirements for prostaglandins in early development. After the period of ubiquitous expression during blastula and early gastrula stages, *cox1* transcripts became restricted to the posterior intermediate mesoderm during somitogenesis and to posterior mesodermal organs at 24 hours post fertilization (hpf), including blood vessel and nephric duct. Partial knockdown of COX-1 expression using moderate doses of MOs or pharmacologic inhibition of its activity after gastrulation both resulted in defective vascular tube formation and shortened intersomitic vessels in the posterior body (Cha et al., 2005). Using pharmacologic inhibition to identify the critical time window of COX-1 function in blood vessel development revealed that COX-1 is required after the 20 somite stage, when the intermediate cell mass (ICM) undergoes morphogenesis to form the dorsal artery and posterior cardinal vein (Cha et al., 2005) (Fig. 2). These findings in zebrafish are consistent with our data in the human cell culture model in which human umbilical vein endothelial cells (HUVECs) treated with COX-1 antisense oligonucleotides exhibited defects in endothelial tube formation (Tsujii et al., 1998), while those treated with COX-2 antisense oligonucleotides did not.

In mammalian systems, mounting evidence supports a critical requirement for cyclooxygenase and its prostaglandin products in angiogenesis. During implantation and decidualization, COX-derived prostaglandins (PGs) are important for uterine vascular permeability and angiogenesis (Matsumoto et al., 2002). Various studies also indicate that NSAIDs inhibited angiogenesis *in vivo* (Majima et al., 1997), primarily by downregulating $PGE_2$ levels. Additionally, $PGE_2$ upregulated the production of angiogenic factors (VEGF, bFGF), which are important for the growth and survival of endothelial cells and stimulates vascular endothelial cell proliferation, migration, and tube formation (Iniguez et al., 2003). In zebrafish, sprouting of the intersomitic vessels from the dorsal aorta occurs by angiogenesis, whereby angioblasts migrate along the dorsal aorta from the posterior lateral mesoderm and sprout at the intersomitic boundaries (Lawson and Weinstein, 2002b). In embryos in which COX-1 activity was inhibited by indomethacin, approximately 70% of the intersomitic vessels were either shortened or absent at 1 day post-fertilization but somite differentiation occurred normally (Cha et al., 2005), leading us to hypothesize that lack or inhibition of COX-1 activity was responsible for shortened intersomitic vessel formation in zebrafish, and not by other secondary defects in development such as somite formation. Zebrafish are an established model for studying angiogenesis (Lawson and Weinstein, 2002a), the role of COX-1 signaling in regulating angiogenesis needs to be further explored in this model system to understand the precise cellular and molecular mechanisms involved.

## Cell Motility in Gastrulation and Cancer Metastasis

Over the past decade, COX-2 derived prostaglandins have been implicated in the development and progression of many types of cancer (Gupta and Dubois, 2001). One aspect of this research involves elucidating the mechanisms by which $PGE_2$ signaling promotes cancer cell invasion and movement during metastasis (Fig. 3). Upon stimulation with $PGE_2$, cancer cells demonstrated increased proliferation and invasion. Furthermore, the stimulatory effects of $PGE_2$ were dependent upon the activation of the phosphatidylinositol 3-kinase/Akt pathway (Sheng et al., 2001), which ultimately induce actin polymerization and, possibly, epithelial-mesenchymal transition, to cause invasion and migration of cancer cells during metastatic spread (Fig. 3). However these studies were primarily from tumor cell lines *in vitro* and the cellular behavior and molecular pathways regulated by $PGE_2$ *in vivo* are not known. $PGE_2$-induced migration and invasion of cancer cells can also occur via rapid transactivation and phosphorylation of the epidermal growth factor receptor (EGFR) both intracellularly (Buchanan et al., 2003b) and extracellularly (Pai et al., 2002), These results supported the notion that the early effects of COX-2-derived $PGE_2$ are mediated, in part, by activation of the EGFR, and this transactivation is responsible for subsequent downstream effects, including the stimulation of cell migration and invasion.

Assaying the prostaglandin levels in zebrafish embryos (Cha et al., 2005) and adults (Grosser et al., 2002) revealed the presence of $PGE_2$, $PGI_2$, $PGF_{2\alpha}$ and low levels of $TxA_2$, suggesting the existence of prostaglandin synthases and receptors. In support of this hypothesis, we and others have recently identified the zebrafish orthologues of microsomal prostaglandin E2 synthase (mPGES) (Pini et al., 2005) and EP receptors in zebrafish (YC, LSK, RND, unpublished data). Moreover using antisense morpholino

oligonucleotides to inhibit the translation and/or splicing of the $PGE_2$ signaling cascade components we demonstrated that $PGE_2$ regulates cell movement during gastrulation via the EP4 receptor in zebrafish (YC, LSK, RND, unpublished data). The similarity between the cancer cell and zebrafish embryo data is quite striking: both models imply that $PGE_2$ stimulation of EP4 is the key step in regulating cell movement, both systems suggest PI3K/Akt as the key mediator downstream of EP4 signaling, and finally, both models do not implicate other EP receptors in this process, demonstrating that $PGE_2$-mediated stimulation of cell movement is restricted to the EP4 receptor. Additionally in zebrafish, EP4 signaling primarily promotes cell motility, without influencing cell shape or directional movement during gastrulation (YC, LSK and RND, unpublished data). Therefore, understanding the mechanism by which EP4 activates PI3K/Akt and, furthermore, elucidating how Akt ultimately allows cell movement to occur in zebrafish may provide insight into how $PGE_2$ regulates cell movement during metastasis.

## Outstanding Questions and Future Directions

Zebrafish is an intriguing and promising model, which may allow for a clearer understanding of the precise role of prostaglandins in regulating embryonic development. One pressing question to address is the unexpected pathology concerning the cardiovascular side effects found in humans after prolonged treatment with COX-2 selective inhibitors. In human studies, COX-2 inhibitors appear to affect the formation of prostacyclin (McAdam et al., 1999), shifting the balance toward a prothrombotic state (Cheng et al., 2002). Hence, it would be important to address whether this also occurs in adult fish by treating them with a wide variety of COX selective inhibitors. Many of the

same genes involved in blood-vessel formation and blood cell development in mammals also have been shown to function in similar ways in zebrafish (Nasevicius et al., 2000). Therefore, we would expect similar cardiovascular side effects in zebrafish as a result of COX inhibition. In this case, it would become critical to carefully quantitate and analyze these cardiovascular defects which may give insight into the pathophysiology of COX-2 inhibition in humans.

Another intriguing question to address is the function of other types of prostaglandins that exist in zebrafish. Our analysis revealed the presence of EP, IP, and FP receptors in the zebrafish EST database. Genetic knockdown experiments and screenings for mutations in genes encoding each prostaglandin synthase and receptor will undoubtedly provide more insight into how prostaglandins function in early development. Furthermore, the detailed mechanism by which prostaglandins signal to downstream effectors warrant further investigation. We have only begun to uncover some of the downstream effectors of prostaglandin receptors in mammalian systems. Any downstream targets identified in zebrafish can be studied in other model systems as well (mouse or cultured cells) to understand the conserved nature of prostaglandin signaling. Studies in developmental model systems have advanced our understanding of major signaling pathways and, if history provides any indication, studies in zebrafish will undoubtedly give much insight into the detailed mechanism for prostaglandin signaling and function. Based on our findings in zebrafish, it is now important to revisit the mammalian models to analyze embryonic defects associated with inhibition of both maternal and fetal production of prostaglandins.

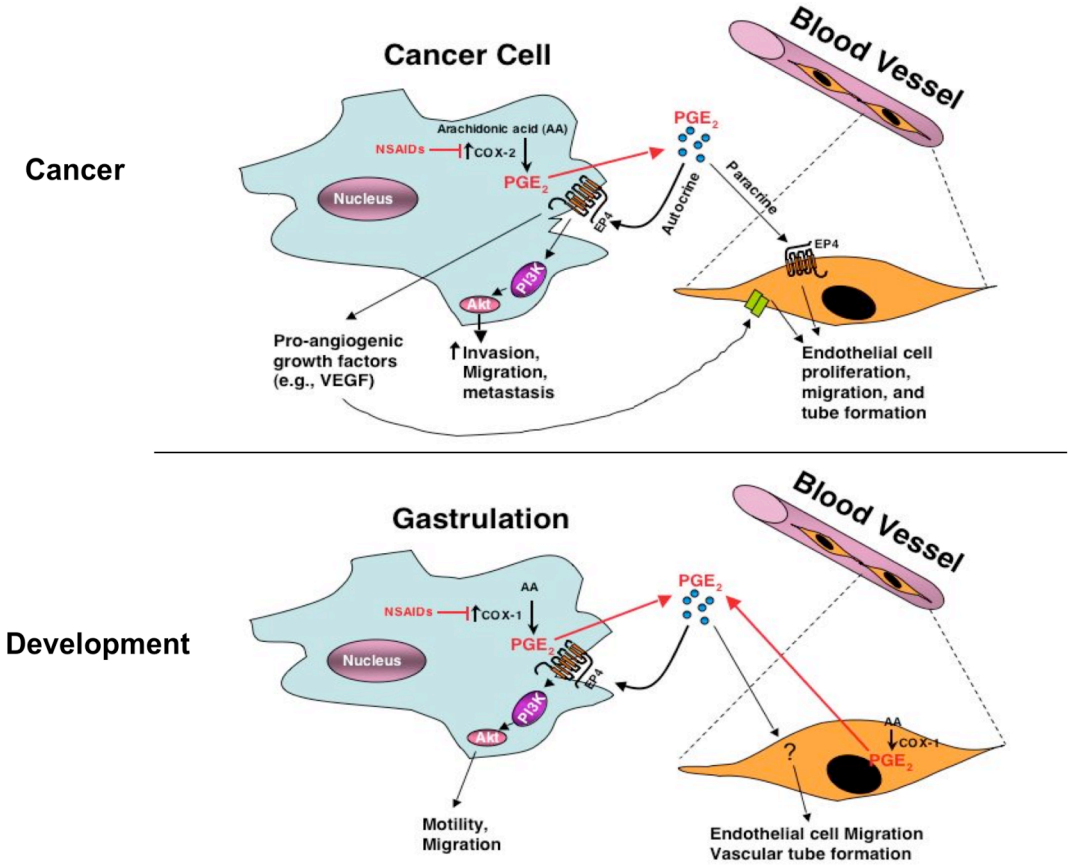


**Figure 27. Conserved Mechanisms of COX-derived $PGE_2$ in Development and Carcinogenesis.** $PGE_2$ stimulation of EP4 receptor results in invasion and migration of cancer cells. In addition, $PGE_2$ can also stimulate production of proangiogenic factors, such as vascular endothelial growth factor, to promote migration and tube formation of endothelial cells. Solid malignancies are made up of multiple types of cells that produce signals that work in both a paracrine and autocrine manner as depicted.

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