Prostaglandin E2 Promotes Embryonic Vascular Development and Maturation in Zebrafish

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Summary Statement: Overproduction of PGE2 causes inflammation and cancer and its blockage results in serious physiological consequences. This study investigated the novel roles of PGE2 in early vascular development of zebrafish.
Abstract

Prostaglandin (PG)-E2 is essential for growth and development of vertebrates. PGE2 binds to G-coupled receptors to regulate embryonic stem cells differentiation and maintains tissue homeostasis. Overproduction of PGE2 by breast tumor cells promotes aggressive breast cancer phenotypes and tumor-associated lymphangiogenesis. In this study, we investigated novel roles of PGE2 in early embryonic vascular development and maturation with microinjection of PGE2 in fertilized zebrafish (Danio rerio) eggs. We injected Texas red-dextran to trace vascular development. Embryos injected with the solvent of PGE2 served as vehicle. Distinct developmental changes were noted from 28-96 hours post fertilization (hpf), showing an increase in embryonic tail-flicks, pigmentation, growth, hatching, and larval movement post-hatching in the PGE2-injected group compared to the vehicle. We recorded a significant increase in trunk vascular fluorescence and maturation of vascular anatomy; embryo heartbeat and blood vessel formation in the PGE2 injected group. At 96hpf, all larvae were sacrificed to measure vascular marker mRNA expression. We observed a significant increase in the expression of stem cell markers efnb2a, ephb4a; angiogenesis markers vegfa, kdrl, etv2 and lymphangiogenesis marker prox1 in the PGE2-group compared to the vehicle. This study shows the novel roles of PGE2 in promoting embryonic vascular maturation and angiogenesis in zebrafish.
Introduction

Prostaglandin (PG) E-2 is a prostanoid, which is endogenously synthesized from the arachidonic acid of vertebrate cell membranes by an enzyme, cyclooxygenase (COX)-2. PGE2 binds to four different G-coupled receptors (EPIs), EP 1-4, which has various cellular functions (Sugimoto Y & Narumiya, 2007). EP2 and EP4 receptors share the cAMP/PKA pathway, however, EP4 additionally signals through the phosphatidylinositol 3-kinase (PI3K)/AKT pathway (Fujino et al, 2003). In vertebrates, PGE2 plays major physiological roles in embryonic development by regulating the homeostatic balance of hematopoietic stem cells during early embryonic growth. The role of PGE synthase (Ptges) in embryonic differentiation and growth in zebrafish was well investigated by Cha et al, 2006. They have displayed that knock down of Ptges in the zebrafish embryo completely abrogated early cell differentiation and cell polarization, which was retrieved with PGE2 addition, as embryos could recover all phenotypes. They have also shown that PGE2 regulates zebrafish growth via EP4/PI3K/Akt pathways (Cha et al, 2006). Supporting this, North et al, 2007 showed that PGE2 regulates the differentiation of embryonic stem cells. This study showed that treatment with chemicals that enhance PGE2 synthesis induced hematopoietic stem cells and in contrast, chemicals, which block prostaglandin synthesis, decreased stem cell numbers (North et al, 2007).

PGE2 induces breast cancer stem like cells (SLCs) via the upregulation of stem cells marker (NANOG and SOX2) in tumors and by stimulation of NOTCH and Wnt genes expression (Majumder et al, 2016). With a COX-2 inhibitor (COX2-I) and specific EP4 antagonist (EP4A) treatment, we could abrogate COX-2/PGE2 induced SLCs in breast cancer. In addition, PI3K/Akt inhibitors also abrogated PGE2 induced NOTCH and WNT genes expression in human breast cancer. Therefore, we have established that COX-2 and PGE2 induce human breast SLCs were
regulated by EP4/PI3K/Akt/NOTCH/WNT pathways (Majumder et al, 2016). However, we never tested the roles of PGE2 in vertebrate vascular development.

Zebrafish are widely used as vertebrate models for pathophysiological studies (North et al, 2007; Zon and Peterson, 2005). The transparency of the zebrafish identifies it as a good model for the investigation of vascular development. Moreover, zebrafish mutually share many structural, functional, and molecular features with other vertebrates and are perfect models for xenotransplantation due to their inability to reject graft within 48hpf (Benyumov et al, 2012; Jung et al, 2017; Mulligan and Weinstein, 2014). In vertebrates, lymphangiogenesis begins from hematopoietic stem cells (HSC) fate determination, through several differentiation steps, to develop arterial and venous progenitors (Nicenboim et al, 2015). The genetic interactions involving HSC homeostasis and replenishment in zebrafish is regulated by PGE2 through the Wnt pathway (Goessling et al, 2009). Similarly, cardiac muscle development and pigmentation in zebrafish was shown to be regulated through Wnt signaling (Dohn and Waxman, 2012; Vibert et al, 2017). In animal models, cell lineage and vascular differentiation stages could be monitored with Texas red low-molecular-weight dextran, a widely used fluorescent dye for tracing vascular lineages in vertebrate development (Zhao et al, 2011). There is no comprehensive report on the roles of PGE2 in zebrafish embryonic angiogenesis, and lymphangiogenesis. Therefore, in this study, we investigated the effects of PGE2 on zebrafish embryonic vascular development and maturation.

The process of vascular development and maturation principally involves the interaction of vascular endothelial growth factors (VEGF) with their cognate receptors (VEGFR) in zebrafish. VEGF/VEGFR interaction plays a critical role in the formation and modification of vascular network during embryonic development in vertebrates. In Zebrafish, primarily, vegfa and
vegfd directly interact with *kdrl* (equivalent to *VEGFR2* in human) to regulate angiogenesis and lymphangiogenesis during early embryonic growth (Covassin et al, 2006; Bahary et al, 2007; Bower et al, 2017). Alternatively, angiogenesis is partly regulated by *vegfa* receptor *flt1* (equivalent to *VEGFR1* in humans), tested in mouse endothelial cell models (Nesmith et al, 2017). In zebrafish, angiogenesis involves a coordinated regulation of *kdrl* and *flt4* (equivalent to *VEGFR3* in human) receptors, partly controlled by Erk and Notch signaling (Phng et al, 2009). Expression of NOTCH transmembrane ligand *efnb2a* and its cognate receptor *ephb4a* on vascular endothelial cells and blood vessels selectively promotes cardiovascular development and angiogenesis in mouse embryos (Gerety, et al, 1999; Chen et al, 2015). However, Krueger et al, 2011 showed that *flt1* negatively regulates *efnb2a* during early angiogenic sprouting and erythropoiesis in zebrafish (Krueger et al, 2011). Furthermore, the roles of another vascular transcription factor *etv2* and its receptor G protein gamma-2 in VEGF-mediated angiogenesis and lymphangiogenesis in vertebrates remains unclear (Leung et al, 2006; Gomez et al, 2009; Davies et al, 2018). Roles of PGE2 in vascular marker expression have never been tested in zebrafish.

Previously, we have shown that in mouse breast cancer cell lines COX-2 induces PGE2 synthesis, which in turn induces tumor-associated angiogenesis and lymphangiogenesis via overproduction of VEGF-C and VEGF-D. This was regulated by EP4/PI3K/Akt pathway, and selective COX-2I and EP4A could inhibit tumor associated angiogenesis, and metastasis in a mouse model (Xin et al, 2012; Majumder et al, 2014; Lala et al, 2018). Furthermore, using rat lymphatic mesenteric lymphatic endothelial cells (RMLEC)s we showed that PGE2 induced lymphangiogenesis *in vitro* could be abrogated with COX2-I and EP4A (Nandi et al 2017). In human breast cancer, we have also shown that PGE2 induces cancer cell migration, invasion, and tumor-associated angiogenesis.
and lymphangiogenesis via upregulation of the EP4/PI3K/AKT pathways. Each of these phenotypes could be abrogated with a specific COX-2I and an EP4A (Majumder M et al, 2016; Lala et al, 2018). Non-steroidal anti-inflammatory drugs (NSAIDs) have emerged as powerful COX-2 inhibitors, which inhibit the synthesis of prostaglandins hence commonly used as pain medication (Ricciotti & FitzGerald, 2011). However, Chronic consumption of NSAIDs by North Americans resulted in severe side effects like gastrointestinal ulcers, perforation, bleeding, cardiac strokes, myocardial infarction, hypertension and renal dysfunction (Harirforoosh et al, 2014; Pai et al, 2018). Most of these effects might be due to blockage of protective physiological functions of PGE2 in human (Guo et al, 2012; Przygodzki et al, 2015). So, in this article, we tested the physiological roles of PGE2 during early embryonic vascular growth and maturation using zebrafish as an \textit{in vivo} model.

\textbf{Results:}

\textbf{PGE2 induces early embryonic movement and tail-flick}

To investigate an early embryonic phenotype in zebrafish, we injected fertilized eggs at the 2-cell stage with either PGE2 (4μM) or vehicle of PGE2 (0.13% BSA) along with Texas-red dextran (2μM) (Fig 1A-1D). Some non-injected embryos served as the control. Almost 99% of embryos in PGE2 and vehicle showed fluorescence in all replicates (data not presented), and the embryos without fluorescence were excluded from the study.

We measured the embryonic tail-flick frequency at 28 hours post fertilization (hpf) in selective embryos. Embryonic tail-flicks for the non-injected (n=17) (image in Fig 1B, Supplementary movie in 1A), vehicle injected (n=26) (image in Fig 1C, Supplementary movie in 1B) and PGE2 injected (n=26) (image in Fig 1D, Supplementary movie in 1C) embryos were recorded and quantified by counting the number of spontaneous tail movement of each embryo for a 30 second
exposure. We conducted an unpaired t-test comparing the mean and standard error of the mean (SEM) in PGE2 and vehicle groups, results showed a significant increase in embryonic tail flicks in PGE2 group, with a P value of 0.004 (Fig 1E). These data suggest that PGE2 enhanced early embryonic movement at 28hpf.

**PGE2 increases embryonic pigmentation and growth**

Along with improved embryonic movement, we also observed a visible change in embryonic pigmentation. So we measured the intensity of embryonic pigmentation at 48hpf and collected data from the selective vehicle (n=11) (Fig 2A) and PGE2 injected (n=10) (Fig 2B) embryos from all replicates. We used vehicle pigmentation as a threshold to measure PGE2 pigmentation (Fig 2C) using ImageJ. Quantification showed a significant increase in the pigmentation in the PGE2 injected embryos compared to the vehicle at 48hpf, with a P value of 0.014 (Fig 2C); data for non-injected is not presented. Increase in the pigmentation is an indicator of progressive development (Kimmel et al, 1995).

Additionally, we noticed that PGE2 embryos growing faster and getting bigger. So we measured yolk to larval area ratio (larval curvature within the embryo). We observed a decrease in yolk sac areas with a corresponding increase of the larval (head to tail) area in PGE2 embryos (Fig 2D) compared to vehicle (Fig 2E) with a significant P value of 0.0086 (Fig 2F). No difference was observed in the yolk to larval area ratios between the non-injected and the vehicle, data not presented. These data further suggest that PGE2 promotes embryonic growth and maturation of zebrafish embryos.
**PGE2 promotes early hatching and movement of embryos at 50hpf**

To further investigate advanced morphological development due to PGE2, we monitored hatching time of non-injected (n=90) (Fig 3A), vehicle (n= 61) (Fig 3B) and PGE2 (n=90) (Fig 3C) embryos in all three replicates. We observed a significant increase in the hatching rate at 50hpf in PGE2 group compared to the vehicle, with a P value of 0.0001. At this time point, no embryo hatched in the non-injected embryos (Fig 3A). Overall, only 5% of the vehicle and approximately 40% of the PGE2 embryos were hatched at 50hpf (Fig 3D) including all replicates. The non-injected embryos hatched at 53h.

Usually, zebrafish embryos follow a resting phase immediately after hatching (Kimmel et al, 1995), which is characterized by physical inactivity. To further associate PGE2-induced early larval development we recorded the swimming activity of zebrafish larvae from 50-53hpf, immediately after hatching. Embryos were resting post-hatching in the non-injected (Supplementary movie 2A) and the vehicle (Supplementary movie 2B) group. However, larvae in PGE2 group were very active immediately after hatching at 50hpf (Supplementary movie 2C). We quantified any movement of the hatched larvae (swimming activity) at 53hpf in all three groups for 30sec. A significant increase in the movement of embryos was recorded in the PGE2 group while only a few hatched larvae could show any activity in the vehicle group, with a significant P value of 0.0001 (Fig 3E). The larvae were more actively swimming in the PGE2 group as a result of an advanced stage of growth (Kimmel et al, 1995) due to PGE2 stimulation. No difference was seen in larval movements between non-injected and vehicle larvae after hatching. The observed early hatching and enhanced swimming activity of the larvae due to PGE2 suggests that PGE2 promotes improved physiological activity in zebrafish, which might need active vascular system.
**PGE2 induced early vascular maturation and increased heartbeat in zebrafish**

Due to the increased embryonic growth and early hatching observed in the PGE2 group, we hypothesized that to support the physiologic activity of hatched larvae, they need a functional vascular supply. Therefore, we sought to characterize the role of PGE2 in the vascular development in zebrafish. We injected Texas-red dextran, a red fluorescent dye that can trace cell lineages in vivo. We monitored fluorescently labeled cell lineages in the hatched embryos in both vehicle and PGE2 groups from 50hpf to 96hpf, with a fluorescent microscope. A more developed vasculature in the trunk areas of the developing larvae in the PGE2 group (Fig 4B) compared to the vehicle (Fig 4A) was recorded. Specifically, fluorescent images showing the trunk vascular fluorescence are illustrated as a more developed dorsal aorta (DA), posterior cardinal vein (PCV) and dorsal longitudinal anastomotic vessel (DLAV) in the PGE2 (Fig 4D) compared to the vehicle (Fig 4C).

We measured total fluorescent intensity in selective embryos (n=9 for vehicle and n=9 for PGE2) with ImageJ and the quantification of images at 96hpf showed a significant increase in mean fluorescence intensity in PGE2 compared to the vehicle, with a P value of 0.0001 (Fig 4E).

It is well established that the zebrafish embryo does not need an active vascular system for 4-5 days post-fertilization (Gore et al, 2012); however, cardiac cells remain the first line of embryonic cells developed in all vertebrates. Furthermore, heart muscle development and heart rate are the indicators of progressed developmental stages in Zebrafish (Kimmel et al, 1995). Thus, we quantified the mean heartbeat of zebrafish larvae at 96hpf. We captured a 30sec movie of the embryonic heartbeat in all three groups. Video data is presented in Supplementary movie 3. The quantitative data of selected embryos showing a significant increase in the mean of heart beating rate of PGE2 larvae (n=15) compared with the vehicle (n=12), with a P value of 0.023 is presented in Fig 4F. We observed no difference between the vehicle and the non-injected group, quantitative
data not presented. The observation of advanced and functional vasculature further suggests that PGE2 promotes early vascular maturation in zebrafish.

**PGE2 induced maturation of vascular anatomy in Zebrafish in a time-dependent manner**

To establish PGE2-induced vascular maturation in zebrafish, we measured the vascular fluorescence in vehicle and PGE2 groups at different time points (53hpf, 72hpf, and 96hpf) (Fig 5). As the embryonic cell lineage gets differentiated during embryonic development; the fluorescence intensity gets diffused with time and it becomes difficult to minimize background interference and maximize fluorescence *in vivo*. The original fluorescent images were added in the Supplementary Fig. 1. To quantify only vascular fluorescence, we converted images to grey scale using ImageJ and highlighted region of interest (ROI) with boxes (Fig 5). We observed a more prominent change in the branching and sprouting of blood vessels during vascular maturation in PGE2 group in a time-dependent manner (Figs 5A-5F). We observed a more developed dorsal aorta (DA), dorsal longitudinal anastomosing vessels (DLAV) and inter-segmental arteries (ISA) in PGE2 larvae (Figs 5B, 5D, 5F) than the vehicle larvae (Figs 5A, 5C, 5E). This observation of vascular anatomy of DA and ISA suggests that PGE2 induces both primary and secondary angiogenesis in zebrafish; data presented with an illustration in Fig 5F and quantitative data of total vasculature is presented in Fig 5G. Quantification (n= 9 for vehicle and n=9 for PGE2 at all time points) showed a significant increase in mean fluorescence intensity in PGE2 embryos compared to the vehicle at 72hpf with P value of 0.0073 and at 96hpf with a P value of 0.0001 (Fig 5G). Although the fluorescence of vascular anatomy was high in PGE2 at 53hpf, the difference was not significant.
**PGE2 regulates angiogenesis and lymphangiogenesis gene expression**

To examine the expression of vascular genes among the non-injected, vehicle and PGE2 injected groups; we extracted total RNA and performed quantitative RT-PCR using TaqMan gene expression assays. We sacrificed embryos at 96hpf and pooled all embryos in the same group in one tube before RNA extraction as a result for gene expression assays our sample size become n=3 for each condition. We observed a drop in gene expression in the vehicle compared to the non-injected group, however, that change was not statistically significant. We hypothesize that this difference was observed due to the microinjection, thus, we decided to compare gene expression fold changes in PGE2 microinjected group to the vehicle-microinjected group. The mRNA fold change results (Fig 6A) showed a marginal (1.2 to 2.1 fold) change in all gene expressions measured between PGE2 and vehicle groups. We observed a significant increase in NOTCH target genes *efnb2* (P = 0.003), *ephb4a* (p=0.01); angiogenesis markers *vegfa* (P =0.02), *kdrl* (P = 0.04), *etv2* (P = 0.02) and lymphangiogenesis marker *prox1* (P = 0.0001) expression in PGE2 group compared to the vehicle (Fig 6A). Increase in vascular gene expression in PGE2 supports vascular anatomy maturation data.

**COX-2 inhibitor (COX-2I) and EP4 antagonist (EP4A) can abrogate PGE2 induced functions in vitro**

We used a rat mesenteric lymphatic endothelial cell line (RMLEC) to test the potential of COX-2I NS398 and an EP4A ONO-AE3208 to inhibit PGE2 induced angiogenesis and lymphangiogenesis *in vitro*. Tube formation assay is a surrogate model to test angiogenesis and lymphangiogenesis *in vitro*. The assay was carried out with RMLEC cells seeded on Matrigel with different treatment conditions (Supplementary Figure 2). Under native serum-free conditions
without any stimulation by virtue of the cells, RMLEC can form very few tubes (a complete network of cells) at 24h, however in the presence of PGE2, numbers of tubes are significantly increased (P of 0.0003). Addition of COX-2I and EP4A significantly abrogated PGE2 induced tube formation of RMLEC P=0.05 and P=0.01 respectively.

Discussion:

It has been established that hematopoietic stem cells (HSC) homeostasis is tightly controlled by PGE2. PGE2 plays a key role in the regulation of embryonic stem cells during embryonic growth and early cell differentiation in zebrafish (Cha et al, 2006). Chemicals that enhance prostaglandin synthesis increases stem cell production, while those that block prostaglandin synthesis decrease stem cell numbers (North et al, 2007). In the adult zebrafish model, PGE2 improved kidney marrow recovery, following an irradiation injury and it plays a regulatory role in spleen and bone marrow formation in a murine model (North et al, 2017). The cyclooxygenase-1 (COX-1) enzyme is responsible for PGE2 synthesis in vertebrates and maintains a physiological level of PGE2 to sustain tissue homeostasis. However, overproduction of PGE2 by COX-2 is reported to promote chronic inflammation and breast cancer (Majumder et al 2018, Lala et al 2018). Overproduction of PGE2 has specifically been shown to be associated with the breast cancer angiogenesis, lymphangiogenesis, and metastasis by upregulation of VEGF-C and VEGF-D (Xin et al, 2012; Majumder et al, 2014).

In the current study, we showed that PGE2 induces embryonic growth, and upregulates angiogenesis and lymphangiogenesis marker expression. Morphological data shows advancement in zebrafish development including an increase in embryonic tail-flicks, pigmentation, larval motility and heart rate in presence of PGE2. This suggests that PGE2 might be promoting
embryonic development. Here, we observed that PGE2 upregulates *efnb2a*, a NOTCH target gene that is involved in angiogenesis and erythropoiesis. It was shown that PGE2 induces embryonic growth in zebrafish by NOTCH/Wnt upregulation (Hogan et al, 2009). Previously, we have shown that overexpression of PGE2 induces *WNT* and *NOTCH* pathway genes in human breast cancer and PGE2 induces cancer stem-like cells (SLCs), which were abrogated with NOTCH/Wnt and PI3K/Akt and Erk inhibitor treatments (Majumder et al, 2016). These results indicate that PGE2 regulates both embryonic and mature stem cells via EP4, PI3k/Akt and ERK pathways (Majumder et al 2016; Lala et al 2018; Cha et al, 2006). Here we found a NOTCH target gene *efnb2a* is upregulated in PGE2 injected embryos, so we further need to investigate other stem cell regulatory pathway genes in zebrafish.

Here we also observed an advancement of zebrafish trunk vascular development with an increase in intersegmental arteries and lymphatic vessel formation in the PGE2 injected group. This suggests that angiogenesis and lymphangiogenesis occurred together during early embryonic development in zebrafish. We observed a marginal fold change in PGE2 injected group compared to vehicle suggests that the effect of externally added PGE2 on vascular gene expression is not so prominent at early embryonic development (96hpf). Our observation is supported by another study showing that different stages of vertebrate growth are associated with different gene expression profiles (Yang et al, 2013). Therefore, we might observe a noticeable fold change in gene expression if the larvae were to develop into adult zebrafish. Never the less, we recorded a significant increase in the percentage of the total vascular fluorescence, with significantly developed vascular anatomy along with the upregulation of angiogenesis genes (*vegfa* and *kdrl*) in the PGE2 injected larvae. Our observation is further supported by another group, which showed that primary angiogenesis sprouts from the dorsal aorta in the trunk of zebrafish are mediated
by \textit{vegfc} and \textit{vegfd} interactions with \textit{kdrl} and \textit{flt4} respectively (Hogan et al, 2009; Bower et al, 2017). Increase in \textit{vegfa} production can also stimulate maturation of vasculature, promote endothelial cells migration and regulate HSC lineages to form red blood cells (Liang et al, 2001). Hence PGE2 induces angiogenesis via upregulation of \textit{vegfa} and \textit{kdrl} in zebrafish. We observed a higher expression of lymphangiogenesis marker, \textit{prox1}, in the PGE2 injected group. This might be due to upregulation of \textit{kdrl}, which is a receptor for both \textit{vegf} and \textit{prox1} to promote angiogenesis and lymphangiogenesis. Above findings are supported by studies showing a significant upregulation of \textit{prox1} in PGE2 treated zebrafish (Bower et al, 2017; Shin et al, 2016; Koltowska et al, 2015).

We propose that PGE2 induced upregulation of \textit{vegf} might be via the PI3K/Akt signaling pathway. Here, we observed that PGE2 upregulated tube formation of RMLEC and it was shown previously that PGE2 regulates tube formation in RMLEC by stimulation of PI3K/Akt signaling (Nandi et al, 2017). We reported earlier that COX2/PGE2 induced VEGF-C/D production and tumor associated angiogenesis and lymphangiogenesis, was inhibited by blocking EP4/PI3K/Akt signaling in breast cancer models (Xin et al, 2012; Majumder et al, 2014; Nandi et al, 2017; Majumder et al, 2018).

In zebrafish, the increase in \textit{vegfa/kdrl} interactions upregulates the vascular transcription gene \textit{etv2} via Map-k signaling (Chetty et al, 2017), which in turn modulates the differentiation of arterial and venous expansions in NOTCH-dependent manner. Overexpression of \textit{vegfa} can repress the venous expansion possibly through the delta-NOTCH pathway, which is independent of \textit{etv2} (Chetty et al, 2017). Here, we observed that PGE2 induces lymphangiogenesis marker \textit{prox1} expression, which facilitates lymphatic differentiation from the venous progenitor stem cells, thus inducing lymphangiogenesis. Our data also suggests that PGE2 induces both angiogenesis and lymphangiogenesis possibly by stimulating the interaction of \textit{vegf} with its
cognate receptor *kdrl*. This interaction, in turn, regulates angiogenesis by upregulating downstream NOTCH target genes, *efnb2a* and *ephb4a*. A suggestive schema of possible mechanisms of PGE2 induced vascular maturation in zebrafish is presented in Fig 6B.

Furthermore, here we demonstrated that COX2-I and EP4A could abrogate PGE2 induced tube formation by RMLEC. High dosages of COX-2 inhibitors are commonly used as anti-cancer and anti-inflammatory drugs to treat human cancers (Harris et al, 2006). Long-term use of COX2-I could cause severe side effects because they block the production of protective prostanoids (Przygodzki et al, 2015) and EP4A was suggested as a better alternative of COX-2I, which spares cardio protective prostanoids (Majumder et al, 2016; Majumder et al, 2018). Hence, this study on the regulatory roles of PGE2 during zebrafish vascular development will help us to better understand the adverse side effects observed with PGE2 inhibitors and will play pivotal roles on the path towards identifying new therapeutic targets in breast cancer.

**Materials and methods:**

**Ethics statement**

The Brandon University Animal Care Committee (BUACC) approved the use of zebrafish in this article and we followed the guidelines of the Canadian Council on Animal Care (CCAC).

**Zebrafish maintenance**

Zebrafish used in this study were housed in the animal facility at Brandon University, maintained by Dr. Christophe LeMoine. The wild-type adult zebrafish (*Danio rerio*) were bred in a plastic tank maintained with Brandon dechlorinated tap water in a 10h:14h light dark cycle, at 28°C. The fish were fed once daily on Adult zebrafish Complete Diet (Zeigler, Gardners, PA). The fertilized zebrafish eggs were harvested in hundreds and raised eggs (1-cell stage) in a glass-plated petri dish filled with E3 embryo medium (in mM: 5 NaCl, 0.17 KCl, 0.33 CaCl, 0.33 MgSO4, and 0.00001%
methylene blue) and kept at 28°C. With the aid of a microinjector (IM 300, Narishige, Long Island, NY) and a pulled 1.0-mm borosilicate glass micropipette (Stutton Instrument, Novato, CA), 1 nanoliter volume of either PGE2 (4μM) or vehicle (0.13% BSA) along with 1μM of red fluorescent dye (Dextran, Texas Red™, 3000 MW, Lysine Fixable, Thermo Fisher, ON) were injected into eggs. Following the microinjection, all groups including, PGE2-injected, vehicle-injected, and the non-injected control were maintained under the same conditions in E3 growth media and incubated at 28°C (LeMoine & Walsh 2013). Phenotypic changes during embryonic growth of zebrafish were monitored and recorded until 96 hours post fertilization (hpf) with a stereoscopic and a fluorescence microscope.

**Drugs**

PGE2 and NS398 were purchased from (Cayman, Ann Arbor, MI, USA); ONO-AE3-208 from ONO Pharmaceuticals, Osaka, Japan. Dr. Peeyush K Lala at the University of Western Ontario kindly provided us with all these chemicals.

**Image and video processing**

Zebrafish embryos were left submerged in a glass-bottomed petri dish filled with E3 medium during data recording. Images and movies of non-injected and the injected (vehicle and PGE2) zebrafish embryos were recorded using a stereoscopic zoom microscope (Nikon SMZ1500) and a fluorescence microscope (Olympus MVX10). Images were further processed and quantified using ImageJ (Simms et al, 2017) and ZFIN (Chavez et al, 2016) software. Movie files were converted and processed using Wondershare Video Converter.

**Zebrafish Assays**

The fertilized zebrafish eggs were obtained at the 1-cell stage, but when we started injecting they reached the 2-cell stage. We replicated microinjections for all conditions at least for three times.
An average of 20 embryos died post injection and the dead embryos were promptly removed. In three experiments number of embryos used is as follows: in experiment 1, non-injected n=30, vehicle injected n=40 and PGE2 injected n=40; in experiment 2, non-injected n=60, vehicle injected n=70 and PGE2 injected n=70; in experiment 3, non-injected n=60, vehicle injected n=30 and PGE2 injected n=60. The eggs injected with 0.13% BSA served as “vehicle” control of PGE2 treatment, and eggs injected with PGE2 (4μM) were considered as the “PGE2” treatment group. We also injected Dextran (red fluorescent dye) in both vehicle and PGE2 groups, to trace the phenotypic changes occurring during early development of the embryos. Also, the fluorescent labeling was employed to aid in the selection of embryos that were successfully injected; embryos without any fluorescence in the vehicle and PGE2 groups were excluded from the study. The vehicle group was first injected, followed by the PGE2 group, with a 1-hour time gap maintained throughout the growth-monitoring period. Phenotypic changes of zebrafish embryonic growth in the three groups were observed and recorded and developmental stages were compared with Kimmel et al (Kimmel et al, 1995).

**RNA extraction and gene expression assays**

At 96hpf, zebrafish larvae were sacrificed by freezing at -80°C for two hours and then we added QIAzol lysis reagent (QIAzol®, Qiagen, ON) followed by homogenization of the tissue with vigorous vortexing. Then we extracted total RNA using miRNeasy Mini Kit (Qiagen, ON) and synthesized cDNA using high-performance TaqMan mRNA cDNA Reverse Transcription Kit (Life Technologies, ON). Quantitative RT-PCR was done with TaqMan Gene Expression Assays (Life Technologies, ON) using a Rotor-Gene 6000 Real-Time PCR (Corbett Research®). Vascular gene expression for *kdrl* (Dr03432884_m1), *vegfa* (Dr03435728_m1), *prox1* (Dr03086822_m1), *efnb2α* (Dr03073975_m1), *ephb4α* (Dr03138278_m1), and *evt2*
(Dr03077892_m1) were normalized to the values of actb1 (Dr03432610_m1) control gene expression by calculating the relative changes between threshold cycle (Ct) of the vascular genes and the control gene (actb1) (delta CT) within each group. The relative fold change in gene expression between the PGE2 and vehicle groups were quantified by calculating del del Ct (the delta CT of treatment group minus delta CT of reference group), followed by fold change of gene expression (2^{del del ct}) as described previously (Majumder et al, 2015, 2016, 2018).

**Tube Formation Assay**

Rat mesenteric lymphatic endothelial cell line (RMLEC) is a spontaneously immortalized LEC isolated from rat mesenteric lymphatic endothelium (Whitehurst et al, 2006), kindly provided by Dr. Peeyush K Lala. RMLEC forms tube-like structure on Matrigel, which is an *in vitro* surrogate of angiogenesis and lymphangiogenesis processes. RMLEC was grown in Dulbecco's Modified Eagle Medium (DMEM) supplemented with 10% FBS, 2mM glutamine, 50 U/ml penicillin and 50μg/ml streptomycin, 1mM Sodium pyruvate, and 1mM nonessential amino acids (all products from Gibco, ON) at 37 °C in a humidity maintained CO2 incubator. Tube formation assay was carried out with RMLEC cells under different treatment conditions on BD Matrigel™ (BD Biosciences, USA). Matrigel was thawed overnight at 4°C, diluted with cold sterile PBS (Gibco) in 1:1 ratio, and 0.25 ml/well was used to coat 24-well culture plates (VWR, ON) and left at 37°C for 6h. After polymerization, 40,000–60,000 cells/well, suspended in DMEM medium were added to each well. Under native serum-free condition, very low levels of tube formation occurred at 24h but PGE2 (20μM) treatment significantly stimulated tube formation. In a separate experiment to test the involvement of COX-2 and EP4 pathways in PGE2 induced tube formation; we added COX-2I NS398 (20μM) and EP4A ONO-AE3208 (20μM) in addition to PGE2. We took 10–15 random pictures per well in all experimental conditions using an
inverted microscope (Nikon). The numbers of total tubes per unit area were quantified using the ImageJ software as reported earlier (Majumder M et al, 2012; Nandi P et al, 2017).

**Statistical Analysis**

Statistical calculations were performed using GraphPad Prism software version 5. Data were presented as mean ± standard error of mean (SEM) for each experiment. Unpaired t-test was used when comparing the mean of two datasets to estimate two-tailed P value. Statistically relevant differences between means were accepted at P<0.05.
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Author contributions:

Concept, project design, and supervision: MM; Experiments and quantifications: MM, AO, KCU, SH, BN; Zebrafish breeding and microinjections: CN; Statistical Analysis: MM, SM, KCU; Image Data Processing: SM, AO, KCU; Video Data Processing: SM; Manuscript writing: MM, KCU, SM.

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Figure 1: Microinjection and embryonic movement at 28hpf: (A) we collected zebrafish eggs at the 2-cell stage and a few non-injected embryos were kept as a reference (B). Only vehicle (C) and PGE2 (D) embryos were fluorescently labeled due to dextran injection. We monitored post-injection growth of embryos with both stereo and fluorescent microscopes and observed an
increase in embryonic tail flicks in the PGE2 group (movie presented in supplementary movie 1). (E) From each biological replicate, we selected some embryos to measure tail-flicks. Chart showing the mean of embryonic tail-flick of non-injected (n=17), vehicle (n=26) and PGE2 (n=26) injected embryos ± SEM. We conducted an unpaired t-test and results showing a significant increase in tail-flicks in PGE2 injected group compared to the vehicle with a two-tailed (indicated as *) P-value of 0.0001.
Figure 2: PGE2 increases zebrafish embryonic development and pigmentation at 48hpf: For the measurement of pigmentation, the gray-scaled images from stereomicroscope were converted to red colored scale using ImageJ. The red color intensity of the pigmented areas in the vehicle (A) was used as a threshold to measurement PGE2-induced pigmentation (B). We selected a few embryos from three biological replicates for this quantification. (C) Data represented as mean of embryonic pigmentation for PGE2 (n=10) and vehicle (n=11) groups ± SEM. An unpaired t-test comparing means showing a significant upregulation of embryonic pigmentation in PGE2 group with a two-tailed P value (*) of 0.0001. (E) PGE2 treated fish is growing bigger with the tail almost
reached the head in the embryonic sac (doted redline with arrow) compared to the vehicle (D). We measured the areas of the fish body (denoted with yellow dotted line) and yolk sac (denoted with green dotted line) and calculated the ratio of the areas as body/yolk. (F) The chart showing a very significant growth difference between the PGE2 and vehicle groups with a two-tailed P value (**) of 0.0086.
Figure 3: PGE2 promotes early hatching of zebrafish at 50hpf. (A-C) showing representative images of hatched embryos in non-injected (A); vehicle (B) and PGE2 injected (C) groups. We analysed n=90 non-injected, n=61 vehicle and n=90 PGE2 embryos. (D) Data presented as the mean of percentages (hatched/total number of eggs) of hatched embryos ± SEM. Hatched embryos were significantly high in PGE2 (33%) than the vehicle (5%), with a two-tailed P value (**) of 0.0001. (E) We also recorded the larval movements as a measure of swimming activity post-hatching in all three groups (video presented in supplementary video 2) at 53hpf. Data presented as the mean of larval movements of hatched embryos [non-injected (n=14), vehicle (n=31) and
PGE2 (n=35) ± SEM. We conducted an unpaired t-test showing significantly higher motility in PGE2 group compared to the vehicle with a two-tailed P value (*) of 0.0008.
Figure 4: PGE2 induced vascular maturation and increased heart rate in zebrafish.

(A and B) shows gray-scaled images of trunk vasculature of developing zebrafish in both vehicle and PGE2 groups at 96hpf. Both vehicle and PGE2 groups were microinjected with Texas-Red dextran dye and grown under the same condition, with vasculature formation captured from 53hpf to 96hpf, data presented only for 96hpf. (C and D) shows fluorescence images of trunk vasculature in the vehicle and PGE2 larvae. Vehicle color was considered as a threshold to measure fluorescence of the PGE2 using ImageJ. (E) The chart showing the mean of trunk vascular fluorescence measured for both vehicle (n=9) and PGE2 (n = 9) larvae ± SEM. The PGE2 group...
showed a significant (*P=0.0001) increase in fluorescence compared to the vehicle group. The PGE2 injected larvae also showed a clear formation of the mature vasculature with dorsal longitudinal anastomosing vessel (DLAV), intersegmental arteries (ISA), dorsal aorta (DA) and posterior cardinal vein (PCV) formation while the vehicle remained premature. (F) The chart shows the mean heart rate of selective embryos from 3 biological replicates (n=12 for vehicles and n=15 for PGE2) ± SEM. An unpaired t-test showed a significantly high heart rate in PGE2 group compared to the vehicle group larvae at 72hpf, with a two-tailed *P value of 0.0221. Corresponding heartbeat videos of all three experimental groups are presented as supplementary movie 3.
**Figure 5: PGE2 induces angiogenesis in zebrafish in a time-dependent manner.** The dextran dye can trace cell lineage to track vascular development in whole fish. However, as the embryo matures, the dye starts to diffuse which makes it difficult to capture vascular maturation beyond 96hpf. (A-F) Represents the developing zebrafish trunk vasculature in both vehicle and PGE2 groups from 53hpf to 96hpf. The region of interest (ROI) in white dotted rectangle boxes showing the difference in vascular fluorescence between groups. (F) In this image, ROI expanded to show dorsal aorta (DA), dorsal longitudinal anastomosing vessel (DLAV), posterior cardinal vein (PCV), and accompanying intersegmental arteries (ISA). (G) We selected a few zebrafish embryos
[PGE2 (n=9) and vehicle (n=9)] in both groups to measure fluorescence intensity with ImageJ. The chart represents the mean fluorescence intensity ± SEM. An unpaired t-test was conducted for each time point and we observed a significant increase in the trunk vasculature fluorescence in the PGE2 compared to the vehicle at 72hpf and 96hpf respectively with *P=0.007 and ** P=0.0001 respectively.
Figure 6: PGE2 regulates vascular marker mRNA expression in zebrafish. (A) Shows the mRNA qPCR expression analysis of different vascular markers from non-injected, vehicle and PGE2 at 96hpf. PGE2 shows an upregulation of vegfa (1.1 fold), kdr1 (1.23 fold), prox1 (1.21 fold), etv2 (1.17 fold), efnb2a (1.62 fold) and ephb4a (fold 2.07) when compared to the vehicle. The tested vascular genes were normalized to actb1 as the control reference gene. For
each group, all embryos were pooled before extraction in each replicate. Data represented as mean of three biological replicates (n=3) ± SEM. Unpaired t-test was conducted to compare fold changes for each gene between PGE2 and vehicle groups showing significant differences with *P<0.05, **P<0.01. (B) Represents the possible signaling mechanism of how PGE2 induces angiogenesis in Zebrafish.
Supplementary Figures

Figure S1. We captured vascular development of hatched zebrafish embryos at different time points 53, 72 and 96hpf with a fluorescent microscope. A region of interest (ROI) for vascular maturation was shown with white dotted rectangle boxes. Vascular development for the vehicle at three-time points are presented in A, C, E and for PGE2 presented in B, D, F.
Figure S2. RMLEC forms tubes on Matrigel. (A) In basal media RMLEC forms very few tubes, (B) PGE2 induced tube formation and in presence of COX-2 inhibitor NS-398 (C) and EP4 antagonist ONO-AE3208 (D) PGE2 induced tube formation abrogated. Scale bar represents 200 µM. (E) We quantified number of total tubes (complete cell network) at 24h and data presented as the mean of three experiments (n=3) ± SEM. Unpaired t-test showing the increase in tube formation in presence of PGE2 (**)P=0.003) and NS398 (*P=0.05) and ONO-AE3208 could significantly block PGE2 induced tube formation.
Supplementary Movies

Movie 1A

Movie 1B
Movie 1C

**Movie 1: PGE2 increases embryonic tail-flick in zebrafish at 28hpf.** (A-C) Shows a 30-second movie representative of embryos from normal, vehicle and PGE2 at 28hpf. The frequency of the embryonic tail-flicks was observed from three replicates of group. No difference was observed in the frequency of embryonic tail-flick between the normal (movie 1A) and the vehicle (movie 1B). The embryonic tail-flick of the PGE2 (movie 1C) was observed to be more frequent than the vehicle.
Movie 2A

Movie 2B
Movie 2: PGE2 promotes swimming activity in zebrafish at 50hpf. (A-C) Shows a 30-second movie of the normal (movie 2A), vehicle (movie 2B) and PGE2 (movie 2C) larvae at 50hpf after hatching. Zebrafish larvae in PGE2 show more active and frequent motility than the vehicle and normal.
Movie 3A

Movie 3B
Movie 3C

**Movie 3: PGE2 promotes increased heartrate in zebrafish at 96hpf.** (A-C) Shows a 30-second movie of the normal (movie 3A), vehicle (movie 3B) and PGE2 (movie 3C) larvae at 96hpf. Zebrafish larvae in PGE2 show faster heart beats than the vehicle and the normal.