Cardiac injury modulates critical components of prostaglandin E₂ signaling during zebrafish heart regeneration

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The inability to effectively stimulate cardiomyocyte proliferation remains a principle barrier to regeneration in the adult human heart. A tightly regulated, acute inflammatory response mediated by a range of cell types is required to initiate regenerative processes. Prostaglandin E₂ (PGE₂), a potent lipid signaling molecule induced by inflammation, has been shown to promote regeneration and cell proliferation; however, the dynamics of PGE₂ signaling in the context of heart regeneration remain underexplored. Here, we employ the regeneration-competent zebrafish to characterize components of the PGE₂ signaling circuit following cardiac injury. In the regenerating adult heart, we documented an increase in PGE₂ levels, concurrent with upregulation of cox2a and ptges, two genes critical for PGE₂ synthesis. Furthermore, we identified the epicardium as the most prominent site for cox2a expression, thereby suggesting a role for this tissue as an inflammatory mediator. Injury also drove the opposing expression of PGE₂ receptors, upregulating pro-restorative ptger2a and downregulating the opposing receptor ptger3. Importantly, treatment with pharmacological inhibitors of Cox2 activity suppressed both production of PGE₂, and the proliferation of cardiomyocytes. These results suggest that injury-induced PGE₂ signaling is key to stimulating cardiomyocyte proliferation during regeneration.

Ischemic heart disease is currently the leading cause of mortality worldwide¹. A fundamental limitation to cardiac regeneration in adult humans is the inability to effectively replenish lost cardiomyocytes (CMs). In stark contrast, multiple vertebrates, including the axolotl, newt, zebrafish, and neonatal mouse, demonstrate the remarkable capacity to regenerate lost or damaged myocardium through the proliferation of resident cardiac muscle cells²–⁵. Therefore, elucidating the cues that promote CM proliferation in these models will be crucial to informing therapies to stimulate regeneration in the injured human heart.

Acute inflammation is a hallmark of the tissue damage response, requiring the meticulous orchestration of signals from multiple cell types. CMs, endothelial cells, immune cells, and more recently, epicardial cells, have been shown to play essential roles in crafting the injury micro-environment⁶–¹¹. A highly regulated inflammatory response is required for regeneration, and abrogating this response blocked regeneration in both zebrafish and neonatal mouse hearts¹²–¹⁴. Among the principal signaling molecules released during acute inflammation are the prostaglandins. Notably, Prostaglandin E₂ (PGE₂) has been shown to promote tissue regeneration by directly stimulating the proliferation of target cells¹⁵,¹⁶, as well as indirectly, by governing the recruitment and polarization of immune cells¹⁷–¹⁹. PGE₂ is synthesized through the sequential catalytic activity of cyclooxygenase (COX) enzymes, prostaglandin-endoperoxide synthase −1 and −2 (colloquially termed COX1 and COX2), followed by the terminal synthase Prostaglandin E synthases (PTGES). There are four membrane-bound PGE₂ receptors. Signaling through these receptors has pleiotropic effects; however upregulation of Prostaglandin E₂ receptor 2 (EP2) and...
EP4 is frequently associated with cancer progression, cell survival and proliferation, while activation of EP3 often stimulates opposing cellular responses.

The multi-faceted effects of PGE2 activity are also mediated by the spatio-temporal dynamics of signal intensity. Although PGE2 has been demonstrated to accelerate skeletal muscle regeneration, it also exacerbated ischemic damage in the brain, and inhibited repair and regeneration of the liver. Of concern, PGE2-pathway genes are also upregulated in many cancers, and while short-term PGE2 treatment promoted regeneration of damaged colon tissues, prolonged exposure-initiated tumor formation. Therefore, it is critical to decode the precise dynamics of pro-regenerative PGE2 signaling to capitalize upon the benefits of this inflammatory mediator.

In the present study, we show that the regenerating zebrafish heart upregulates enzymes essential for PGE2 synthesis, and modulates PGE2 receptor expression to optimize pro-regenerative signaling. Moreover, we identify the epicardium as a focus of PGE2 expression, implicating this tissue as a source of inflammation-associated PGE2 signaling. Importantly, pharmacological inhibition of Cox2 activity suppressed both levels of PGE2, and CM proliferation early in the regenerative process. Taken together, our data suggest that PGE2 promotes CM proliferation during the acute inflammatory response, and holds potential therapeutic promise for humans.

Materials and Methods

Zebrafish husbandry and heart amputation. All animal studies were performed in accordance with an approved protocol (Protocol 16–14) by the Institutional Animal Care and Use Committee at MDI Biological Laboratory. Wild type animals were used of the Ekkwill (EK) or EKxAB mixed background strains. Transgenic lines used in this study were Tg(cmlc2:EGFP), Tg(fli1a:EGFP), Tg(lfli1a:EGFP), and Tg(mpeg1:YFP). Adult zebrafish 4–18 months of age were anaesthetized in a 1:1,000 dilution of 2-phenoxyethanol. Approximately 20% of the ventricular apex was resected with iridectomy scissors, as previously described.

Intraperitoneal microinjection of selective Cox2 inhibitors. Zebrafish were injected daily, beginning on the day of surgery, with 220 ng/g of NS-398 or 1 μg/g Celecoxib (Cayman Chemical, Ann Arbor, MI #70590, #10008672); vehicle controls were 0.1% DMSO, or 0.44% EtOH, respectively.

Immunohistochemistry. Hearts were extracted, fixed in 4% paraformaldehyde, equilibrated in 30% sucrose, then embedded in TBS tissue freezing medium (Fisher Scientific, Hampton, NH #TFM-C) and sectioned to 10 μm. Primary antibodies used were rabbit anti-Mef2 (Santa Cruz Biotechnology, Santa Cruz, CA #SC-313; 1:75) and mouse anti-Pcna (Sigma-Aldrich, Nadick, MA #P8825; 1:400). Secondary antibodies used were Alexa Fluor 488 goat anti-rabbit IgG (H + L) for anti-Mef2, and Alexa Fluor 594 goat anti-mouse IgG (H + L) for anti-Pcna (Invitrogen - Thermo Fisher Scientific, Waltham, MA #A11034, #A11020). Images were captured at 20X using an Olympus BX53 microscope and Retiga 2000 DC camera. CM proliferation indices were calculated as a percentage of Mef2(+)/Pcna(+) cells relative to the total number of Mef2(+) cells in a defined area adjacent to the injury.

In situ hybridizations. Hearts were processed as described above, and serial sections were subjected to in situ hybridization studies with DIG-labelled RNA probes directed against cox2a, cox2b and ptger2a using previously described methods. cDNA fragments corresponding to the first 900-bp of each gene were synthesized (www.IDTDNA.com) and cloned into the pMiniT vector (www.NEB.com). Antisense probes were synthesized with either SP6 or T7 Polymersase using the Roche DIG Labelling Kit (SP6/T7) in accordance to the manufacturer's suggested protocol (www.Roche.com). Negative controls included cox2a riboprobe but no secondary antibody, and secondary antibody only. Images were captured using settings described under the Immunohistochemistry methods section.

Gene expression studies of whole heart tissues. Ventricles were isolated in ice-cold PBS, placed immediately in Ambion TRIzol Reagent (Invitrogen - Thermo Fisher Scientific, Waltham, MA #2302700), and homogenized using an electric homogenizer. RNA was extracted using Zymo Direct-zol RNA Microprep Kit, (Zymo Research Corp, Irvine, CA #R2069), followed by treatment with DNaseI (New England Biolabs, Ipswich, MA #M0303), and final purification with RNA Clean & Concentrator-5 Kit (Zymo Research Corp, Irvine, CA #R1014). cDNA was synthesized using ProtoScript® II First Strand cDNA Kit (New England Biolabs, Ipswich, MA # E6560S). qPCR studies were performed in technical triplicate using Brilliant III Ultra-Fast SYBR® Green QPCR Master Mix (Agilent, Santa Clara, CA #600882); vehicle controls were 0.1% DMSO, or 0.44% EtOH, respectively.

Fluorescence-Activated Cell Sorting (FACS) and gene expression. Single-cell suspensions were prepared from 1–25 hearts of zebrafish reporter strains as previously described, with modifications. Briefly, ventricles were isolated and washed in ice-cold PBS to remove blood cells. Tissues were digested in Hank’s Balanced Salt Solution plus 0.13 U/ml Roche Liberase DH (Thermo Fisher Scientific, Waltham, MA # 5401054001) at 37°C, while stirring gently with a magnetic spinbar. Supernatants were collected every 5 minutes and neutralized with 10% sheep serum. Dissociated cells were spun down, washed in PBS, and resuspended in PBS plus 2% fetal bovine serum. The cell suspension was then strained through a 35 μm mesh and DAPI added as a viability dye. Sorting was performed on a FACSARia II (BD Biosciences, Franklin Lakes, NJ), gated to exclude doublets, debris, and dead cells. Viable cells were sorted directly into Ambion TRizol LS Reagent (Invitrogen - Thermo Fisher Scientific, Waltham, MA # 10296–010). RNA was extracted using Zymo Direct-zol RNA Microprep kit, (Zymo Research Corp, Irvine, CA #R2069) as directed by the manufacturer. Total RNA was amplified with the Ovation® PicoSL WTA Kit (NuGEN, Redwood City, CA #3312) to generate cDNA for downstream qPCR analysis.
Enzyme-Linked Immunosorbent Assay (ELISA) for PGE₂. ELISA for PGE₂ was conducted using the Prostaglandin E₂ Human ELISA Kit (Invitrogen - Thermo Fisher Scientific, Waltham, MA # KHL1701) according to manufacturer’s instructions. Briefly, ventricles were collected from six weight-matched clutchmates, washed in ice-cold PBS, and homogenized in 500 µl TRIS buffer. Homogenate was spun down at 1200 rpm for four min at 4 °C to eliminate particulate, and supernatant collected for ELISA. Assays were run in technical triplicate.

LC-MS/MS analysis: Lipid isolation. For each biological replicate, ventricles from five weight-matched clutchmates were isolated, washed in ice-cold PBS, flash frozen in liquid nitrogen and stored at −80 °C until shipment on dry ice to the University of Iowa for sample preparation and analysis; samples were processed and analyzed in a blinded manner. Tissues were homogenized in 1 ml sucrose buffer (250 mM sucrose, 50 mM HEPES pH 7.1, 1 mM EDTA, 1 mM EGTA with fresh protease inhibitors) using a Dounce homogenizer. A small aliquot was set aside to quantify proteins for sample normalization. Protein was precipitated and separated from homogenates with the addition of 2 ml HPLC grade methanol (Fisher Scientific, Hampton, NH) and centrifugation at 2,200 × g at 4 °C for 15 minutes. Supernatants containing lipids were diluted with 12.5 ml HPLC grade H₂O (Fischer Scientific, Hampton, NH), and supplemented with 100 µl of internal standard stock including the following deuterated eicosanoids (Cayman Chemical, Ann Arbor, MI): 0.2 ng/µl d8 arachidonic acid, 0.1 ng/µl d4 6-keto-prostaglandin (PG)F₁α, 0.1 ng/µl d4 PGD₂, 0.1 ng/µl d4 PGE₂, 0.1 ng/µl d4 PGF₂α, 0.1 ng/µl d4 thromboxane (TX)B₂, 0.1 ng/µl d8 15-deoxy-Δ12,14-PGJ₂, 0.1 ng/µl d8 5-(S)-hydroxycisatoeatrienoic acid (HETE), 0.1 ng/µl 15(S)-HETE, and 0.1 ng/µl d12(S)-HETE diluted in 50% HPLC grade ethanol (Fisher Scientific, Hampton, CA). Samples were loaded onto a Strata-X 33 µ Polymeric Reversed Phase column 60 mg/3 ml (Phenomenex, Torrance, CA) that was previously conditioned with HPLC grade methanol and equilibrated with HPLC grade water. Lipids were washed with HPLC grade water and eluted in HPLC grade methanol. Eluate was dried using a SpeedVac and samples were suspended in 100 µl of liquid chromatography (LC) solvent A (water-acetonitrile-formic acid (63:37:0.2, v/v/v)) for LC-MS/MS analysis. Samples were stored at −80 °C until analyzed.

Liquid chromatography and mass spectrometry. An ACQUITY UPLC BEH C18 column, 130 Å, 1.7 µm, 2.1 mm × 100 mm with in-line filter (Waters, Milford, MA) was equilibrated at 35 °C at a flow rate of 300 µl/min with LC solvent A on an ACQUITY UPLC H-Class system (Waters, Milford, MA). A small portion of each sample, 30 µl, was loaded onto the column at a flow rate of 300 µl/min of 100% LC solvent A followed by a linear gradient to 20% LC solvent B (acetoni-trile-isopropanol(50:50, v/v)) over six min, then increased to 55% LC solvent B over 0.5 min and 100% LC solvent B over 5.5 min and held for four min. Column was primed with 100% LC solvent A for three min between samples. Metabolites were directed to an ACQUITY TQ detector mass spectrometer equipped with an electrospray ionization source set to negative ion mode (Waters, Milford, MA). Specific analytes were detected via multiple reaction monitoring and quantified using a standard curve of known concentration using MassLynx software (Waters, Milford, MA). Each standard (Cayman Chemical, Ann Arbor, MI) and internal standard was resolved at the following retention time (RT) with the following mass transitions: arachidonic acid, RT 12.52 min, m/z 303 → 259; 6-keto- PGF₁α, RT 1.47 min, m/z 369 → 207; PGD₂, RT 3.42 min, m/z 351 → 189; PGE₂, RT 2.94 min, m/z 351 → 189; PGF₂α, RT 2.63 min, m/z 353 → 193; TXB₂, RT 2.12 min, m/z 369 → 169; 15-deoxy-Δ12,14-PGJ₂, RT 8.62 min, m/z 315 → 271; 5-(S)-HETE, RT 9.58 min, m/z 319 → 115; 12-(S)-HETE, RT 9.24 min, m/z 319 → 179; 15-(S)-HETE, RT 9.15 min, m/z 319 → 175; d8 arachidonic acid, RT 12.45 min, m/z 311 → 267; d4 6-keto- PGF₁α, RT 1.46 min, m/z 373 → 231; d4 PGD₂, RT 3.36 min, m/z 355 → 193; d4 PGE₂, RT 2.95 min, m/z 355 → 193; d4 PGF₂α, RT 2.51 min, m/z 357 → 197; d4 TXB₂, RT 2.13 min, m/z 373 → 173; d4 15-deoxy-Δ12,14-PGJ₂, RT 8.61 min, m/z 319 → 275; d8 5-(S)-HETE, RT 9.55 min, m/z 317 → 116. Results were further normalized to sample protein concentrations using Microsoft Excel (Microsoft, Redmond, WA).
Results

Cardiac injury triggers an elevation in PGE₂ synthesis. Prostaglandins are powerful lipid signals synthesized at the site of injury that regulate the inflammatory response. To profile cardiac prostaglandins and quantify changes associated with injury, we used Liquid Chromatography Tandem Mass Spectrometry (LC-MS/MS). In uninjured hearts, PGE₂ concentrations were significantly higher than those detected for other prostanoids. PGD₂ and 6-keto PGF₁α, a stable metabolite of prostacyclin, barely registered above background levels, while TXB₂, a Thromboxane A metabolite, and PGF₂α, were undetected (Fig. 1A). To define prostaglandin levels during regeneration, we amputated ~20% of the adult ventricle, allowed regeneration to proceed for 3 days, and extracted ventricles for analyses. At 3 days post-amputation (dpa), concentrations of PGE₂ were again, significantly higher than all other prostanoids examined (Fig. 1B). Furthermore, we found that at 3 dpa, concentrations of PGE₂ increased by more than a 60% relative to uninjured hearts (Fig. 1C). Together, these experiments identify PGE₂ as the most abundant prostanoid in the zebrafish heart, and establish an injury-induced increase in PGE₂ concentrations.

Enzymes critical to PGE₂ synthesis are upregulated in regenerating adult hearts. Having shown that PGE₂ is elevated in the heart after injury, we next asked how enzymes critical to PGE₂ synthesis are modulated during regeneration. COX enzymes catalyze the first rate-limiting step in prostanoid synthesis. Mammals have two COX isozymes, constitutively expressed COX1, and inducible COX2. Three Cox isoforms have been identified in the zebrafish, a single ortholog of mammalian COX1, and two orthologs of mammalian COX2: Cox2a and -2b.43 Downstream of the Cox enzymes, prostaglandin E synthase (Ptges), the zebrafish ortholog of mammalian PTGES, catalyzes the terminal step of PGE₂ biosynthesis44.

To characterize relative expression of the Cox enzymes, we conducted qPCR studies in uninjured and 3 dpa regenerating hearts. These assays showed that in uninjured ventricles, cox1 expression was significantly elevated relative to both cox2a (>-log, 6.5-fold) and cox2b (>-log, 2.5-fold) (Fig. 2A). At 3 dpa however, cox2a levels had increased more than log, 2.5-fold relative to uninjured hearts. No significant changes were observed in cox2b or cox1 transcripts (Fig. 2B). Mirroring the response of cox2a, expression of the terminal prostaglandin synthase ptges was also significantly upregulated (>log, 0.5-fold) after amputation injury when compared to uninjured levels (Fig. 2C).

To define the spatial distribution of cox2a and cox2b during heart regeneration, we performed in situ hybridizations on uninjured, 1, 2 and 3 dpa regenerating hearts using DIG labelled RNA probes. While cox2a expression was observed in CMs at all time points, expression became enriched within the resection injury zone at 2 and 3 dpa (Fig. 2D). This localized signal suggests potential expression in immune, epicardial and/or endocardial cells. Expression of cox2b displayed a more uniform pattern and intensity across the early stages of heart regeneration.
(Fig. 2E). These signals, however, were absent when heart sections were hybridized with cox2a riboprobe in the absence of an anti-DIG antibody (Fig. 2D) or with anti-DIG independently (Fig. 2E).

Together, these results demonstrate that the zebrafish dynamically responds to cardiac injury by upregulating both cox2a and ptges, two enzymes critical to PGE2 synthesis. Importantly, increased expression of these genes correlates directly with increased levels of PGE2 observed in ventricles subsequent to injury.

Cox2a expression is highest in epicardial cells at 3 dpa. Multiple cell types contribute to the injury-stimulated production of prostaglandins, the end products of Cox activity. To localize expression of the Cox enzymes in the injured heart, and identify potential sites of PGE2 synthesis, we utilized Fluorescence-Activated Cell Sorting (FACS) to isolate distinct cell populations for qPCR studies. CMs, epicardial cells, endocardial/endothelial cells, and macrophages were sorted from the dissociated ventricles of Tg(cmlc2:EGFP); (tcf21:DsRed), Tg(fli1a:EGFP), and Tg(mpeg1.YFP) strains, respectively (Fig. 3A–C). Purity of the sorts was validated by qPCR, which revealed a log 2 6 to 7-fold enrichment of cell-specific markers in fluorescent(+) vs. fluorescent(−) cells (Fig. 3D).

qPCR analysis showed that at 3 dpa, cox2a expression was lowest in cmlc2(+) CMs and fli1a(+) endocardial/endothelial cells. Relative to cmlc2(+) cells, we observed a trend towards increased levels of cox2a transcripts in mpeg1(+) macrophages, which have classically been associated with cox2 expression45,46. Unexpectedly, we found cox2a expression was ~log 2 2-fold higher in tcf21(+) epicardial cells relative to macrophages (Fig. 3E). Expression of cox2b, which showed a muted response to injury, was highest in fli1a(+) cells (Fig. 3F). There was no significant difference in cox1 expression among the resident cardiac cells assayed; however, Cox1 expression in mpeg1(+) cells was significantly lower than that observed in fli1a(+) and tcf21(+) cells (Fig. 3G). From these studies, we identified tcf21(+) epicardial cells as the primary site of inducible cox2a expression during the early stages of zebrafish heart regeneration.

Injury activates a differential shift of PGE2 receptor expression in the heart. Having shown that the zebrafish heart responds to injury by upregulating the expression of cox2a and downstream production of PGE2, we next examined the expression of PGE2 receptors. Mammals express four G protein-coupled PGE2 receptors, EP1, EP2, EP3, and EP4. Zebrafish orthologs of the mammalian EP1-4 receptors are Ptger1a, Ptger2a,
Ptger3, and Ptger4a, respectively. Examination of receptor expression in the uninjured heart demonstrated that the level of \( \text{ptger3} \) was higher by a log 2 factor of ~2 relative to both \( \text{ptger2a} \) and \( \text{ptger4a} \) (Fig. 4A). Ptger1a was undetected in the ventricles (data not shown).

Injury induced a differential shift in receptor expression, and at 3 dpa, \( \text{ptger2a} \) was upregulated by more than log2 0.6-fold while \( \text{ptger3} \) was downregulated by more than log2 1-fold relative to uninjured hearts. We observed no significant change in expression for \( \text{ptger4a} \) (Fig. 4B).

In situ hybridization studies revealed \( \text{ptger2a} \) expression was similar to the spatial dynamics of \( \text{cox2a} \) levels. \( \text{Ptger2a} \) was expressed throughout the heart and enriched within the wounded apex in 1, 2 and 3 dpa hearts (Fig. 4C). These results demonstrate that in the zebrafish, cardiac injury activates a dynamic shift in PGE2 receptor expression, upregulating the proliferation-associated EP2 ortholog \( \text{ptger2a} \), while downregulating the functionally opposing receptor, \( \text{ptger3} \).

**Activation of the Cox2-PGE2 circuit stimulates cardiomyocyte proliferation.** PGE2 has been shown to promote cell proliferation in multiple contexts. To better understand the impact of Cox2 activity on PGE2 synthesis and CM proliferation, we subjected animals to ventricular amputation and treated them with either NS-398, a small molecule shown to selectively inhibit Cox2 activity in the zebrafish\(^47\), or DMSO control. At 3 dpa, ELISA assays demonstrated that PGE2 concentrations were reduced by more than 47% in NS-398 treated hearts relative to controls (Fig. 5A). To determine effects of suppressed Cox2 activity upon heart regeneration, we quantified CM proliferation indices at 3 dpa, a time during regeneration previously demonstrated to exhibit high levels of proliferative activity\(^48\). Immunostaining for Myocyte enhancer factor −2 (Mef2-green), a nuclear CM marker, and Proliferating cell nuclear antigen (Pcna-red), a nuclear marker for proliferating cells, showed that NS-398 treatment suppressed CM proliferation by approximately 70% relative to controls at 3 dpa (Fig. 5B–D).
To confirm these findings, we examined the effects of another selective Cox2 inhibitor, Celecoxib. At 3 dpa, Celecoxib treatment also had a significant effect, reducing CM proliferation indices by almost 50% relative to controls (Fig. 5E). Collectively, these studies demonstrate that, in the injured zebrafish heart, Cox2 activity drives both PGE2 synthesis and CM proliferation during the early stages of heart regeneration.

Discussion

Prostaglandin E2 (PGE2) is a potent inflammatory mediator, with pleiotropic effects. In the present study, we have identified a pro-regenerative role for PGE2 during cardiac regeneration in adult zebrafish. After ventricular amputation, the injured zebrafish heart revealed increased levels of PGE2, as well as upregulated expression of cox2a and ptges, two enzymes critical to PGE2 synthesis (Figs. 1, 2). Importantly, pharmacologic inhibition of Cox2 activity suppressed both PGE2 and CM proliferation, supporting a crucial role for the Cox2-PGE2 circuit in initiating the regenerative response (Fig. 5).

In addition to the wound environment, we unexpectedly identified the epicardium as a potential source of inflammation-associated prostaglandin signaling in the regenerating heart. During the inflammatory response, inducible Cox2 has canonically been associated with immune cells recruited to the site of injury, notably macrophages19. However, during conditions of homeostasis, Cox2 is constitutively maintained in some tissues including the vascular endothelium, where it supports the synthesis of prostanoid vasodilators49,50. The zebrafish genome encodes two Cox2 genes, Cox2a and Cox2b. During regeneration, FACS studies revealed expression of the most inducible Cox enzyme, cox2a, was highest in the epicardium, while more constitutively expressed Cox2b was most prominent in endocardial/endothelial cells (Fig. 3). These findings raise the intriguing possibility that Cox2a and Cox2b have divergently evolved in specific tissues, such that Cox2b is expressed in endothelial cells to maintain vascular tone, while in the epicardium, Cox2a facilitates a rapid inflammatory response.

An emerging role for the epicardium as a source of inflammatory signaling has been highlighted in both mammalian and zebrafish models. In mice, epicardial cells were found to be a source of YAP-mediated IFNγ production following myocardial infarction, orchestrating the recruitment of immune-suppressive regulatory T cells (Tregs)43. PGE2 has been implicated in both YAP activation36, and Treg recruitment37, suggesting that in the epicardium, crosstalk between these pathways could mediate the inflammatory response. Our current work supports this study, identifying the epicardium as a potential locus of reparative, inflammatory signaling.

In comparison to our qPCR studies (Figs. 2B and 4B), in situ hybridizations revealed more uniform expression for cox2a, −2b and ptger2a in response to injury (Figs. 2D,E and 4C). One potential explanation for the expression differences observed between these two methodologies is their relative sensitivity. Highly sensitive qPCR assays are more likely to amplify subtle differences in transcript levels, compared to the semi-quantitative nature of in situ hybridizations. Interestingly, we also noted that while in situ studies identified cox2a transcripts in both injury and remote zones of the adult heart, CM proliferation appeared to be limited to the wound area (Fig. 5B). It is possible this localized proliferation may be attributable to additional factors, either related, or unrelated to Cox2 activity, that are spatially restricted during regeneration.
In the downstream PGE\(_2\) signaling circuit, we found that PGE\(_2\) receptor expression was dynamically modulated in the regenerating heart. PGE\(_2\) signals through the autocrine and paracrine activation of four G protein-coupled receptors to initiate diverse downstream pathways. Activation of EP2, the ortholog of zebrafish Ptger2a, directly promotes cell proliferation in the context of regeneration and cancer\(^{23,51}\), polarizing neutrophils and macrophages towards a reparative phenotype\(^{18,19}\). By contrast, EP3, the ortholog of zebrafish Ptger3, appears to activate pathways in opposition to EP2, inhibiting cell proliferation\(^{25,26}\) and exacerbating pathologic inflammation\(^{13,52}\). Our profiling studies of PGE\(_2\) receptor expression revealed that injury drives the upregulation of \(\text{ptger2a}\) and reciprocal downregulation of \(\text{ptger3}\) in cardiac tissues (Fig. 4), suggesting that in the zebrafish heart, PGE\(_2\) signaling is directed towards a restorative response.

Whether modulation of the PGE\(_2\) circuit after injury has an enduring impact on cardiac regeneration remains an open question. Our work showed that in the regenerative zebrafish heart, injury triggers the upregulation of genes critical to PGE\(_2\) synthesis (Fig. 2). We furthermore demonstrated that activation of the PGE\(_2\) signal is essential to stimulate CM proliferation, a major driver of heart regeneration (Fig. 5).

In other model systems, PGE\(_2\) has been shown to directly promote the proliferation of multiple cell types, including skeletal muscle stem cells\(^{28}\) and primary human CMs\(^{15}\). However, a large body of work also supports a role for PGE\(_2\) in governing the recruitment, retention, and pro-regenerative polarization of neutrophils, macrophages, and T cells\(^{17,38,35}\). Therefore, it is likely that PGE\(_2\) stimulates CM proliferation directly, as well as indirectly, through orchestrating restorative immune cell activity. This study, documenting the injury-induced modulation of PGE\(_2\) signaling during cardiac regeneration, seeds the field for future research to define the mechanisms through which PGE\(_2\) promotes the early, regenerative response.
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Acknowledgements

We thank Stephanie Anderson, Ari Dehn, Karlee Markovich, Austin Orecchio and Anne Yu for all zebrafish animal care, Ken Poss for providing the Tg(cmlc2:GFP), Tg(tcf21:Dsred) and Tg(fli1a:GFP) reporter strains. William Schott at the Jackson Laboratory provided the technical support for FACS experiments. The Yin laboratory research on heart regeneration is supported by grants from the National Institute of General Medical Sciences (P20 GM104318, P20 GM103423), the American Heart Association Scientist Development Grant (11SDG7210045) and the MacKenzie Foundation (17003).

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Competing interests

The authors declare no competing interests.

Additional information

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