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Prolonged neutrophil retention in the wound impairs zebrafish heart regeneration after cryoinjury

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ABSTRACT

Neutrophils are the first line defenders in the innate immune response, and rapidly migrate to an infected or injured area. Recently, bidirectional migration of neutrophils to the wound and the corresponding functions have become popular research pursuits. In zebrafish larvae, CXCR1/CXCL8 is the predominant chemoattractant pathway to recruit neutrophil to wound, while CXCR2/CXCL8 pathway mediate neutrophil dispersal in wound after injury. Here, we found that both CXCR1/CXCL8 and LTB4/BLT1 signals are activated in zebrafish heart after cryoinjury. And with a CXCR1/2 selective inhibitor (SB225002) treatment, the recruitment of neutrophils was not affected, but reverse migration of neutrophils was inhibited after cryoinjury of heart. We suggested that the neutrophil recruitment to cryoinjured area might be mediated by LTB4/BLT1 signals at the presence of SB225002. Therefore, SB225002 treatment resulted more accumulation and long retention of neutrophils in the injured heart. The long retention of neutrophils in the wound promoted revascularization in the injured heart; however, the AKT/mTOR pathway was inhibited and the regeneration was impaired. Our findings suggest that retention of neutrophils is a well-orchestrated process and might regulate regeneration by the AKT/mTOR pathway.

1. Introduction

Neutrophils act as one of the first inflammatory cells in removing infection or harmful agents in response to tissue injury or pathogen invasion [1]. It is widely believed that migration and retention of neutrophils to the injured or infected tissue is directly or indirectly orchestrated by chemokines, such as CXCL8, LTB4, etc. [1,2]. Neutrophils in zebrafish share many characteristics with mammalian neutrophils, such as similar morphology, biochemistry and functional features. The zebrafish provides several advantages for studying neutrophil migration, such as transparent larvae, easy genetic manipulation, and many transgenic lines to utilize [3,4]. The chemokine CXCL8 is one of the most potent and best-studied neutrophil chemoattractants in both humans and zebrafish [2,5]. In zebrafish larvae, it is reported that CXCR1/CXCL8 mediates neutrophil recruitment to the fin wound, and CXCR2/CXCL8 mediates neutrophil recruitment to infection. Additionally, neutrophil reverse migration is regulated through the CXCR2/CXCL8 signal pathway [2,6,7] and crosstalk with macrophage redox signaling [8]. These observations suggest that the mechanism of neutrophil recruitment varies by signal pathway. However, both the recruitment and dispersal of the neutrophil can be inhibited by CXCR1/2 selective inhibitor (SB225002) [2,6,9]. Moreover, LTB4 is an additional neutrophil chemoattractant in zebrafish, and the BLT1/LTB4 signal is independent of the CXCR1/CXCL8 pathway recruiting neutrophils [2,10–12].

Neutrophils are predominately phagocytic and produce reactive oxygen species (ROS) and cytotoxic granule components which can eliminate infection [3]. Besides their role in phagocytosis, mature neutrophils contain many proteins including growth factors or pro-angiogenic factors, which can be rapidly released after activation and directly contribute to tissue repair and angiogenesis [13]. Interestingly, heart function is worsened and mice progressively develop heart failure after myocardial infarction when neutrophils are absent [14]. Neutrophils are also necessary for the blastemal formation in axolotl limb

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Abbreviations

IA	injured area
PCNA	Proliferating cell nuclear antigen
embCMHC	embryonic cardiomyocyte heavy chain
TUNEL	Terminal deoxynucleotidyl transferase dUTP nick end labeling
DMSO	Dimethyl sulfoxide
qRT-PCR	quantitative real time polymerase chain reaction
ECM	Extracellular matrix
Tnf-α	Tumor necrosis factor-α
Il-1β	Interleukin 1 beta
LTB4	Leukotriene B4
BLT1	leukotriene B4 receptor 1
ROS	Reactive Oxygen Species
MI	Myocardial infarction
Dpc	Days post cryoinjury
alox5a	arachidonate 5-lipoxygenase activity
CXCL	CXC chemokine ligand
CXCR	CXC chemokine receptor
AKT	Protein kinase B
mTOR	mechanistic target of rapamycin
vegf	vascular endothelial growth factor
mpx	myeloperoxidase
mepg	macrophage-expressed gene
NRG1	Neuregulin 1
HRP	Horseradish peroxidase
PVDF	polyvinylidene difluoride

regeneration [15]. However, excessive neutrophils in tissue can result in chronic inflammation, such as in atherosclerosis, MI, autoimmune disease, and cancer [6,16]. In zebrafish larvae and mouse, inhibition of neutrophils in the wound accelerates regeneration in the injured fin and liver, respectively [2,17,18]. In our previous study, we demonstrated that neutrophils are rapidly recruited to the wound after injury, peak at 1 day post cryoinjury (dpc) and then gradually disperse by 4 dpc [19]. Additionally, we found excessive leukocyte accumulation in the wound promoted apoptosis and prevented regeneration in cryoinjury heart [20]. However, the dispersal of neutrophils is mainly through “reverse migration” and “apoptosis” in zebrafish and mammals, respectively. The dispersal of neutrophils in the tissue is critical for wound healing and maintenance of tissue homeostasis [21]. Neutrophils rapidly infiltrate the ischemic border zone of the ischemia/reperfusion (I/R) injury site producing ROS and other inflammatory cytokines, resulting in acute inflammation, cardiomyocyte apoptosis, and worsening heart functioning. A high number of neutrophils retention in heart is considered detrimental for patients with MI [22], and is an indicator of adverse clinical outcomes in patients with acute coronary syndrome [23,24]. However, the mechanism of neutrophil mediated-inflammation worsening cardiac function after MI is not clear.

In the present study we demonstrate that inhibition of the CXCR1/2/CXCL8 signal pathway using SB225002 didn't impair neutrophils recruitment to the cryoinjured area. However, we find retention of neutrophils in the wound was prolonged. The expression of *cxcl8, cxcr1, cxcr2, alox5a, blt1* was induced after cryoinjury, suggesting that BLT1/LTB4 signals might be another alternative pathway independent of the CXCR1/2/CXCL8 mediated neutrophil recruitment to the wound after heart cryoinjury. Here we found the prolonged retention of neutrophils in the wound promoted revascularization. Finally, we revealed that the AKT/mTOR signal pathway is inhibited and heart regeneration is blocked with prolonged retention of neutrophils in the injured area.

2. Materials and methods

2.1. Zebrafish maintenance

Zebrafish wild-type (AB) strain, *Tg* (*fli1a*: eGFP) and *Tg* (*coro1a*: GFP; *lyz*: Dsred) were maintained at The City University of Hong Kong fish facility at 28 ± 1 °C under a 14:10 light/dark cycle [20]. All experimental procedures and animals in the present study were approved by the Department of Health, Hong Kong, SAR, China (refs (14–118) in DH/HA&P/8/2/5 Pt.3). The experiments were conducted in accordance with the relevant guidelines and regulations in Hong Kong, SAR, China.

SB225002 (SML0716; Sigma-Aldrich) was dissolved in DMSO at 20 mM and stored at −20 °C. Subsequently, SB225002 was diluted to 5 μM in the fish water just prior to use [2,6,9]. Fish water and the inhibitor SB225002 were changed once a day.

2.2. Cryoinjury

Zebrafish (12–15 months old) were anesthetized with 0.05% Tricaine (E10521; Sigma-Aldrich) and placed on a moist sponge for surgery. The ventricle was exposed and subjected to cryoinjury using methods described by Chablais et al. [25]. Immediately after injury, the fish were put in the fish water with 5 μM SB225002 or 0.25% DMSO as the vehicle control.

2.3. Histological techniques

Zebrafish were sacrificed with an overdose of tricaine treatment, and the heart was collected at different timepoints. The hearts were fixed with 4% PFA at room temperature for 2 h, and paraffin sections were prepared. The injured area of the ventricle was determined by Picrosirus red staining (ab150681, Abcam) and percentage of scar volume in the ventricle was calculated as described previously [19].

2.4. Immunofluorescence and TUNEL staining

Following deparaffinization and rehydratization, antigen retrieval was performed by heating the sections in 10 mM sodium citrate buffer (0.05% Tween 20, pH 6.0) at 95 °C for 15 min. Non-specific binding sites were blocked by incubating the sections in blocking buffer (5% BSA, 10% goat serum, 0.5% Tween-20, 0.5% Triton X-100) for 1 h at room temperature. The primary antibodies used in the study were mouse anti-PCNA (sc-56; Santa Cruz), rabbit anti-PCNA (sc-7907; Santa Cruz), rabbit polyclonal anti-GFP (ab13970; Abcam), mouse anti-vimentin (ab8978; Abcam), mouse anti-RFP (ab62341; Abcam), mouse anti-myosin heavy chain (MF-20; DSHB), and mouse anti-embCMHC (N2.261; DSHB), anti-Collagen Type I (SP1.D8, DSHB). The secondary antibodies used were Cy3-conjugated goat anti-mouse (A10521; Invitrogen) or Alexa Fluor 488-conjugated goat anti-rabbit (A11034; Invitrogen) antibodies. Apoptotic cells were determined by TUNEL staining using the DeadEnd™ Fluorometric TUNEL System kit (G3250; Promega) according to the manufacturer's instructions [20]. Nuclei were stained with DAPI. Images were captured using an Olympus BX61 microscope.

2.5. qRT-PCR

Total RNA extraction and qRT-PCR were performed as described previously [20]. The expression of each gene was analyzed in four biological replicates using $2-\Delta\Delta Ct$ method. The primer sequences of each genes are listed in Table 1.

2.6. Western blotting

Three zebrafish hearts were pooled together and treated with RIPA

Table 1
qRT-PCR primer sequences of the genes.

tnf-α-F	ACAAGGCAATTTCACTTCCA
tnf-α-R	AGCTGATGTGCAAAGACACC
il-1β-F	TTGTGGGAGACAGACAGTGC
il-1β-R	GATTGGGGTTTGATGTGCTT
β-actin-F	GCTGACAGGATGCAGAAGGA
β-actin-R	TAGAAGCATTTGCGGTGGAC
mpx-F	AGAACCATCCTCAGTTTCCCAGT
mpx-R	CCAATCAATCCACGGAGCA
blt1-F	GGGAGTTTGGACCTGTCGTT
blt1-R	TGGTTTGGTAACAGCGAGCC
cxcr1-F	TTCAGTTCGGCTGCACTATG
cxcr1-R	GGAGCAACTGCAGAAACCTC
cxcr2-F	TGACCTGCTTTTTTCCCTCACT
cxcr2-R	TGACCGGCGTGGAGGTA
mpeg-F	GTGAAAGAGGGTTCTGTTACA
mpeg-R	GCCGTAATCAAGTACGAGTT
alox5a-F	CCAGCTACACGGTGACTGTT
alox5a-R	TATGAGTCCACCGCTCCTCT
vegf-c-F	GGCCTCAACAGAGCTTCAAC
vegf-c-R	TCTCTTGGGGTCCACGTTAC

Lysis and Extraction Buffer (89901, Thermo scientific) according to manufacturer's instructions. Four biological replicates were performed. Protein concentration was determined with the BCA Protein Assay kit (23225; Thermo Scientific). A sample of 10 μg protein was separated on 10% SDS-PAGE gel, and transferred onto polyvinylidene difluoride (PVDF) membrane (10600023; GE Healthcare life science). Membranes were blocked with 5% no fat milk in PBST (0.05% Tween 20 in PBS), incubated in the appropriate primary antibody overnight at 4 °C, followed by incubation in the corresponding HRP-conjugated secondary antibody at room temperature for 2 h. Proteins were detected with EMD Millipore Luminata Western HRP chemiluminescence substrate (WBLUF0500; Millipore). The following primary antibodies were used: mouse anti-GAPDH (60004–1; Proteintech Group, Inc.) at 1:10000, rabbit anti-NRG1 antibody (ab27303, Abcam), rabbit anti-Foxp3 antibody (GTX16590, Gentex), rabbit anti-Akt (pan) (C67E7) (4691, CST), rabbit anti-Phospho-Akt (Ser473) (D9E) (4060, CST), rabbit anti-Phospho-mTOR (Ser2448) (2971, CST), rabbit anti-Caspase-3 (9662, CST). For secondary antibodies, HRP-conjugated rabbit anti-mouse IgG (AP160P; Millipore), and HRP-conjugated goat anti-rabbit IgG (AP307P; Millipore) were used.

2.7. Statistical analysis

Angiogenesis was measured by quantification the ratio of GFP expression area to the injured area of the ventricle. The expression of vimentin, embCMHC, Collagen Type I was determined by quantification the ratio of the expression area of these proteins to the injured area with Image J. The hue value of the band in Western blotting was quantified using Image J, and the expression level of the protein in control group was normalized to 1 using GAPDH. Statistical analysis was calculated using two-tailed *t-test* (Microsoft Excel 2013, GraphPad prism 8), and a p value < 0.05 was considered statistically significant. Data are presented as means ± S.D. (standard deviation).

3. Results

3.1. SB225002 prolonged neutrophil retention in the wound after cryoinjury

The CXCR1/2 selective inhibitor (SB225002) has been shown to inhibit neutrophil recruitment into the wound of the zebrafish larvae fin after amputation by the CXCR1/CXCL8 signal pathway, and inhibit reverse migration by the CXCR2/CXCL8 signal pathway [2,6,9]. Recently, we demonstrated neutrophils rapidly migrate into the cryoinjured area of the heart at 1 dpc [19]. Therefore, we wanted to know whether SB225002 could inhibit neutrophil recruitment into the wound of the heart after cryoinjury. To investigate this hypothesis, the fish of *Tg* (*coro1a*: GFP; *lyz*: Dsred) were subjected to heart cryoinjury with concurrent SB225002 treatment. Subsequently, the number of neutrophils in the wound at 1 dpc was quantified. Unexpectedly, our immunofluorescent results revealed that SB225002 treatment did not block recruitment of neutrophils into the injured area of the heart at 1 dpc (Fig. 1A and B). Instead, the number of neutrophils in the wound of SB225002 treated fish was slightly higher than the control group (albeit non-significant), and the number of macrophages was lower than the control group (Fig. 1A–C). To confirm this observation, the expression of *mpx* and *mpeg*, markers of neutrophil and macrophages, respectively, were quantified. Results of the qRT-PCR analysis showed expression of *mpx* was upregulated, and *mpeg* was downregulated in the SB225002

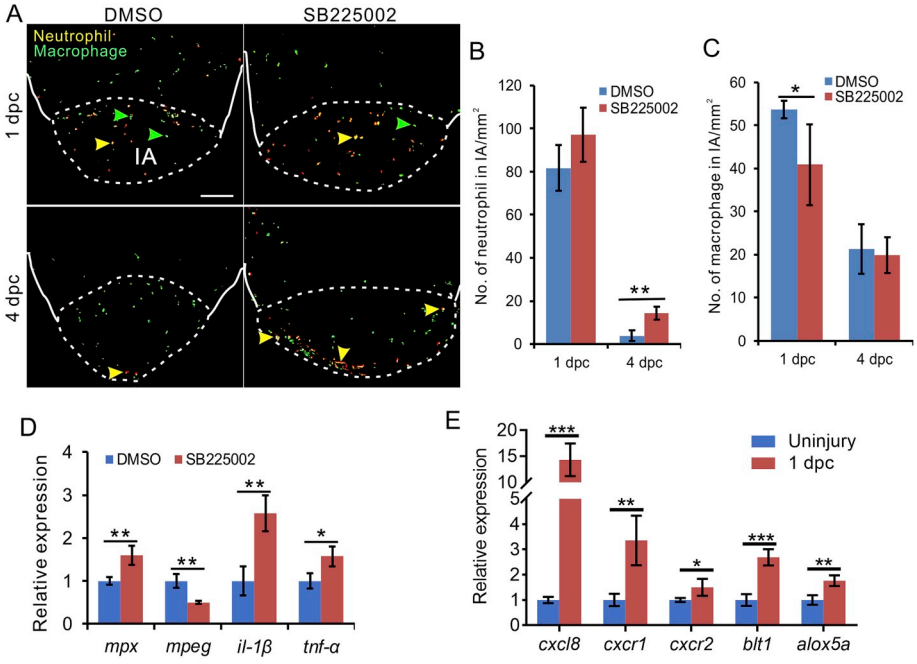

Fig. 1. SB225002 inhibited neutrophil reverse migration but not recruitment in zebrafish heart after cryoinjury. **A**. Representative immunofluorescent images showing neutrophils (yellow, yellow arrowhead) and macrophages (green, green arrowhead) in the injured area (IA, dashed line bounded area) of the heart at 1 and 4 dpc with DMSO and SB225002 treatment. Three times were performed of the experiment. Scale bar: 100 μm. **B, C**. Bar chart showing the quantification of neutrophils (B) and macrophages (C) in panel A, using a two-tail *t-test*. Asterisk represents a significant difference at $p < 0.05$ (*) or $p < 0.01$ (**) level (n = 5–6). **D, E**. qRT-PCR bar chart result showing the relative gene expression in the heart of DMSO and SB225002 treated fish (D), and in the heart of fish with uninjury and cryoinjury heart at 1 dpc (E) using a two-tail *t-test*. Asterisk represents a significant difference was observed at $p < 0.05$ (*), $p < 0.01$ (**) or $p < 0.001$ (***) level (n = 4). (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

treated group, consistent with the previous immunofluorescent results in Fig. 1A (Fig. 1D).

We have previously demonstrated neutrophil accumulation in the wound at 1 dpc is rapidly dispersed by 4 dpc [19]; however, the SB225002 inhibitor did not block neutrophil recruitment at 1 dpc in the cryoinjured heart. Therefore, we wanted to know whether the SB225002 inhibitor affects neutrophil reverse migration. We investigated the number of neutrophils in the wound at 4 dpc. Here, the number of neutrophils in the cryoinjured heart of SB225002 treated fish was significantly more than in control fish (Fig. 1A). Interestingly, macrophages were unaffected. These results suggest SB225002 inhibits reverse migration of neutrophils in the cryoinjured heart, hence, prolongs accumulation and retention of neutrophils in the cryoinjured area, and results in higher expression of pro-inflammatory cytokines *il-1β* and *tnf-a* in the heart (Fig. 1D). This pathogenesis is similar to human chronic inflammation after MI.

In order to explain why SB225002 can not inhibit the neutrophil recruitment into the cryoinjured area of zebrafish heart, we first investigated the expression of *cxcl8* and *cxcr1*, which mediate neutrophil recruitment in the fin wound [6]. Similar to fin amputation [9], the qRT-PCR results indicate that the expression of *cxcl8* and *cxcr1* were strongly induced at 1 dpc. Since SB225002 is a CXCR1/2 selective inhibitor, and did not inhibit neutrophil recruitment in our study, we hypothesized that there were additional signals which mediate neutrophil recruitment in the cryoinjured heart. Subsequently, BLT1/LTB4 was investigated due to its importance in neutrophil recruitment in zebrafish [2,7]. We investigated the expression of *alox5a*, which is involved in the biosynthesis of LTB4 [26], and *blt1* using qRT-PCR. Our results indicate that *blt1* and *alox5a* were highly expressed in the heart after cryoinjury (Fig. 1E). This result suggests that BLT1/LTB4 might be another pathway of neutrophil recruitment in the wound of the cryoinjured heart.

3.2. SB225002 treatment promoted revascularization in the cryoinjured area of heart

It has been widely reported that neutrophils play an important role in angiogenesis by releasing proangiogenic factors (i.e. VEGF) in chronic inflammatory diseases and in tumors [27,28]. Thus, we wondered whether SB225002 induced prolonged neutrophil retention in the wound could promote angiogenesis in the cryoinjured heart. To ascertain this relation, *Tg* (*fli1a*: eGFP) zebrafish were subjected to cryoinjury and blood vessels were visualized using anti-GFP staining at 7 dpc in both SB225002 treated and DMSO control fish groups. We found new blood vessel density in the SB225002 treated group was increased compared to the control group (Fig. 2A and B). Additionally, the percentage of double positive cells ($GPF^+/PCNA^+$), which represent proliferating endothelial cells, was significantly higher in the SB225002 treated group compared to the DMSO control group (Fig. 2C). Furthermore, we determined the expression of *vegf-c*, due to VEGF gene expression levels peaking at 1 dpc in the heart [29]. The qRT-PCR results demonstrate that *vegf-c* expression was higher in the cryoinjured heart of SB225002 treated fish compared to DMSO control fish at 1 dpc (Fig. 2D). Thus, these results reveal that SB225002 promotes revascularization in the injured area of the heart.

3.3. Prolonged neutrophil retention induced apoptosis in the injured area

SB225002 inhibits reverse migration of neutrophils, which leads to neutrophil accumulation in the injured area and a high expression of pro-inflammatory cytokines, such as *tnf-a* and *il-1β* (Fig. 1). It has been reported that high levels of *tnf-a* and *il-1β* expression in zebrafish induce excessive apoptosis [20,30]. Thus, we quantified apoptotic cells in the injured area of the heart at 1 dpc and 7 dpc in both SB225002 treated and DMSO control fish groups using TUNEL staining. Our results show that the number of apoptotic cells in the SB225002 treated group was significantly higher than in the DMSO control group at 7 dpc; however, 1 dpc was non-significant (Fig. 3A and B). Subsequently, the level of Caspase 3 expression was investigated in the heart using Western blot, as this protein has been shown to mediate the apoptotic cascade in zebrafish [31]. The results reveal that the level of Caspase 3 expression was markedly increased in the heart of the fish treated with SB225002 at 7 dpc (Fig. 3C).

3.4. SB225002 inhibited heart regeneration after cryoinjury

Neutrophils are necessary for orchestrating wound healing by promoting macrophage polarization in the mammalian heart after MI [14]. Furthermore, the accumulation of neutrophils in the ischemic border zone after ischemia/reperfusion (I/R) injury results in inflammation and deterioration in cardiac tissue and function [22]. Thus, we wondered whether prolonged retention of neutrophils in the injured area affects heart regeneration. First, we assessed the expression of embryonic cardiomyocyte heavy chain (embCMHC), which is an important marker in zebrafish heart regeneration [32]. The immunofluorescence results demonstrate that SB225002 inhibits the expression of embCMHC in the border zone of the cryoinjured area of the heart (Fig. 4A and B). In agreement with this observation, we found that proliferating embCMHC (double positive $embCMHC^+$ and $PCNA^+$) was significantly less in the heart of SB225002 treated fish compared to

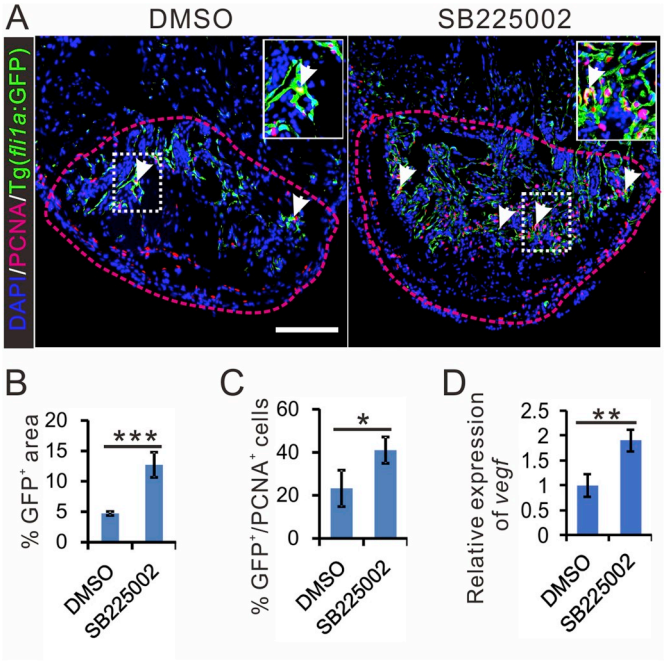

Fig. 2. SB225002 treatment promoted angiogenesis in the heart after cryoinjury. A. Blood vessels were visualized in heart of *Tg* (*fli1a*: eGFP) fish by anti-GFP immunostaining, and the proliferating endothelial cells were double labeled with GFP (green) and PCNA (red). Representative images showing blood vessels (green) and proliferating endothelial cells ($GFP^+/PCNA^+$, white arrow) in the injured area. Scale bar: 100 μm. Three times were performed of the experiment. The white square in the right corner of each image exhibits the magnification of the corresponding dash line area in the main images. **B, C.** Bar charts showing the quantification of blood vessel density (B) and percentage of proliferating endothelial cells (C) in panel A. Asterisk means a significant difference was observed between SB225002 treated and DMSO control fish groups at $p < 0.05$ (*) or $p < 0.001$ (***) level with a two-tail *t-test* ($n = 6$). **D.** qRT-PCR bar chart result showing relative gene expression of *vegf-c* in the heart of DMSO and SB225002 treated fish at 1 dpc. Asterisk (**) represents a significant difference was observed at $p < 0.01$ level with a two-tail *t-test* ($n = 4$). (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

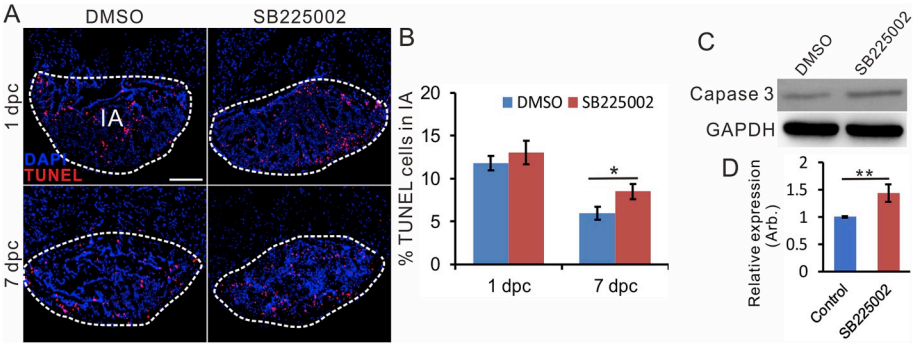

Fig. 3. SB225002 induced excessive apoptosis in the cryoinjured heart. A. Representative images showing apoptotic cells (pink) in the injured area (IA) of heart at 1 dpc and 7 dpc in both SB225002 treated and DMSO control fish using TUNEL staining. Scale bar:100 μm. Two times were performed of the experiment. **B.** Bar charts showing the percentage of apoptotic cells in panel A. Asterisk (*) represents a significant difference was observed at $p < 0.05$ level with a two-tail *t-test* (n = 6). **C.** Western blot showing the level of expression of Caspase 3 in the heart at 7 dpc, with GAPDH as the reference. Two times were performed of the experiment. **D.** Bar chart showing the quantification of the relative expression of Caspase 3 in panel C, and the expression of Caspase 3 in control group was normalized to 1 using GAPDH. Asterisk (**) represents a significant difference was observed at $p < 0.01$ level with a two-tail *t-test* (n = 4). (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

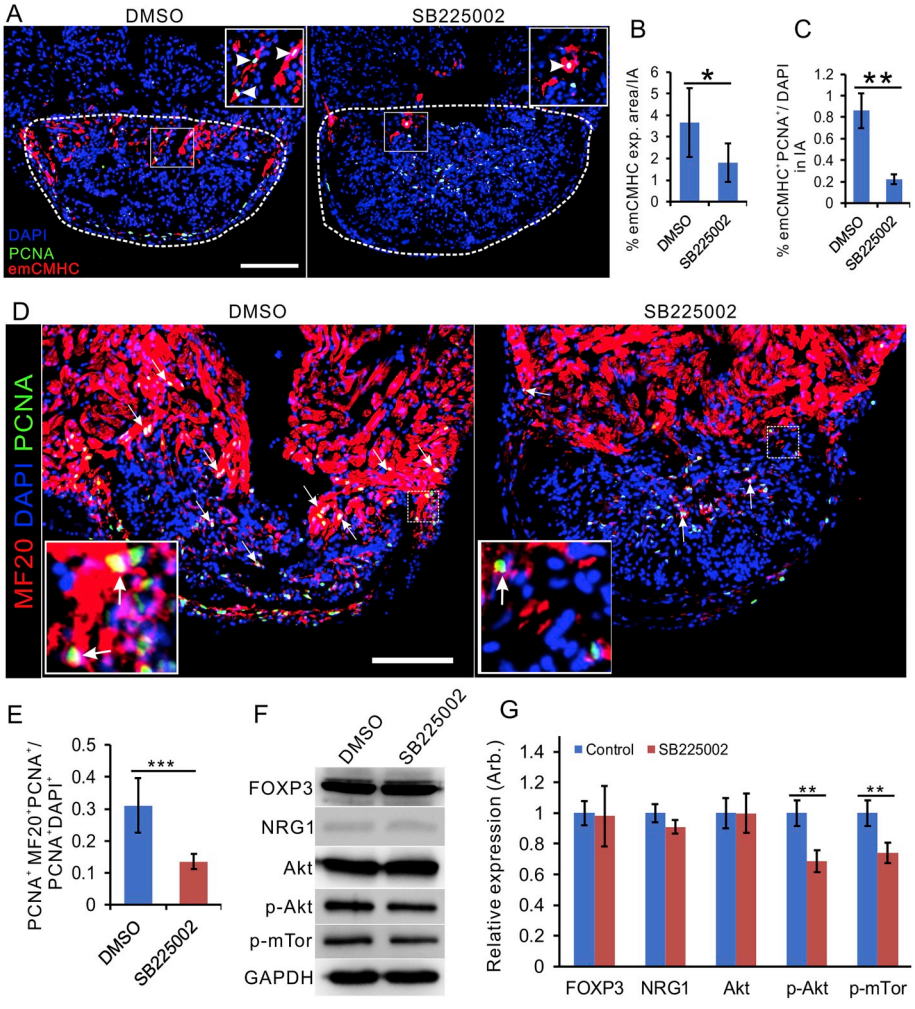

Fig. 4. SB225002 inhibited proliferating embCMHC and cardiomyocytes by the AKT/mTOR signal pathway. A. Representative images showing the expression of embCMHC (red) and PCNA (green) in the heart of both SB225002 treated and DMSO control fish at 7 dpc. White arrowheads indicating proliferating embCMHC. Scale bar: 100 μm. **B, C.** Bar chart showing the quantification of the expression of embCMHC (B) and the percentage of proliferating embCMHC (C) in panel A. Asterisk represents a significant difference was observed at $p < 0.05$ (*) or $p < 0.01$ (**) level with two-tail *t-test* (n = 6). **D.** Representative images showing proliferating cardiomyocytes (white arrow, positive $MF20^+/PCNA^+/DAPI^+$ cells) in the heart of both SB225002 treated and DMSO control fish at 7 dpc. Scale bar: 100 μm. Two times were performed for the experiment A and D. **E.** Bar chart showing the quantification of proliferating cardiomyocytes in the border (within 100 μm) of the injured area in panel A. Asterisk (***) represents a significant difference was observed at $p < 0.001$ level with two-tail *t-test* (n = 6). **F.** Western blot showing the level of expression of each protein in the heart of SB225002 treated and DMSO control fish at 7 dpc, with GAPDH as the reference. And two times were performed of the experiment. **G.** Bar chart showing the quantification of the relative expression of proteins in panel F, and the expression of proteins in control group was normalized to 1 using GAPDH. Asterisk (**) represents a significant difference was observed at $p < 0.01$ level with a two-tail *t-test* (n = 4). (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

DMSO control fish (Fig. 4A, C). Subsequently, proliferating cardiomyocytes were investigated in both SB225002 treated and control groups, as they are believed to restore lost or damaged tissue in the injured heart [33]. We found the number of proliferating cardiomyocytes, double labeled PCNA and MF20, was significantly reduced by SB225002 (Fig. 3D and E).

In order to investigate the mechanism involved, the expression of FOXP3 and NRG1 was assessed, which has been reported to play a crucial role in cardiomyocyte proliferation [34,35]. However, no clear difference of the expression level of FOXP3 and NRG1, between the heart of SB225002 treated and control fish was noted (Fig. 4F). Subsequently, AKT/mTOR signaling, which also plays a key role in regulating cardiomyocyte proliferation [36,37], was examined in the heart of SB225002 treated fish after cryoinjury. Our data reveal that SB225002 inhibits the expression of p-Akt and p-mTor, but not pan-Akt, in the cryoinjured heart. These results suggest that the AKT/mTOR signal pathway was blocked by SB225002 in the heart after injury.

One of the most serious consequence of MI is formation a permanent collagen-rich scar which impairs heart function and even results in heart failure [38]. In zebrafish, the collagen-rich scar is transiently formed and then degraded by a regenerating process [25,39]. Thus, we asked if scar formation and regression were affected by SB225002.

First, the accumulation of vimentin positive fibroblasts, which are involved in extracellular matrix production in the injured heart [39], was investigated in the injured area at 7 dpc. We found that the accumulation of vimentin positive fibroblasts was remarkedly increased in the injured heart of SB225002-treated fish compared to control fish (Fig. 5A and B). Collagen is a prominent extracellular matrix component produced by fibroblasts in the wound after injury [39]. In accordance with this observation, collagen deposition in the injured area of the heart in SB225002 treated fish was significantly more than in control fish (Fig. 5C and D). Additionally, there was a larger collagen rich scar in the heart of fish treated with SB225002 compared to control fish at 30 dpc (Fig. 5E and F). Collectively, our data demonstrate that SB225002 blocked scar degradation after cryoinjury.

4. Discussion

Tissue injury followed by an inflammatory response plays a crucial role in wound healing and regeneration. Increasingly, studies show inflammatory cells, such as macrophages and neutrophils, determine scaring or regeneration after injury or physiological diseases (i.e. MI) [40]. On the one hand, neutrophils involve in regulating macrophages polarized to towards a reparative phenotype, which is essential to wound healing; and inhibition the neutrophil recruitment via CXCR1/CXCL8 signals blocked tissue regeneration [14,15]. One the other hand, neutrophils exert harmful effects on MI and tissue regeneration, exacerbating cardiac function by eliciting acute and chronic inflammation [41]. However, the mechanism behind neutrophils in heart function deterioration after MI is not clear. Here, we showed the CXCR1/2 selective inhibitor SB225002 does not block neutrophils recruitment into the wound of zebrafish heart after cryoinjury, but prevent reverse migration during the dispersal phase, hence prolonging neutrophil retention in the infarcted area of the heart. The prolonged neutrophil retention in the wound contributed to angiogenesis, apoptosis, reduction of cardiomyocyte proliferation and AKT/mTOR signals; therefore, blocking heart regeneration (Fig. 6).

In zebrafish larvae, CXCR1/CXCL8 and CXCR2/CXCL8 mediate neutrophil recruitment to the wound in fin amputation and infection, respectively; additionally, CXCR2/CXCL8 regulates neutrophil reverse migration [6]. Using the selective CXCR1/2 inhibitor 5 μM SB225002, neutrophil recruitment to the fin wound and infection site in zebrafish larvae is blocked [2,6,7,9]. For example, SB225002 inhibits neutrophil recruitment into the damaged limb in axolotl [15]. However, our study shows that SB225002 does not affect recruitment of neutrophils into the cryoinjured area of the heart, although the expression of *cxcl8*, *cxcr1*, *cxcr2, blt1* and *alox5a* (synthesis LTB4) was induced (Fig. 1). It is known

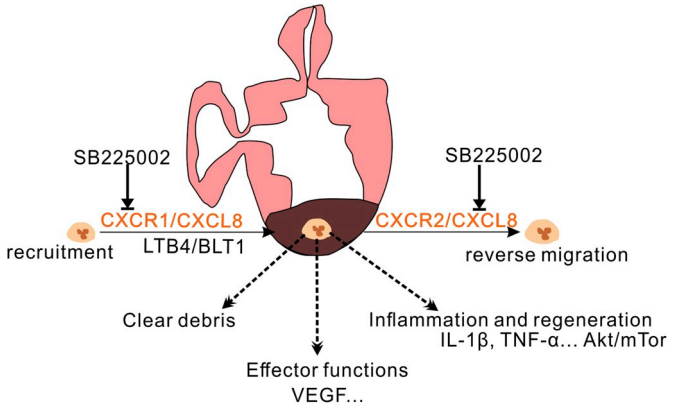

Fig. 6. Proposed model showing migration and function of neutrophils in cryoinjured heart. Upon heart cryoinjury in zebrafish, CXCR1/CXCL8 and LTB4/BLT1 signal pathways are induced. SB225002 inhibit CXCR1/CXCL8 but not LTB4/BLT1 signals. LTB4/BLT1 signals recruit neutrophils to the injured area independent CXCR1/CXCL8. Thus, the recruitment of neutrophil to the injured area of heart is not affected at the presence of SB225002. However, CXCR2/CXCL8 is inhibited by SB225002, which blocks the reverse migration of neutrophil and results more neutrophils accumulates in the injured area. The neutrophils in the injured area removing debris, promoting angiogenesis, secreting pro-inflammatory cytokines, and regulating regeneration.

that BLT1/LTB4 is another pair of chemokines that regulate neutrophil recruitment in zebrafish [2,7]. Thus, we suggest BLT1/LTB4 chemokines might be another independent CXCR1/CXCL8 signal which mediates neutrophil recruitment in the heart after cryoinjury (Fig. 6). In the present study, SB225002 inhibited reverse migration of neutrophils, which resulted in neutrophil accumulation in the wound and prolonged inflammation. The pathogenesis is similar to that seen in humans with MI [1,41]; therefore, this model is valuable for studying the mechanism and therapies for myocardial infarction.

VEGF mediates revascularization in the injured area of the heart, occurring as early as 15 h post cryoinjury [42]. Additionally, neutrophils are one of the first inflammatory cells that respond to damage. Besides removing debris, neutrophils can release VEGF to support the angiogenesis in the wound [27,43]. Our observation that the prolonged retention of neutrophils in the injured area promotes revascularization and expression of *vegf-c* in the heart (Fig. 2), is consistent with the previous study [27]. The prolonged retention of neutrophils in the wound elicits upregulation in the expression of pro-inflammatory cytokines, such as *il-1β* and *tnf-α* (Fig. 1). Additionally, apoptosis in the injured area of the heart was significantly increased (Fig. 3). These

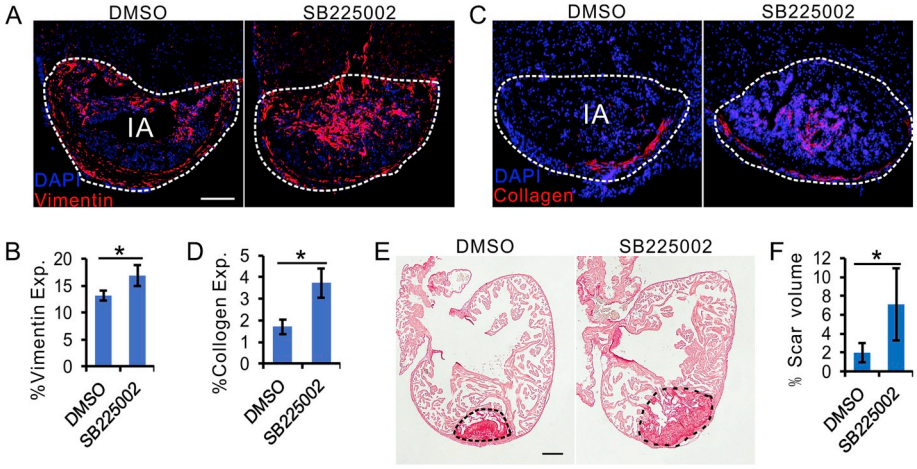

Fig. 5. SB225002 reduced collagen-rich scar degradation in the heart after cryoinjury. A-D. Representative immunofluorescent sections showing the expression of vimentin positive fibroblasts (A, red) and collagen type I (C, red) in the injured area (IA) of the heart at 7 dpc. Bar chart showing the expression level of vimentin positive fibroblasts and collagen in B and D, respectively. Asterisk (*) represents a significant difference was observed at $p < 0.05$ level with two-tail *t-test* (n = 6). Two times were performed of the experiment. **E**. Representative histological sections showing the percentage of scar volume (dashed line bound area) in the heart at 30 dpc using picrosirius red staining. Scale bar: 100 μm. **F**. Bar chart presenting the percentage of scar volume in panel E. Asterisk (*) represents a significant difference was observed at $p < 0.05$ level with two-tail *t-test* (n = 6). (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

results are in agreement with a previous study indicating neutrophils in the infarcted heart promote cell death of cardiomyocytes [41].

AKT/mTOR signals are involved in protein synthesis, cell growth and proliferation in cardiovascular physiology and pathology [37]. The mTOR signal is activated in the heart during development and MI [44]. Hearts of mTOR knockout mice exhibit attenuation of cardiomyocyte proliferation, decrease in cardiomyocyte number and an increase in apoptosis, fibrosis, cardiac dysfunction and heart failure [44]. Our data reveal that SB225002 inhibited reverse migration of neutrophils and reduced proliferating cardiomyocyte (including embryonic cardiomyocytes), and increased apoptosis and fibrosis, meanwhile p-Akt and p-mTor was inhibited (Figs. 4 and 5). These results suggest prolonged neutrophil retention in the wound of the heart blocked regeneration, possibly through the AKT/mTOR signal pathway. In agreement with our observation, it has been reported that heat shock protein A12B (HSPA12B) reduces the infiltration of neutrophil to the wound and attenuates cardiac dysfunction and improves cardiomyocyte survival via PI3K/AKT/mTOR signal pathway [45]. Additionally, our results were also consistent with a previous study that SB225002 inhibited cancer cell proliferation by phosphorylation of AKT and mTOR [46]. Although reports of mTOR signals regulating neutrophil-mediated inflammation are known [47,48], the mechanisms behind prolonged neutrophil retention affected mTOR signals in the present study should be investigated further.

5. Conclusion

In the present study, we report for the first time that the CXCR1/2 selective inhibitor SB225002 did not block the recruitment of neutrophils, but prolonged neutrophil retention by preventing reverse migration. We found that excessive accumulation of neutrophils in the injured heart blocked AKT/mTOR signals and impaired heart regeneration. The present findings provide a potential mechanism that neutrophil mediated inflammation worsens heart functioning in patients with MI.

Conflicts of interest

The authors declare that they have no conflicts of interest with the contents of this article.

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References

[1] J. Wang, Neutrophils in tissue injury and repair, Cell Tissue Res. 371 (3) (2018) 531–539.
[2] S. de Oliveira, et al., Cxcl8 (IL-8) mediates neutrophil recruitment and behavior in the zebrafish inflammatory response, J. Immunol. 190 (8) (2013) 4349–4359.
[3] J.W. Leiding, Neutrophil evolution and their diseases in humans, Front. Immunol. 8 (2017) 1009.
[4] S.A. Renshaw, et al., A transgenic zebrafish model of neutrophilic inflammation, Blood 108 (13) (2006) 3976–3978.
[5] E. Hoffmann, et al., Multiple control of interleukin-8 gene expression, J. Leukoc. Biol. 72 (5) (2002) 847–855.
[6] D. Powell, et al., Chemokine signaling and the regulation of bidirectional leukocyte migration in interstitial tissues, Cell Rep. 19 (8) (2017) 1572–1585.
[7] Q. Deng, et al., Localized bacterial infection induces systemic activation of neutrophils through Cxcr2 signaling in zebrafish, J. Leukoc. Biol. 93 (5) (2013) 761–769.
[8] S. Tauzin, et al., Redox and Src family kinase signaling control leukocyte wound attraction and neutrophil reverse migration, J. Cell Biol. 207 (5) (2014) 589–598.
[9] C. Zuñiga-Traslaviña, et al., Cxcl8b and Cxcr2 regulate neutrophil migration through bloodstream in Zebrafish, J. Immunol. Res. (2017) 2017.
[10] F. Ellett, et al., Defining the phenotype of neutrophils following reverse migration in zebrafish, J. Leukoc. Biol. 98 (6) (2015) 975–981.
[11] T. Okuno, T. Ishitani, T. Yokomizo, Biochemical characterization of three BLT receptors in zebrafish, PLoS One 10 (3) (2015) e0117888.
[12] S. de Oliveira, E.E. Rosowski, A. Huttenlocher, Neutrophil migration in infection and wound repair: going forward in reverse, Nat. Rev. Immunol. 16 (6) (2016) 378.
[13] J. Dalli, et al., Heterogeneity in neutrophil microparticles reveals distinct proteome and functional properties, Mol. Cell. Proteom. 12 (8) (2013) 2205–2219.
[14] M. Horckmans, et al., Neutrophils orchestrate post-myocardial infarction healing by polarizing macrophages towards a reparative phenotype, Eur. Heart J. 38 (3) (2016) 187–197.
[15] S.L. Tsai, C. Baselga-Garriga, D.A. Melton, Blastemal progenitors modulate immune signaling during early limb regeneration, Development 146 (1) (2019) dev169128.
[16] D.R. Powell, A. Huttenlocher, Neutrophils in the tumor microenvironment, Trends Immunol. 37 (1) (2016) 41–52.
[17] S. Kuboki, et al., Hepatocyte signaling through CXC chemokine receptor-2 is detrimental to liver recovery after ischemia/reperfusion in mice, Hepatology 48 (4) (2008) 1213–1223.
[18] L. Li, et al., Live imaging reveals differing roles of macrophages and neutrophils during zebrafish tail fin regeneration, J. Biol. Chem. 287 (30) (2012) 25353–25360.
[19] S. Xu, et al., Matrix metalloproteinases (MMPs) mediate leukocyte recruitment during the inflammatory phase of zebrafish heart regeneration, Sci. Rep. 8 (1) (2018) 7199.
[20] S. Xu, et al., Excessive inflammation impairs heart regeneration in zebrafish breakdance mutant after cryoinjury, Fish Shellfish Immunol. 89 (2019) 117–126.
[21] A.L. Robertson, et al., A zebrafish compound screen reveals modulation of neutrophil reverse migration as an anti-inflammatory mechanism, Sci. Transl. Med. 6 (225) (2014) 225ra29-225ra29.
[22] S.-L. Puhl, S. Steffens, Neutrophils in post-myocardial infarction inflammation: damage versus resolution? Front. Cardiovasc. Med. 6 (2019) 25.
[23] S. Chia, et al., Association of leukocyte and neutrophil counts with infarct size, left ventricular function and outcomes after percutaneous coronary intervention for ST-elevation myocardial infarction, Am. J. Cardiol. 103 (3) (2009) 333–337.
[24] L. Guasti, et al., Neutrophils and clinical outcomes in patients with acute coronary syndromes and/or cardiac revascularisation, Thromb. Haemost. 106 (10) (2011) 591–599.
[25] J.M. González-Rosa, et al., Extensive scar formation and regression during heart regeneration after cryoinjury in zebrafish, Development 138 (9) (2011) 1663–1674.
[26] A. Chatzopoulou, et al., Glucocorticoid-induced attenuation of the inflammatory response in zebrafish, Endocrinology 157 (7) (2016) 2772–2784.
[27] Y. Gong, D.-R. Koh, Neutrophils promote inflammatory angiogenesis via release of preformed VEGF in an in vivo corneal model, Cell Tissue Res. 339 (2) (2010) 437–448.
[28] X. Huo, et al., Transcriptomic profiles of tumor-associated neutrophils reveal prominent roles in enhancing angiogenesis in liver tumorigenesis in zebrafish, Sci. Rep. 9 (1) (2019) 1509.
[29] S.-L. Lai, et al., Reciprocal analyses in zebrafish and medaka reveal that harnessing the immune response promotes cardiac regeneration, Elife 6 (2017) e25605.
[30] T. Hasegawa, et al., Transient inflammatory response mediated by interleukin-1β is required for proper regeneration in zebrafish fin fold, Elife 6 (2017) e22716.
[31] T. Weber, et al., Caspase-mediated apoptosis induction in zebrafish cerebellar Purkinje neurons, Development 143 (22) (2016) 4279–4287.
[32] A.-S. de Preux Charles, et al., Preconditioning boosts regenerative programmes in the adult zebrafish heart, Open Biol. 6 (7) (2016) 160101.
[33] C. Jopling, et al., Zebrafish heart regeneration occurs by cardiomyocyte dedifferentiation and proliferation, Nature 464 (7288) (2010) 606.
[34] S.P. Hui, et al., Zebrafish regulatory T cells mediate organ-specific regenerative programs, Dev. Cell 43 (6) (2017) 659–672 e5.
[35] M. Gemberling, et al., Nrg1 is an injury-induced cardiomyocyte mitogen for the endogenous heart regeneration program in zebrafish, Elife 4 (2015) e05871.
[36] A. Bonifacio, et al., The AKT/mTOR signaling pathway plays a key role in statin-induced myotoxicity, Biochim. Biophys. Acta Mol. Cell Res. 1853 (8) (2015) 1841–1849.
[37] S. Sciarretta, M. Volpe, J. Sadoshima, mTOR signaling in cardiac physiology and disease: Sciarretta et al. mTOR signaling in the cardiovascular system, Circ. Res. 114 (3) (2014) 549.
[38] M.A. Laflamme, C.E. Murry, Heart regeneration, Nature 473 (7347) (2011) 326.
[39] F. Chablais, A. Jaźwińska, The regenerative capacity of the zebrafish heart is dependent on TGFβ signaling, Development 139 (11) (2012) 1921–1930.
[40] S.A. Eming, T.A. Wynn, P. Martin, Inflammation and metabolism in tissue repair and regeneration, Science 356 (6342) (2017) 1026–1030.
[41] M.J. Schloss, et al., The time-of-day of myocardial infarction onset affects healing through oscillations in cardiac neutrophil recruitment, EMBO Mol. Med. 8 (8) (2016) 937–948.
[42] R. Marín-Juez, et al., Fast revascularization of the injured area is essential to support zebrafish heart regeneration, Proc. Natl. Acad. Sci. 113 (40) (2016) 11237–11242.
[43] M. Gaudry, et al., Intracellular pool of vascular endothelial growth factor in human neutrophils, Blood 90 (10) (1997) 4153–4161.
[44] Y. Zhu, et al., Mechanistic target of rapamycin (Mtor) is essential for murine embryonic heart development and growth, PLoS One 8 (1) (2013) e54221.
[45] Q. Kong, et al., HSPA12B attenuated acute myocardial ischemia/reperfusion injury

via maintaining endothelial integrity in a PI3K/Akt/mTOR-dependent mechanism, Sci. Rep. 6 (2016) 33636.
[46] M. Xu, et al., SB225002 inhibits prostate cancer invasion and attenuates the expression of BSP, OPN and MMP-2, Oncol. Rep. 40 (2) (2018) 726–736.
[47] Y. Bao, et al., mTOR and differential activation of mitochondria orchestrate neutrophil chemotaxis, J. Cell Biol. 210 (7) (2015) 1153–1164.
[48] W. Ratajczak-Wrona, et al., PI3K-Akt/PKB signaling pathway in neutrophils and mononuclear cells exposed to N-nitrosodimethylamine, J. Immunotoxicol. 11 (3) (2014) 231–237.