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Excessive inflammation impairs heart regeneration in zebrafish *breakdance* mutant after cryoinjury

Shisan Xu[a], Chichi Liu[a], Fangjing Xie[a], Li Tian[a], Sinai HC. Manno[a], Francis A.M. Manno III[b], Samane Fallah[a], Bernd Pelster[c,*], Gary Tse[d,e,f,**], Shuk Han Cheng[a,g,h,***]

[a] Department of Biomedical Sciences, College of Veterinary Medicine and Life Science, City University of Hong Kong, Hong Kong SAR, PR China
[b] Department of Physics, College of Science and Engineering, City University of Hong Kong, Hong Kong SAR, PR China
[c] Institut für Zoologie, Universität Innsbruck, Center for Molecular Biosciences, Universität Innsbruck, Innsbruck, Austria
[d] Department of Medicine and Therapeutics, Faculty of Medicine, The Chinese University of Hong Kong, Hong Kong SAR, PR China
[e] Li Ka Shing Institute of Health Sciences, Faculty of Medicine, The Chinese University of Hong Kong, Hong Kong, PR China
[f] Shenzhen Research Institute, The Chinese University of Hong Kong, Shenzhen, PR China
[g] State Key Laboratory of Marine Pollution (SKLMP) at City University of Hong Kong, Hong Kong SAR, PR China
[h] Department of Materials Science and Engineering, College of Science and Engineering, City University of Hong Kong, Hong Kong SAR, PR China

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ABSTRACT

Inflammation plays a crucial role in cardiac regeneration. Numerous advantages, including a robust regenerative ability, make the zebrafish a popular model to study cardiovascular diseases. The zebrafish *breakdance* (*bre*) mutant shares several key features with human long QT syndrome that predisposes to ventricular arrhythmias and sudden death. However, how inflammatory response and tissue regeneration following cardiac damage occur in *bre* mutant is unknown. Here, we have found that inflammatory response related genes were markedly expressed in the injured heart and excessive leukocyte accumulation occurred in the injured area of the *bre* mutant zebrafish. Furthermore, *bre* mutant zebrafish exhibited aberrant apoptosis and impaired heart regenerative ability after ventricular cryoinjury. Mild dosages of anti-inflammatory or prokinetic drugs protected regenerative cells from undergoing aberrant apoptosis and promoted heart regeneration in *bre* mutant zebrafish. We propose that immune or prokinetic therapy could be a potential therapeutic regimen for patients with genetic long QT syndrome who suffers from myocardial infarction.

1. Introduction

The inflammatory response is essential for fighting infection, maintaining normal tissue homeostasis and initiating wound healing. Wound healing is a highly dynamic and well-orchestrated process in which the inflammatory response is involved in determining tissue injury leading to regeneration or a fibrotic scar [1]. Inflammation appears to play a dual role, with positive and negative effects on cardiac regeneration [1–3]. In zebrafish and neonatal mice, inflammation is indispensable for initiating the regeneration process [4,5]. However, inflammatory cytokines, such as TNF-α and IL-1β, also result in apoptosis and impair regeneration [1,4–7]. It has been reported that in fish, an excessive immune response or immune deficiency causes a failure of heart regeneration [6,7]. Previously, we have shown that matrix metalloproteinases (MMPs) act as inflammatory mediators during zebrafish heart regeneration. During the early stages after injury, MMPs could modulate leukocyte recruitment via chemokines, and thereby regulate zebrafish heart regeneration [8].

Congenital long QT syndrome (LQTS) is a potential life-threatening cardiac arrhythmia caused by mutations in different ion channel genes, such as KCNQ1, KCNH2, and SCN5A, resulting in action potential prolongation [9]. It is estimated that LQTS affects 1 in 3000 newborns and it results in approximately 4000 deaths yearly in the United States of America [10]. Zebrafish share several characteristics with human cardiac repolarization, as well as similar electrical phenotypes in response to pharmacological and genetic manipulation [10–12]. In zebrafish *breakdance* (*bre*), an I59S missense mutation occurs in the KCNH2 gene [also termed the human Ether-à-go-go-Related Gene, HERG],

* Corresponding author.
** Corresponding author. Department of Medicine and Therapeutics, Faculty of Medicine, The Chinese University of Hong Kong, Hong Kong SAR, PR China.
*** Corresponding author. Department of Biomedical Sciences, College of Veterinary Medicine and Life Science, City University of Hong Kong, Hong Kong SAR, PR China.
E-mail addresses: Bernd.pelster@uibk.ac.at (B. Pelster), Tseg@cuhk.edu.hk (G. Tse), bhcheng@cityu.edu.hk (S.H. Cheng).

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Abbreviations

Bre	Breakdance
IA	injured area
MMP	Matrix metalloproteinase
MMP in	Matrix metalloproteinase inhibitor
Dex	Dexamethasone
Cis	cisapride
pH3	phospho-Histone H3
PCNA	Proliferating cell nuclear antigen
emCMHC	embryonic cardiomyocyte heavy chain
TUNEL	Terminal deoxynucleotidyl transferase dUTP nick end labeling
Arb	Arbitrary
TnnT	Troponin T
DMSO	Dimethyl sulfoxide
α-SMA	α-smooth muscle actin
qRT-PCR	quantitative real time polymerase chain reaction
ECM	Extracellular matrix
ERG	Ether-à-go-go-Related gene
apop	apoptotic
cas	caspase
Tnf-α	Tumor necrosis factor-α
Il-1β	Interleukin 1 beta
KCNQ1	Potassium Voltage-Gated Channel Subfamily Q Member 1
KCNH2	Potassium voltage-gated channel subfamily H member 2
SCN5A	sodium voltage-gated channel alpha subunit 5
QT	Q wave T wave

which encodes for a rapidly activating-delayed rectifier potassium channel, leading to a trafficking defect and a lower number of channels on the cell membrane [13–15]. Ultimately, these alterations lead to a 2:1 atrioventricular block in the larvae stage and LQTS in adult fish are observed. These phenotypes closely recapitulate clinical findings and enable zebrafish to be a suitable model for studying the mechanisms of cardiac arrhythmias and screening for potential drugs. For instance, flurandrenolide and 2-methoxy-N-(4-methylphenyl) benzamide reverse action potential prolongation in *bre* mutant zebrafish [10,11,15]. Interestingly, a study showed that flurandrenolide suppressed the LQT phenotype via the glucocorticoid signaling pathway [10], which indicates that LQTS might be related to inflammation. In this regard, a few studies have investigated inflammation in LQTS patients, and tumor necrosis factor receptor 1 (TNFR-α1) was reposted as a promising predictor of cardiovascular survival [16,17]. However, previous studies focused on the reduction of arrhythmia in LQTS patients, while seldom reports have assessed the inflammatory response of LQTS patients after myocardial infarction (MI). Moreover, previous research has neglected how the inflammatory response is modulated in *bre* mutant zebrafish cardiac regeneration.

In the present study, we demonstrate an excessive inflammatory response after cryoinjury in *bre* mutant hearts result in aberrant apoptotic regenerative cells and impair fibrotic scar degradation. We also find a higher number of proliferating cells, dedifferentiated cardiomyocytes (i.e. embryonic cardiomyocyte) and more extracellular matrix accumulation in the injured area of the *bre* mutant heart. A moderate dose of anti-inflammatory reagents (dexamethasone, MMP9/MMP13 inhibitor I) and prokinetic drugs (cisapride) attenuate the inflammatory response and apoptotic cells, thereby rescuing the regeneration and promoting scar degradation in the heart of *bre* mutant zebrafish. The present results suggest downregulating the inflammatory response during heart injury might assist in cardiac regeneration in *bre* mutant zebrafish.

2. Materials and methods

2.1. Zebrafish maintenance

Zebrafish wild-type (AB) strain and *bre* mutant were maintained in a controlled environment at The City University of Hong Kong fish facility, at 28 ± 1 °C with a daily 14:10 day-light cycle [14]. All experimental procedures were approved by the Department of Health, Hong Kong, SAR, China (refs (14–118) in DH/HA&P/8/2/5 Pt.3), and the experiments were conducted in accordance with the relevant guidelines and regulations in Hong Kong, SAR, China.

Dexamethasone (Dex, D-2915; Sigma), MMP9/MMP13 inhibitor I (MMP in, 21265; Cayman) and cisapride (Cis, CDS021610; Sigma-Aldrich) were dissolved in DMSO at 100 mM, 1 mM and 10 mM, respectively and stored at −20 °C. Subsequently, dexamethasone, MMP9/MMP13 inhibitor I, and cisapride were diluted to 100 μm, 1 μm, and 10 μm in fish water prior to use, respectively. In one series of experiments, the *bre* mutant ventricle was cryoinjured and treated with 0.1% DMSO (vehicle control), 100 μm dexamethasone, 1 μm MMP9/MMP13 inhibitor I, or 10 μm cisapride, respectively. Water for zebrafish and chemicals were changed every two days.

2.2. Zebrafish heart cryoinjury and fin amputation

Adult zebrafish (6–12months) were individually anesthetized by immersion in 0.04% tricaine solution (E10521; Sigma-Aldrich), and subsequently placed in a groove within a wet sponge. Once the heart was exposed, cryoinjury was performed on the ventricle using methods described previously [18]. A sham operation was performed on zebrafish, in which hearts were exposed but not subjected to cryoinjury. Zebrafish larvae at 3 dpf were also used, anesthetized using 0.01% tricaine solution, and transferred to a 1.5% agar gel plate where a fin amputation was performed using a sharp blade.

2.3. RNA sequencing analysis and qRT-PCR

Total RNA was extracted using a Trizol reagent (9109; Takara Bio. Inc.), and decontaminated with RQ1 RNA-free DNase (M6101; Promega). For RNA sequencing, a paired-end 50-bp read length sequencing was performed using an Illumina Hiseq 2000 platform, three bio-replicates were performed. Sequencing and the primary analysis were performed by BGI (Shenzhen, China). For qRT-PCR, 1 μg total RNA was used to synthesize cDNA using the PrimeScript reverse transcription (RT) reagent kit (6210B; Takara) according to the manufacturer's instructions. The expression of *ifn-γ, irg1, il-1β, ccl2, cxcl11, tnf-α, l-plastin, mmp13a, caspase a, caspase 3a, caspase 3b, caspase 7-l, caspase 8* and *bcl-2 like* were determined by qRT-PCR using SYBR Premix Ex Taq (RR402A; Takara), and *β-actin* was used as a reference gene. Each gene analysis was performed in triplicate and the results were analyzed using the $2^{-\Delta\Delta}$Ct method. The primer sequences were listed in Table S1.

2.4. Histological techniques

Zebrafish were sacrificed, hearts were harvested and subsequently fixed with 4% PFA at 4 °C overnight. A series of ~5 μm paraffin sections were prepared. The volume of the scar in the ventricle was determined by Picrosirus red staining (ab150681, Abcam) and morphometric analysis conducted as described previously [8].

2.5. Immunohistochemistry

Paraffin sections were dewaxed and antigens were retrieved by incubating the sections in 10 mM sodium citrate buffer (0.05% Tween 20, pH 6.0) at 95 °C for 15 min. The staining and microscopy method was

performed as previously described, and images of larvae were captured using a Zeiss Lightsheet Z.1 3D microscope [8]. The primary antibodies used in the study were mouse anti-L-plastin (sc-133218; Santa Cruz), rabbit anti-PCNA (sc-7907; Santa Cruz), mouse anti-troponin T (MA5-12960; Thermo), rabbit anti-phospho-Histone H3 (Ser10) (06–570; Millipore), rabbit anti-α-smooth muscle actin (GTX25694; Gentex), mouse anti-myosin heavy chain (MF-20; DSHB), and mouse anti-embCMHC (N2.261; DSHB). The secondary antibodies used were Cy3-conjugated goat anti-mouse (A10521; Invitrogen) or Alexa Fluor 488-conjugated goat anti-rabbit (A11034; Invitrogen) antibodies.

2.6. TUNEL staining

Paraffin sections were deparaffinized and rehydrated. Apoptotic cells were detected using the DeadEnd™ Fluorometric TUNEL System kit (G3250; Promega) according to the manufacturer's instructions. Briefly, rehydrated sections were equilibrated with 0.85% NaCl and PBS, refixed with 4% PFA in PBS (15 min, RT), washed with PBS, and treated with 10 mg/ml Proteinase K in PBS (8–10 min, RT). The samples were further refixed with 4% PFA in PBS (5 min, RT), washed with PBS, and then incubated in equilibration buffer. The sample was incubated with the TUNEL reaction mixture at 37 °C for 1 h, counterstained with DAPI and images were acquired using fluorescent microscopy. The TUNEL-positive cells in the injured area were quantified with Image J.

2.7. Statistical analysis

Statistical analysis was performed using Student's two-tailed *t*-test (Microsoft Excel 2010) and one way-ANOVA (SPSS 13.0) with LSD post-hoc test. For statistical testing, $p < 0.05$ was considered to be statistically significant. Data are presented as means ± S.D. (standard deviation).

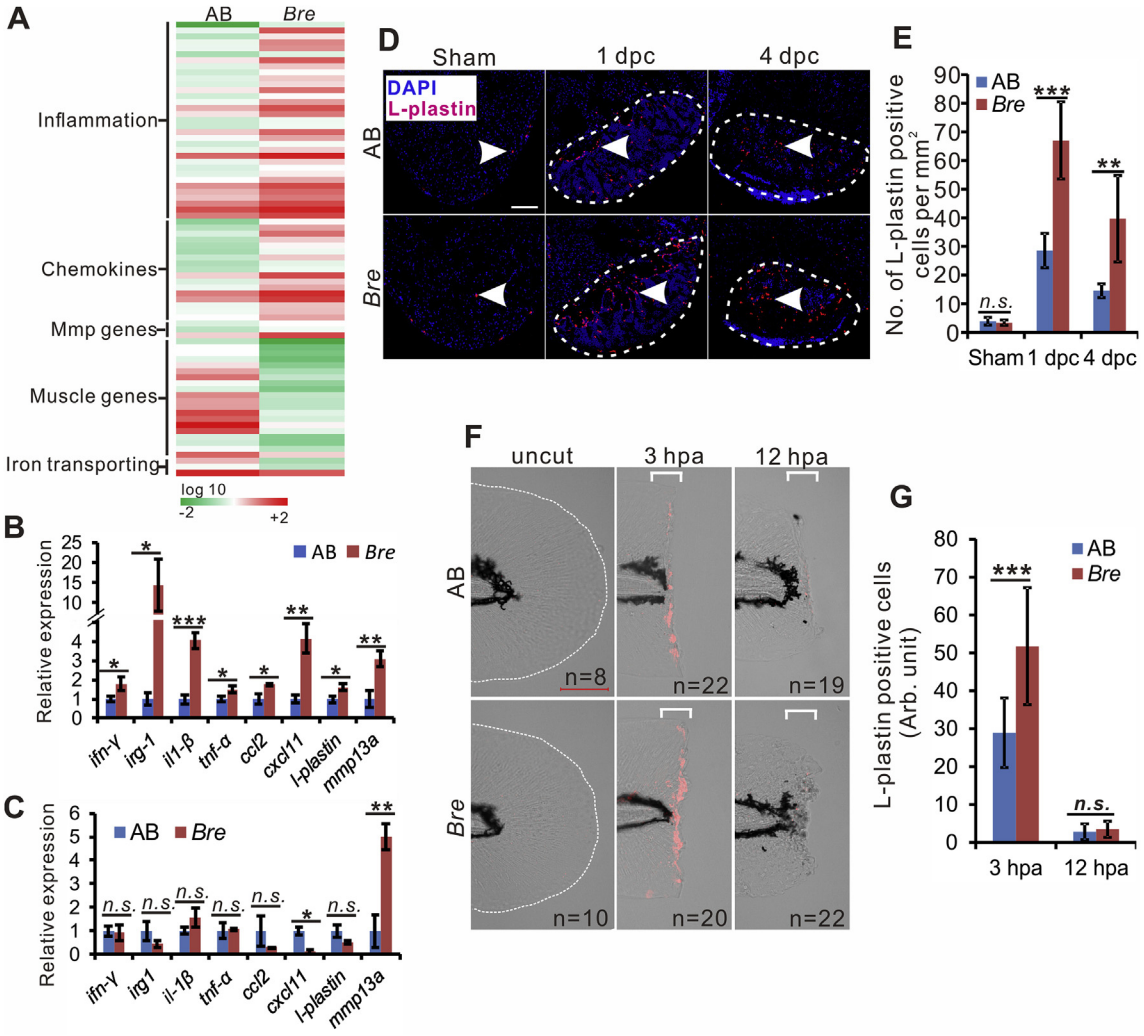

Fig. 1. Excessive inflammation is induced in the injured tissue of *bre* mutant zebrafish. **(A)**. Heat map shows transcriptome analysis of AB and *bre* mutant heart at 4 dpc, n = 3. (**B**, **C**). The qRT-PCR analysis of gene expression in the heart of AB and *bre* mutant zebrafish at 4 dpc (B), and untreated heart (C). The asterisk indicates significant difference observed at $p < 0.05$ (*) and $p < 0.01$ (**) between AB and *bre* mutant zebrafish, while the '*n.s*' indicates no significant difference, n = 3, two tail *t*-test. **(D)**. Representative heart sections of sham-operated, 1 dpc and 4 dpc AB wild-type and *bre* mutant zebrafish showing L-plastin positive cells (red, white arrowhead) in the injured area (bounded by a white dashed line). Scale bar: 100 μm. (**E**). Bar chart shows the quantification of L-plastin positive cells (n = 4–6) in panel (C) was significantly different at $p < 0.01$ (**) and $p < 0.001$ (***), two tail *t*-test. (**F**). Representative images displaying L-plastin positive cells (red) in the fin of *bre* mutant larvae. The white dash line in the panel indicates the shape of the uncut fin. Scale bar: 200 μm. (**G**). Bar chart shows the quantification of L-plastin positive cells in panel E was significantly different at $p < 0.001$ (***), two tail *t*-test. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

3. Results

3.1. Cryoinjury induced excessive inflammation in bre mutant zebrafish heart

To investigate the differential expression of genes between in the hearts of AB wild-type and *bre* mutant zebrafish after cardiac damage, cryoinjury was applied to the ventricles and RNA sequencing analysis was performed at 4 dpc in both AB and *bre* mutant fish. Transcriptome analysis identified approximately 26,450 genes in both groups. Among these genes, 53 candidate genes were identified as encoding proteins or chemokines and were known to be involved in inflammation. Their expression levels were significantly higher in *bre* mutant than in wild-type zebrafish (Fig. 1A and Table S2-3). These genes included *mpeg1*, *coro1a*, *l-plastin etc.* which are leukocyte markers; *ifn-γ*, *irg-1*, *il-1β*, *tnf-α etc.* which are inflammatory molecules; and chemokines *ccl2*, *cxcl11 etc.* which regulate leukocyte migration. Furthermore, three genes related to matrix metalloproteinase (*mmp*), including *mmp13a,* were highly expressed in *bre* mutant compared with AB zebrafish (Fig. 1A and Table S4). By contrast, there were 20 genes that coded for proteins known to be involved in muscle cell differentiation (Fig. 1A and Table S5), such as troponin and myosin, and 3 genes which coded for proteins involved in ion transport (i.e., Ca^{2+}, Na^{+}; Fig. 1A and Table S6). These genes were significantly downregulated in the heart of *bre* mutant compared with AB wild-type zebrafish at 4 dpc, and this suggests that the regenerative ability of the *bre* mutant zebrafish might be impaired. To confirm the RNA-seq data, we determine the expression of *ifn-γ*, *irg-1*, *il-1β*, *tnf-α, ccl2, cxcl11, l-plastin, and mmp13a* in AB wild type and *bre* mutant heart by q RT-PCR at 4 dpc. The results showed that the expression of this set of genes were upregulated in *bre* mutant fish compared with AB (Fig. 1B), consistent with RNA-seq data.

To test whether untreated *bre* mutant zebrafish also express high levels of inflammatory genes, qRT-PCR was performed to determine the expression of *ifn-γ*, *irg-1*, *il-1β*, *tnf-α, ccl2*, *cxcl11, l-plastin, and mmp13a* in the heart of untreated *bre* mutant and AB zebrafish (Fig. 1C). Interestingly, except for *mmp13a* and *ccl2,* no significant difference in expression level was found between *bre* mutant and AB zebrafish. Furthermore, the expression of *ccl2* was lower in *bre* mutant.

To follow-up the gene expression assays, we assessed the accumulation of inflammatory cells (i.e. leukocytes) in the lesion site of *bre* mutant hearts after cryoinjury. The immunofluorescence analysis revealed a higher number of leukocytes at 1 dpc and 4 dpc in *bre* mutant zebrafish compared with AB zebrafish, but no significant difference was observed between sham-operated groups (Fig. 1D and E).

To examine whether injury could also induce an excessive inflammatory response in other tissue, we assessed leukocytes in the amputated fin using the L-plastin antibody. In the intact fin, the detection of L-plastin positive leukocytes was less in both *bre* mutant and AB larvae (Fig. 1F). After 3 h post amputation (hpa), L-plastin positive leukocytes were accumulated in the amputated fin site, and there were more leukocytes in *bre* mutant than in AB zebrafish (Fig. 1G). By 12 hpa, the number of leukocytes was decreased, and no significant

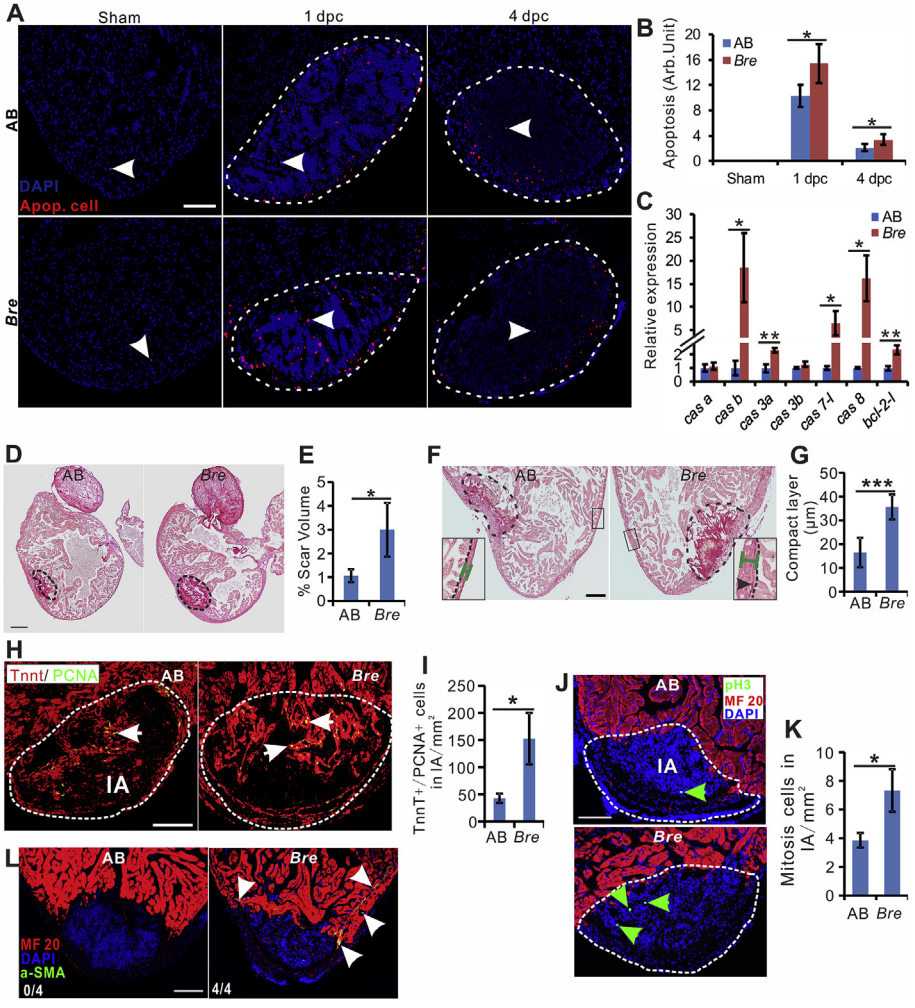

Fig. 2. Comparative analysis of cardiac regenerative ability in AB and *bre* mutant zebrafish after heart cryoinjury. (A). Representative TUNEL staining sections of AB and *bre* mutant zebrafish indicating the apoptotic cells (red, white arrow) in the injured area (bounded by the white dashed line) of the heart at 1 dpc and 4 dpc, and whole ventricle of sham-operated zebrafish. Scale bar: 100 μm. **(B).** Bar chart shows the quantification of apoptotic cells (n = 3–6) in panel (A) was significantly different at $p < 0.05$ (*), two tail *t*-test. **(C)** qRT-PCR analysis expression levels of apoptotic cytokine in AB and *bre* mutant heart at 4 dpc. The asterisk indicates significant difference observed at $p < 0.05$ (*) and $p < 0.01$ (**) between AB and *bre* mutant zebrafish, n = 3, two tail *t*-test. **(D-G)** Representative picrosirus red staining paraffin sections of AB and *bre* mutant zebrafish heart displaying scar volume (D) and thickness of compact layer (F) at 30 dpc. The black dashed line indicates the injured scar area. Black arrowhead (F) indicates the fibrotic subepicardial layer in *bre* mutant zebrafish. Scale bar: 100 μm. Bar chart (E, G) shows the quantification of the percentage of scar volume in panel D and thickness of the epicardial layer in panel F was significantly different between AB and *bre* mutant at $p < 0.05$ (*) (n = 5), two tail *t*-test. **(H)** Representative images of immunofluorescent labeling of cardiomyocyte (red) and PCNA (green) in the injured area of AB and *bre* mutant zebrafish at 7 dpc. White arrow indicates the double positive $TnnT^{+}/PCNA^{+}$ cells in the injured area (IA). Scale bar: 100 μm. **(I).** Bar chart shows the quantification of double positive $TnnT^{+}/PCNA^{+}$ (n = 4) in panel H, two tail *t*-test, $p < 0.05$ (*). **(J).** Representative images of immunofluorescent labeling of mitotic cells (green, green arrowhead) in the injured area of AB and *bre* mutant zebrafish at 7 dpc. Scale bar: 100 μm. **(K).** Bar chart shows the quantification of mitotic cells (n = 4) in panel J, two tail *t*-test, $p < 0.05$ (*). **(L).** Representative images of immunofluorescent labelling of α-SMA (green, white arrowhead) in the heart of AB (n = 4) and *bre* mutant (n = 4) zebrafish. Scale bar: 100 μm. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

difference was observed between *bre* and AB zebrafish larvae (Fig. 1G). These results indicate that fin injury induced an increase in the number of inflammatory cells in the wound of *bre* mutant zebrafish larvae.

Overall, the data indicate that KCNH2 deficiency might be involved with modulating the immune response after tissue injury in *bre* mutant zebrafish. Furthermore, myocardium infarction (MI) induced by cryoinjury results in excessive inflammatory response in the heart which is accompanied by a high expression of genes involved in inflammation, chemokines, and leukocytes infiltration.

3.2. Aberrant apoptosis and impaired heart regeneration in bre mutant zebrafish after cryoinjury

Inflammation and pro-inflammatory chemokines have been suggested to directly or indirectly induce cell death during wound healing and MI [19,20]. In the heart of *bre* mutant zebrafish, cryoinjury caused overexpression of inflammatory response genes, including *tnf-α* and *il-1β*, which has been shown to lead to chronic inflammation and thereby apoptosis in zebrafish [6].

To test whether excessive inflammatory response causes aberrant cell death in *bre* mutant heart after cryoinjury, we performed TUNEL staining in both *bre* mutant and AB zebrafish heart at 1 dpc, 4 dpc and in sham-operated groups. Consistent with our previous study [8], TUNEL-positive cells were detected at a peak level at 1 dpc, and then decreased gradually (Fig. 2A). However, a significantly increased number of apoptotic cells was found in the injured area of heart in *bre* mutant zebrafish compared with AB zebrafish at 1 dpc and 4 dpc, while no significant change was observed in sham-operated groups (Fig. 2A and B). In addition, our qRT-PCR results showed that the expression of caspase b, caspase 3a, *caspase 7-like, caspase 8* and anti-apoptotic gene *bcl-2 like* was higher in *bre* mutant than in AB heart at 4 dpc (Fig. 2C). Furthermore, our transcriptome analysis showed that the expression level of apoptotic cytokines such as *caspase b*, *caspase 7-like, caspase 8* and was markedly high in *bre* mutant heart compared with AB at 4 dpc, while anti-apoptotic gene *bcl-2 like* was also markedly expressed in *bre* mutant heart, which might be a *in vivo* self-regulation mechanism (Table S7). These results indicate that highly expressed inflammatory genes in *bre* mutant zebrafish heart might cause aberrant apoptosis after injury.

An overactive immune response to cardiac injury in adult mice can negatively affect regeneration by promoting fibrotic scar formation [4,21]. To further analyze the heart regenerative ability in *bre* mutant zebrafish, we cryoinjured the heart of AB and *bre* mutant zebrafish utilizing an identical cryoprobe and time paradigm, to ensure the ventricle had a comparable scale of cardiac damage. After 30 days, the zebrafish were sacrificed and the hearts were extracted. Histological analysis revealed that there was a larger collagen-rich scar in the heart of *bre* mutant zebrafish compared with AB zebrafish (Fig. 2D and E). In addition, we found the compact layer in the heart of *bre* mutant zebrafish was significantly thicker than in AB zebrafish. Here apparently collagen was observed in the subepicardial layer of heart in *bre* mutant zebrafish (Fig. 2F and G). These data suggest that the heart regenerative

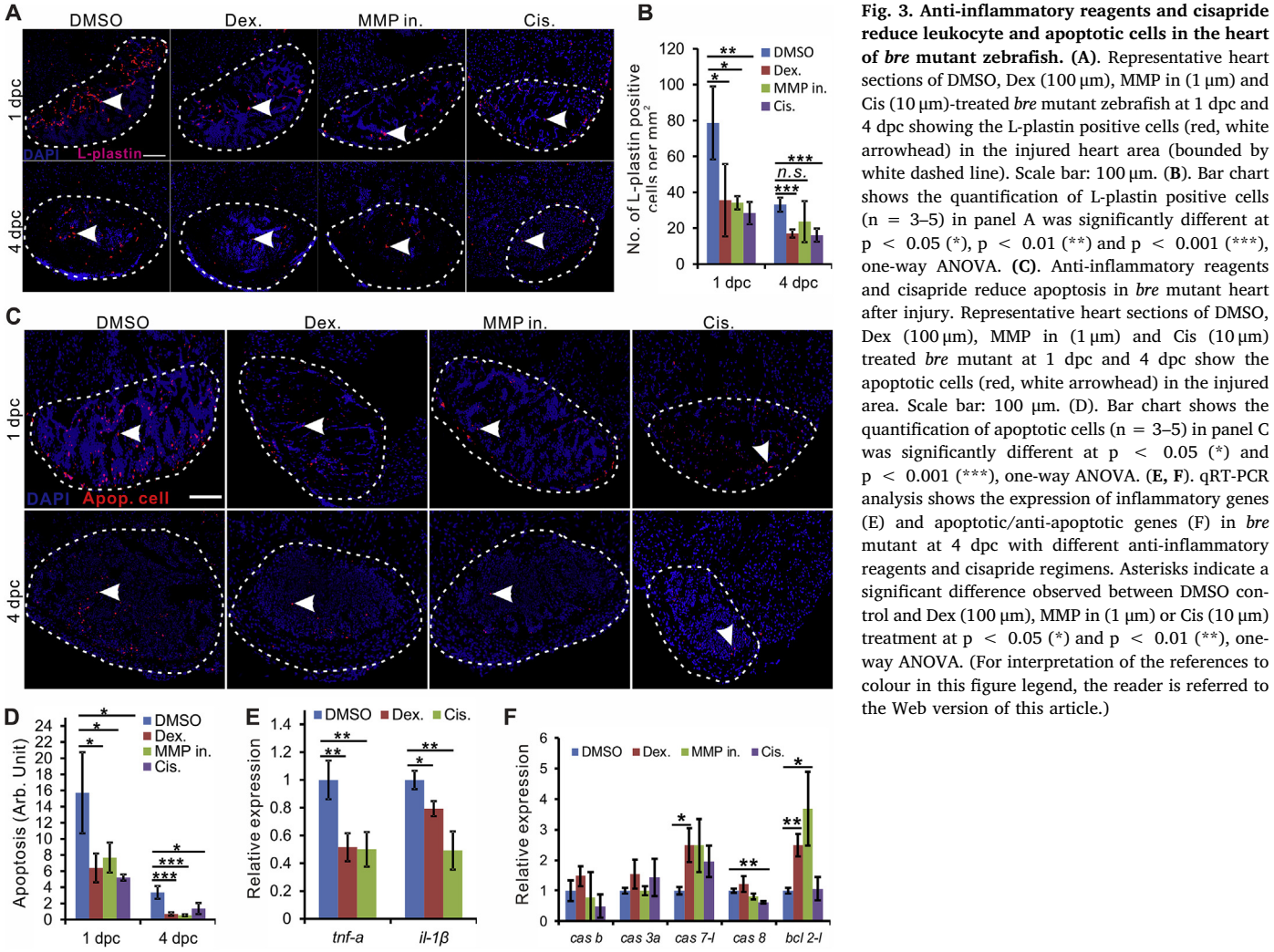

Fig. 3. Anti-inflammatory reagents and cisapride reduce leukocyte and apoptotic cells in the heart of *bre* mutant zebrafish. (A). Representative heart sections of DMSO, Dex (100 μm), MMP in (1 μm) and Cis (10 μm)-treated *bre* mutant zebrafish at 1 dpc and 4 dpc showing the L-plastin positive cells (red, white arrowhead) in the injured heart area (bounded by white dashed line). Scale bar: 100 μm. (**B**). Bar chart shows the quantification of L-plastin positive cells (n = 3–5) in panel A was significantly different at $p < 0.05$ (*), $p < 0.01$ (**) and $p < 0.001$ (***), one-way ANOVA. **(C)**. Anti-inflammatory reagents and cisapride reduce apoptosis in *bre* mutant heart after injury. Representative heart sections of DMSO, Dex (100 μm), MMP in (1 μm) and Cis (10 μm) treated *bre* mutant at 1 dpc and 4 dpc show the apoptotic cells (red, white arrowhead) in the injured area. Scale bar: 100 μm. (D). Bar chart shows the quantification of apoptotic cells (n = 3–5) in panel C was significantly different at $p < 0.05$ (*) and $p < 0.001$ (***), one-way ANOVA. (**E, F**). qRT-PCR analysis shows the expression of inflammatory genes (E) and apoptotic/anti-apoptotic genes (F) in *bre* mutant at 4 dpc with different anti-inflammatory reagents and cisapride regimens. Asterisks indicate a significant difference observed between DMSO control and Dex (100 μm), MMP in (1 μm) or Cis (10 μm) treatment at $p < 0.05$ (*) and $p < 0.01$ (**), one-way ANOVA. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

ability was impaired in *bre* mutant zebrafish after cardiac damage.

Cell proliferation is crucial for heart regeneration and provides new cell sources to restore the lost or damaged tissue. Interestingly, cell proliferation is also regulated by inflammation [4,22]. Thus, we explored whether proliferating cells were affected in the *bre* mutant. We stained the heart at 7 dpc with anti-troponin T (TnnT) and PCNA to label proliferating cardiomyocytes. Surprisingly, we found that the number of double positive of $TnnT^+$ and $PCNA^+$ proliferating cardiomyocytes was higher in *bre* mutant zebrafish heart than in AB wild type zebrafish (Fig. 2H and I). To further confirm the proliferating cells in *bre* mutant and AB zebrafish heart, we quantified the number of mitotic cells in the injured area of the heart at 7 dpc using immunofluorescence analysis. Consistent with the $TnnT^+/PCNA^+$ staining, we observed more pH3-positive mitotic cells in the injured area of the heart in *bre* mutant compared with AB zebrafish (Fig. 2J and K). Then, we determined the expression of embryonic cardiac myosin heavy chain (embCMHC), which is used as a marker of undifferentiated CMs [23]. Our data revealed that embCMHC was markedly induced in close vicinity to the injured area of the heart in *bre* mutant when compared with AB zebrafish (Fig. S1).

To visualize the myofibroblasts and smooth muscle cells, we used an antibody against α-smooth muscle actin (α-SMA), which is involved in the ECM and blood vessel formation [24,25]. In the injured heart of AB zebrafish at 4 dpc, an insignificant quantity of α-SMA labeled cells were detected (Fig. 2L). In contrast, a considerable number of α-SMA positive cells were induced in the subepicardial layer and near the inner border in the injured area of the heart in *bre* mutant (Fig. 2L).

Therefore, our data indicate that *bre* mutant zebrafish displayed aberrant apoptosis and impaired heart regeneration after cryoinjury. Interestingly, the number of proliferating cells was also higher in the injured area of the *bre* mutant heart than in AB wild type zebrafish.

3.3. Anti-inflammatory and prokinetic drugs suppress excessive inflammation and aberrant apoptosis in bre mutant zebrafish after injury

It has been suggested that inflammation and pro-inflammatory chemokines can directly or indirectly induce cell death during wound healing and MI [19,20]. In heart of *bre* mutant zebrafish, cryoinjury caused overexpression of inflammatory response genes, including *tnf-α* and *il-1β*, which has been shown to lead to chronic inflammation and thereby apoptosis in zebrafish [6]. The literature indicates that anti-inflammatory glucocorticoids such as dexamethasone (Dex) can attenuate leukocyte infiltration, reduce the expression of *il-1β* and decrease apoptosis in the wound [6,26]. To test whether dexamethasone can reduce aberrant apoptosis in the heart of *bre* mutant after injury, we first assessed the number of leukocytes in the injured area of the heart using anti-L-plastin staining after dexamethasone treatment. Compared with the control group treated with DMSO, dexamethasone significantly attenuated the number of L-plastin positive cells in the injured area at 1 and 4 dpc (Fig. 3 A, B). Furthermore, the number of L-plastin positive cells in the injured area of the heart of *bre* mutant zebrafish treated with dexamethasone was similar to AB zebrafish at 1 dpc and 4 dpc (Fig. 1D and E). Moreover, similar to dexamethasone treatment, MMP9/MMP13 inhibitor I and cisapride also reduced the number of L-plastin positive leukocytes in the injured area of the heart in *bre* mutant zebrafish (Fig. 4 A, B).

The subsequent procedure we performed was the evaluation of dexamethasone to induce a reduction of aberrant apoptosis in the wound. The TUNEL-staining analysis revealed that, compared to the vehicle control, fish treated with dexamethasone had a significantly reduced number of apoptotic cells in the injured area (Fig. 3C and D). In addition, our qRT-PCR results showed that dexamethasone reduced the expression level of *tnf-α* and *il-1β*, and promoted the expression of anti-

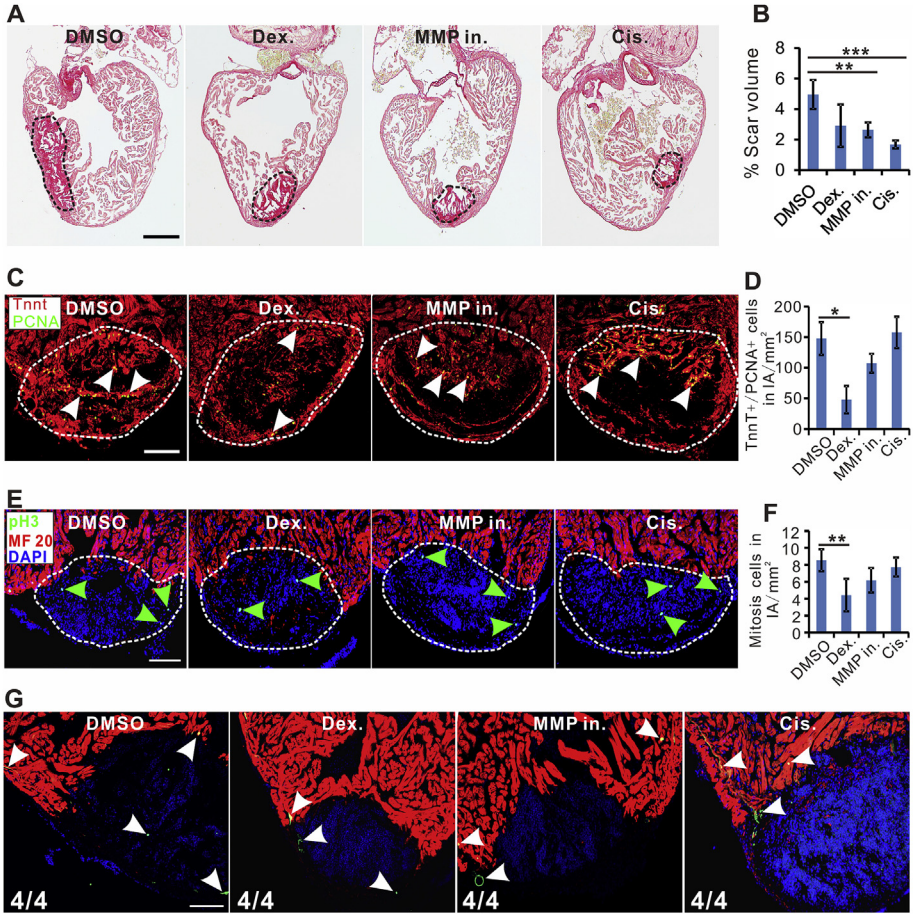

Fig. 4. Anti-inflammation reagents and cisapride rescue the heart regenerative capability in *bre* mutant after cryoinjury. (A). Representative picrosirus red staining paraffin sections of heart in *bre* mutant treated with DMSO (vehicle control), Dex (100 μm), MMP in (1 μm) and Cis (10 μm) illustrate the scar volume at 30 dpc. The black dashed line bounded area indicates the injured scar area. Scale bar: 100 μm. **(B)**. Bar chart (n = 4) shows the quantification of the percentage of scar volume in panel A was significantly different between control and each treatment group at $p < 0.01$ (**) and $p < 0.001$ (***), two tail *t*-test. **(C)** Representative images of immunofluorescent labeling of cardiomyocyte (red) and PCNA (green) in the injured area of *bre* mutant zebrafish treated with DMSO (vehicle control), Dex (100 μm), MMP in (1 μm) and Cis (10 μm) at 7 dpc. The white arrow indicates the double positive $TnnT^+/PCNA^+$ cells in the injured area (IA). Scale bar:100 μm. **(K)**. Bar chart shows the quantification of double positive $TnnT^+/PCNA^+$ (n = 4) in panel H, two tail *t*-test, $p < 0.05$ (*). **(E)** Representative heart sections of *bre* mutant with different treatment, displaying mitotic cells (green) in the injured area at 7 dpc. Scale bar: 100 μm. **(F)**. Bar chart shows the quantification of mitotic cells (n = 3–4) in panel C was significantly different between each group at $p < 0.01$ (**), two tail *t*-test. **(G)**. Representative heart sections of *bre* mutant zebrafish with different treatment show the α-SMA (green, white arrowhead) at 4 dpc. Scale bar: 100 μm. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

apoptotic gene *bcl-2-like* (Fig. 3E and F).

Accordingly, our data are consistent with a previous study reporting that dexamethasone can attenuate leukocytes and reduce apoptotic regenerative cells in the wound [6]. It has been reported that MMP13 was a target for glucocorticoids and the expression is involved in apoptosis processes [27–29]. We then investigated whether MMP9/MMP13 inhibitor I could suppress aberrant apoptosis in *bre* mutant zebrafish. Here, the results were similar with dexamethasone-treated zebrafish, where apoptotic cells were reduced in the injured area of heart with the MMP9/MMP13 inhibitor I treated zebrafish compared with vehicle control zebrafish (Fig. 3C). Additionally, MMP9/MMP13 inhibitor I increased the expression level of the anti-apoptotic gene *bcl-2-l* in heart of *bre* mutant zebrafish compared with vehicle control zebrafish (Fig. 3F).

Defective ERG protein trafficking reduces the number of potassium channels in the cell membrane, thereby predisposing to ventricular arrhythmias in *bre* mutant [15]. Meder et al. and colleagues found that cisapride can rescue the ERG protein trafficking defect in *bre* mutant zebrafish, thereby suppressing arrhythmogenesis [15]. In this regard, we investigated whether cisapride could rescue the excessive inflammation and aberrant apoptosis in the injured area of the heart. Interestingly, treatment with 10 μm cisapride not only reduced the number of L-plastin positive leukocytes and cell death in the injured area of the heart (Fig. 3A, C), but also suppressed the level of expression of *tnf-α*, *il-1β* and *caspase 8* (Fig. 3E and F) in *bre* mutant at 4 dpc compared with vehicle control.

Collectively, these data suggest that a mild dosage of anti-inflammatory chemicals (i.e. dexamethasone and MMP9/MMP13 inhibitor I) and a pro-kinetic drug (cisapride) reduces excessive inflammation and aberrant apoptosis in the injured heart, which might rescue the heart regenerative capability in *bre* mutant zebrafish.

3.4. Anti-inflammatory and prokinetic drugs rescue the heart regenerative ability in bre mutant zebrafish after cryoinjury

It has been reported that an overactive immune response to cardiac injury in adult mice can negatively affect regeneration by promoting the formation of a fibrotic scar [4,21]. Hence, we used anti-inflammatory glucocorticoids dexamethasone (Dex) treatment to investigate if a rescue of fibrotic scar degradation occurs in the ventricle of *bre* mutant zebrafish. We treated *bre* mutant zebrafish with dexamethasone in the first week after cardiac cryoinjury. Subsequently, those zebrafish were placed back in the standard conditions of fish water. Histological analysis demonstrated that scar volume was partly reduced ($p = 0.078$) in zebrafish treated with dexamethasone compared to the vehicle control group (DMSO) at 30 dpc (Fig. 4A). It is known that MMP13 was a target for glucocorticoids in zebrafish [27–29]. Our results show that the expression of *mmp13* was higher in both the untreated and injured heart of *bre* mutant zebrafish compared with AB zebrafish (Fig. 1B and C). To investigate whether downregulating activity of MMP13 could rescue the heart regenerative capability in *bre* mutant zebrafish, we cryoinjured the ventricle and administrated a mild dosage of MMP9/MMP13 inhibitor I (MMP in.; 1 μm) dissolved in the zebrafish water in the first week. The histological results indicated that MMP9/MMP13 inhibitor I promoted fibrotic scar degradation in *bre* mutant (Fig. 4A). Our data shows that cisapride could rescue the excessive inflammation and aberrant apoptosis in *bre* mutant zebrafish (Fig. 3). Therefore, we investigated whether cisapride could promote heart regeneration in *bre* mutant zebrafish. Interestingly, treatment with 10 μm cisapride significantly reduced the scar volume at

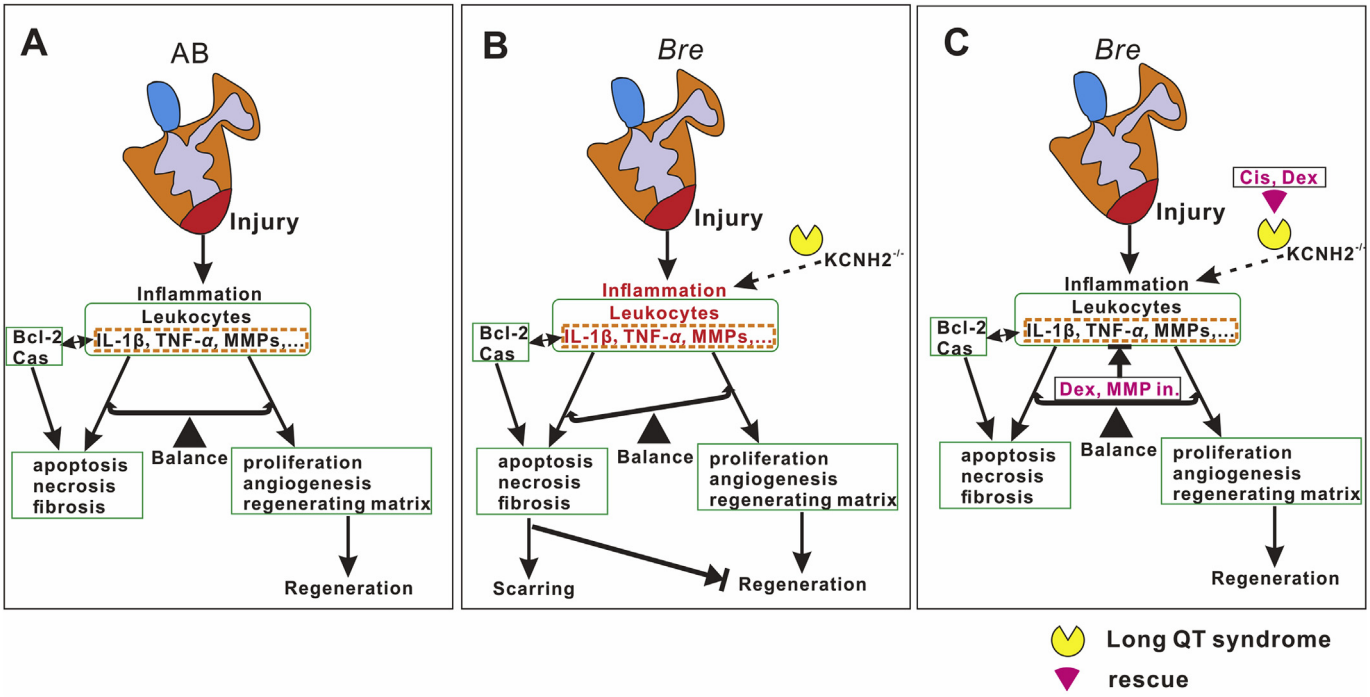

Fig. 5. Proposed model describing inflammation and regeneration after heart cryoinjury. **A**. Cryoinjury triggers an inflammatory response in zebrafish heart. Inflammatory cytokines induce apoptosis and apoptotic factors (i.e. caspases) can activate cytokines. Regenerative process is initiated by inflammatory cytokines. Inflammation orchestrates the balance between apoptosis and regeneration, thereby promoting heart regeneration in AB zebrafish. **B**. Cryoinjury induces excessive leukocyte accumulation and inflammatory gene expression in *bre* mutant ($KCNH2^{-/-}$) hearts. An excessive inflammatory response plays a positive role in regenerating cell proliferation and dedifferentiation. However, highly expressed inflammatory cytokines (i.e. TNF-α, IL-1β) in inflammatory cells induce apoptotic genes (caspase family) and result in aberrant apoptosis directly or indirectly, which impairs heart regenerative ability (i.e. negative role of inflammation). **C.** The anti-inflammatory drug dexamethasone (Dex) and pro-kinetic agent cisapride (Cis) attenuate the excessive inflammation, downregulating the apoptotic genes (*caspase*), upregulating the anti-apoptotic genes (*bcl-2*) and reducing the aberrant apoptotic regenerating cells thereby promoting heart regeneration. Anti-inflammation drugs (Dex and MMP inhibitor) downregulate inflammation and reduce apoptosis, which contributes to improved heart regenerative ability in *bre* mutant.

30 dpc compared with vehicle control (Fig. 4A and B).

It is known that a high concentration of dexamethasone and MMP 9/MMP 13 inhibitor I can suppress the inflammation response, inhibit cell proliferation and the regenerative process [4,5,8]. Thus, we tested the effect of dexamethasone, MMP 9/MMP 13 inhibitor I, and cisapride on regenerative cells in the injured heart. Our data revealed that cisapride didn't affect the $TnnT^+/PCNA^+$ cardiomyocytes and mitosis cells in the injured area, and the MMP 9/MMP 13 inhibitor I slightly, but not significantly, reduced the proliferating cells. However, the treatment with dexamethasone significantly reduced the proliferating cardiomyocytes and mitotic cells (Fig. 4C–F). Furthermore, the number of proliferating cells in the dexamethasone-treated *bre* mutant was similar with that in AB zebrafish (Fig. 2H–K), suggesting that dexamethasone (100 μm) could regulate cell proliferation in *bre* mutant. In addition, dexamethasone, MMP 9/MMP 13 inhibitor I and cisapride did not show significant reduction in the expression of α-SMA in *bre* mutant (Fig. 4G).

Thus, our results indicate that dexamethasone, MMP 9/MMP 13 inhibitor I and cisapride to a large extent promote fibrotic scar degradation in the heart of *bre* mutant zebrafish, albeit affecting cell proliferation.

4. Discussion

Recent studies have reported the essential role of inflammation for cardiac regeneration, but excessive or inadequate inflammatory response can impair this process [6,30]. The mechanisms underlying inflammation-mediated regeneration remain incompletely understood. Here, we showed that in *bre* mutant zebrafish, excessive inflammation occurred after cryoinjury and acted as a double-edged sword during heart regeneration, inducing aberrant apoptosis and causing impairment in the heart regeneration, as well as eliciting more regenerative cells. Immune therapy with a mild dosage of dexamethasone, MMP inhibitors or cisapride promoted cardiac regeneration by reducing apoptosis (Fig. 5). Thus, our study reinforces the notion that heart regeneration is a finely orchestrated process regulated by inflammation, which determines fibrotic scar-based repair and complete cardiomyocyte-based regeneration.

Genetic mutated KCNH2 in *bre* mutant results in life-threatening long QT syndrome cardiac arrhythmia. Recently, accumulating data indicate that inflammation and immunity may be also involved in long QT syndrome [31]. In the present study we revealed that *bre* mutant displays impaired heart regenerative capability; and an overactive immune response following cardiac cryoinjury. Here, inflammatory genes (i.e. *ifn-γ, il-1β, tnf-α, l-plastin, mmp13a etc.*) were markedly upregulated in the heart and an excess number of leukocytes accumulated in the injured area of the heart. The excessive inflammation might further aggravate cardiac arrhythmogenesis in *bre* mutant. Interestingly, it has been reported that the anti-inflammatory glucocorticoid dexamethasone can suppress the LQTS phenotype [10]. Additionally, it is known that KCNH2 and inflammatory cytokine TNF receptor-1 are co-expressed on the cytoplasmic membrane in various cancer cells [32]. Furthermore, our results provide evidence that fin amputation induces excessive inflammatory cells in the injured site in *bre* mutant. These results indicate that KCHN2 might be involved in the inflammation response after tissue injury. Nevertheless, the mechanisms of KCNH2 regulating inflammation in the heart are not well understood, justifying further pursuits in this field.

Inflammation exhibits a seemly paradoxical function to wound healing and regeneration [33]. There is an increasing body of literature indicating contradictory findings where inflammation is both harmful and essential for tissue regeneration [1–3]. Scientific research and clinical data point out that the numbers of cytokines and inflammatory cells accumulated in the injured heart exert cytotoxic action on cardiomyocytes, thereby promoting fibrotic scar formation, cardiac remodeling and worsening cardiac function [4,21]; X. [34,35]. In our study, we found that the level of expression of inflammatory cytokine genes is extremely high, and associated with aberrant apoptosis and larger fibrotic scar in *bre* mutant heart after injury. This finding is consistent with reports that an overactive immune response to cardiac injury in adult mice can negatively affect regeneration by promoting fibrotic scar formation [4,21]. However, recent studies have indicated that neonatal mice lose their heart regenerative ability after cardiac injury when inflammatory cells (i.e. macrophages) are reduced [33,36]. Pertinent to the present study, the robust regenerative capability in zebrafish can be impaired by inhibiting of the inflammatory response [5,22,26,37]. Furthermore, previous studies have found that inflammatory cells play a crucial role in distinct regenerative periods. In these studies, depletion or inhibition of macrophages in the early inflammatory phase, but not the tissue outgrowth stage, tissue remodeling or scar resolution stage, impair tissue regeneration in the salamander and zebrafish [8,37,38]. In addition, more and more studies revealed that inflammation can promote heart regeneration in zebrafish, medaka, and mouse via stimulating cardiomyocyte proliferation [7,33,39]. Lastly, our findings in *bre* mutant further confirm that inflammation can enhance cardiomyocyte proliferation, which is consistent with previous studies. These results suggest that regeneration is well regulated and controlled by inflammation, and disturbances in the inflammatory response can lead to aberrant tissue regeneration [1].

Inflammatory cytokines, such as TNF-α, induce apoptosis as well as promote cell proliferation [32,40]. Here, our data indicate that inflammation plays both a positive and negative role in heart regeneration in *bre* mutant zebrafish. Our transcriptome analysis identified 53 immune-related genes markedly upregulated in the heart of *bre* mutant compared with AB zebrafish. In addition to these genes, *tnf-α* and *il-1β* were considered to be involved in apoptosis and impaired tissue regeneration [1,6], which was also supported by our data (Fig. 1A). It is also reported that gene families such as the *capases* and the *B cell lymphoma* (*bcl*)-2 family are involved in the process of apoptosis, which is at least partly regulated by *tnf-α* signals [41]. Intriguingly, our qRT-PCR and RNA-seq data showed that the level of expression of *caspases b*, *caspase 3a, caspases 7-like*, *caspase 8* and *bcl-2-like* was markedly higher in *bre* mutant heart compared with AB zebrafish at 4 dpc (Fig. 2C, Table S7). In zebrafish, it is reported that *caspase 3* and *caspase 8* mediate the apoptosis process [42,43], and *caspase b* was also involved in activating IL-1β, which further regulates apoptosis in zebrafish [6,44]. The aberrant apoptosis might result in the impaired heart regeneration in *bre* mutant zebrafish. This is consistent with our RNA-seq data which demonstrated that the expression level of muscle-related genes was lower in *bre* mutant heart than in AB zebrafish and the scar degradation was also slower in *bre* mutant zebrafish (Figs. 1A and 2D, S5). However, regenerative cells, such as proliferating cardiomyocyte, mitotic cells, embryonic cardiomyocytes, and α-SMA positive cells, were markedly induced in the heart of *bre* mutant zebrafish after cryoinjury (Fig. 2H-L, S1). Recent data has revealed that leukocytes play a key role in promoting cell proliferation, angiogenesis and other regenerative processes [1,7,45]. Therefore, these results suggest that regenerative cells in *bre* mutant heart might directly or indirectly be associated with the substantial leukocyte accumulation in the injured area.

We demonstrated that a mild dosage of glucocorticoid (dexamethasone) and MMP9/MMP13 inhibitor I reduce the number of leukocyte and apoptotic cells in the injured area of the heart and promote cardiac regeneration in *bre* mutant zebrafish (Figs. 3 and 4). Our study (Fig. 3E) is consistent with previous research reporting that glucocorticoids can suppress the expression of *tnf-α* and *il-1β*, and hence reduce apoptosis [6]. Furthermore, we found that dexamethasone increased the expression of the anti-apoptotic gene *bcl-2-l* in the heart of *bre* mutant. However, dexamethasone not only reduced apoptosis, but suppressed cell proliferation (Fig. 4C–F). Moreover, the number of proliferating cells in dexamethasone-treated *bre* mutant fish was reduced to a similar level as AB zebrafish. Additionally, the MMP9/MMP13 inhibitor treated group had slightly reduced proliferating cells

in the injured area, although this change did not reach significance (Fig. 4C–F). Here, we report for the first time, cisapride can reduce the inflammatory response and apoptosis, without affecting cell proliferation. Therefore, we speculated that dexamethasone and cisapride could rescue the heart regenerative ability in *bre* mutant zebrafish by moderating the expression of *tnf-α, il-1β*, and anti-apoptotic gene *bcl-2-like,* hence reducing aberrant apoptosis (Fig. 5). Nevertheless, treating normal zebrafish with glucocorticoids or MMP9/MMP13 inhibitor also has been known to impair tissue regeneration capability [6,8,26], suggesting that inflammation should be modest and spatiotemporally orchestrated in tissue regeneration. Additionally, it has been demonstrated that dexamethasone and cisapride can rescue the cardiac arrhythmia in *bre* mutant via the glucocorticoid signaling pathway and glycosylation protein trafficking, respectively [10,15]. In fact, our data demonstrate that cisapride and dexamethasone can rescue the heart regenerative ability in *bre* mutant zebrafish via immunosuppression, thus substantiating that KCNH2 might play a role in the regulation of immune system (Fig. 5).

5. Conclusions

In the present study, we report for the first time, anti-inflammatory and pro-kinetic drugs can protect regenerative cells from aberrant apoptosis and promote fibrotic scar degradation in *bre* mutant zebrafish, thereby rescuing the heart regeneration ability after cardiac damage. The present findings provide evidence that KCHN2 is involved in inflammation in zebrafish, in addition to potential immune therapeutic regimens for human genetic LQT patients who suffer from MI.

Conflicts of interest

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

Acknowledgments

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.fsi.2019.03.058.

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