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# Regeneration of infarcted myocardium with resveratrol-modified cardiac stem cells

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## Abstract

The major problem in stem cell therapy includes viability and engraftment efficacy of stem cells after transplantation. Indeed, the vast majority of host-transfused cells do not survive beyond 24–72 hrs. To increase the survival and engraftment of implanted cardiac stem cells in the host, we developed a technique of treating these cells with resveratrol, and tested it in a rat model of left anterior descending (LAD) occlusion. Multi-potent clonogenic cardiac stem cells isolated from rat heart and stably transfected with EGFP were pre-treated with 2.5 μM resveratrol for 60 min. Rats were anaesthetized, hearts opened and the LAD occluded to induce heart attack. One week later, the cardiac reduced environment was confirmed in resveratrol treated rat hearts by the enhanced expression of nuclear factor-E2-related factor-2 (Nrf2) and redox effector factor-1 (Ref-1). M-mode echocardiography after stem cell therapy, showed improvement in cardiac function (left ventricular ejection fraction, fractional shortening and cardiac output) in both, the treated and control group after 7 days, but only resveratrol-modified stem cell group revealed improvement in cardiac function at the end of 1, 2 and 4 months time. The improvement of cardiac function was accompanied by enhanced stem cell survival and engraftment as demonstrated by the expression of cell proliferation marker Ki67 and differentiation of stem cells towards the regeneration of the myocardium as demonstrated by the expression of EGFP up to 4 months after LAD occlusion in the resveratrol-treated stem cell group. Expression of stromal cell-derived factor and myosin conclusively demonstrated homing of stem cells in the infarcted myocardium, its regeneration leading to improvement of cardiac function.

**Keywords:** cardiac stem cells • resveratrol • redox • Nrf2 • Ref-1 • NFκB • SDF • heart • ischaemia

## Introduction

Although there is a great potential for stem cell therapy for regeneration of injured myocardium, major challenge remains the low rate of survival and differentiation of the implanted cells to the injured myocardium. In fact, majority of the implanted stem cells do not survive beyond a few hours [1–3]. The leading cause of cell death after implantation is the microenvironment of the injured tissue including oxidative stress, inflammatory response and development of pro-apoptotic factors. Several attempts have been made to improve the stem cell survival after the cell therapy [4–6]. For example, atorvastin treatment was found to improve the survival and effects of implanted mesenchymal stem cells in post-infarct swine hearts [7]. In another study, haemeoxygenase modified the stem cells to increase their survival [8]. In a more recent study, Pim-1 kinase overexpression modified the cardiac progenitor cell activities [9]. Thus, protection of the implanted cells from the adverse environment of the injured tissue is of utmost importance for a successful cell therapy.

Recently, we were successful in improving survival, homing and engraftment of cardiac stem cells transplanted into the infarcted myocardium by changing the oxidizing environment of the heart into a reducing environment with resveratrol, a redox sensitive polyphenolic antoxidant [10]. In that study, a group of

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rats were fed resveratrol for a period of 21 days that modified the intracellular redox status of the animals. When cardiac stem cells were implanted in these nutritionally modified hearts, the cells survived up to 3 days, and underwent differentiation and proliferation leading to the improvement of cardiac function.

This study was undertaken to examine whether pre-treatment of the cardiac stem cells with resveratrol could promote their engraftment and therapeutic efficacy after transplantation to myocardium subjected to the left anterior descending (LAD) occlusion. The results of our study demonstrated that the resveratrol-modified stem cells survived up to 120 days, underwent differentiation and proliferation leading to improved cardiac function.

# Materials and methods

## Animals

All animals used in this study received humane care in compliance with the Animal Welfare Act and other federal statutes and regulations relating to animals and experiments involving animals and adheres to the principles stated in the Guide for the Care and Use of Laboratory Animals, NRC Publication, 1996 edition. Sprague–Dawley male rats weighing between 250 and 300 g were fed *ad libitum* regular rat chow with free access to water until the start of the experimental procedure. The rats were randomly assigned to one of the following three groups: LAD occlusion in control group, LAD occlusion and stem cell treatment in control group and LAD occlusion and stem cell treatment in resveratrol treated group.

## Resveratrol-treated cardiac stem cells

The multi-potent adult clonogenic cardiac stem cells isolated from rat heart (a gift from Dr. P. Anversa) were cultured in 10 cm dishes, and further treated with resveratrol (Sigma-Aldrich, St. Louis, MO, USA), a polyphenolic phylotoxin present in grapes and red wine, at the dose of 2.5 $\mu$M in culture medium for 2 weeks time [11]. The medium was changed once in 2 days with fresh medium containing resveratrol 2.5 $\mu$M. At the end of 2 weeks of resveratrol treatment, the cardiac stem cells were collected by trypsinization followed by centrifugation at $400 \times g$ for 5 min, and the cells (about $2 \times 10^6$ collected from each 10 cm dish) were suspended in 70 $\mu$l volume of PBS pH 7.4. The cell suspension was directly injected into the border zone of the myocardium immediately after the ligation of the left anterior coronary artery.

## Surgery, LAD occlusion and stem cell therapy

The rats were anaesthetized with ketamine (100 mg/kg)/xylazine (10 mg/kg, i.p.) in combination with buprenorphine (0.5–2.5 mg/kg, s.c., b.d.), intubated and ventilated at a rate of 70 breaths/min. A left lateral thoracotomy was performed under painless and aseptic conditions. After the chest was opened, the pericardium was cut. A 6-0 polypropylene suture was passed under the LAD at the level of the left atrial appendage. Myocardial ischaemia (MI) was produced by permanently occluding the LAD. Then stem cells were delivered directly on the myocardium into the bordering regions of ischaemia in the second (control) and the third (resveratrol-treated) group. The injection needle was introduced 1 mm into the myocardium at an angle (10–20°) in a cranial direction. After completion of all the surgical procedures, the chest was closed with 4-0 nylon sutures and the ventilation rate was reduced to 50 breaths/min till the spontaneous respiration starts. The rats were then extubated and kept in a temperature controlled environment to prevent hypothermia while the rats were continuously monitored. Buprenorphine (0.1 mg/kg s.c.) was administered as an analgesic and gentamycin (1 mg/kg) as an antibiotic to prevent post-surgical infections.

## M-mode echocardiography

After 7 days, 2 months and 4 months of cell therapy, rats were sedated using isoflurene (3%, inhaled), shaved and placed on a heated pad [10]. Ultrasound gel was spread over the precordial region, and ultrasound biomicroscopy (Vevo 770, Visual-Sonics Inc., Toronto, ON, Canada) and transthoracic two-dimensionally (2D) guided M-mode echocardiography were performed using a Vevo 770 ultrasound system (Agilent Technologies, Andover, MA, USA) equipped with a 25-MHz transducer as described by us earlier. In brief, the left ventricle was analysed in apical, parasternal long axis and parasternal short axis views for left ventricular inner diameters (LVIDs) in systole (FS) and cardiac output (CO) determined. 2D directed M-mode images of the left ventricular short axis were taken just below the level of the papillary muscles to analyse ventricular wall thickness and chamber diameter. All left ventricular parameters were measured according to the modified American Society of Echocardiography recommended guidelines. All measurements from different animals were averaged and represented in bar diagram.

## Immunofluorescence and confocal microscopy

Heart tissue samples were collected at the end of experiments: (1) control [implant of resveratrol-non-treated cardiac stem cells (EGFP-labelled) only without the LAD occlusion] and pre-treatment with reservatrol); (2) LAD occlusion followed by implantation with resveratrol-treated cardiac stem cells (EGFP-labelled), fixed in 2% buffered paraformaldehyde (pH 7.4), embedded and frozen in O.C.T. compound, and subjected to cryosectioning [12]. The obtained specimens (5-$\mu$m cuts) were processed for immunofluorescence analysis as described previously [12]. The primary antibody against (i) p65-$\kappa$B (mouse monoclonal IgG), (ii) Nrf2 (nuclear factor E2-related factor) (rabbit polyclonal IgG), (iii) myosin (rabbit polyclonal IgG), (iv) Ref-1 (redox effector factor 1) (rabbit polyclonal IgG) and (v) SDF-1 (goat polyclonal IgG) were from Santa Cruz Biotechnology, Inc. Goat polyclonal IgG against Ki67 were from eBiosciences. Mouse IgG antibody against c-Kit was from Cell Signaling Technology, Inc. The primary antibodies were used in 1:250 dilutions. This was followed by incubation with secondary Alexa-Fluor® fluorochrome-conjugated antibody and nuclei counterstaining with Hoechst 33342 (Molecular Probes, Inc., Eugene, OR, USA) diluted 1:3000. The secondary antibodies used were (i) ALEXA-Fluoro 594-conjugated donkey anti-rabbit IgG, (ii) ALEXA-Fluoro 647-conjugated donkey anti-goat IgG and (iii) ALEXA-Fluoro 594-conjugated donkey antimouse IgG (Molecular Probes, Inc.). Negative controls for non-specific binding included normal donkey serum without primary antibody or with secondary antibody alone. The labelled specimens were rinsed, mounted in Gelvatol (Monsanto Corp., St. Louis, MO, USA), and cover-slipped for fluorescence microscopy. Confocal microscopic images were

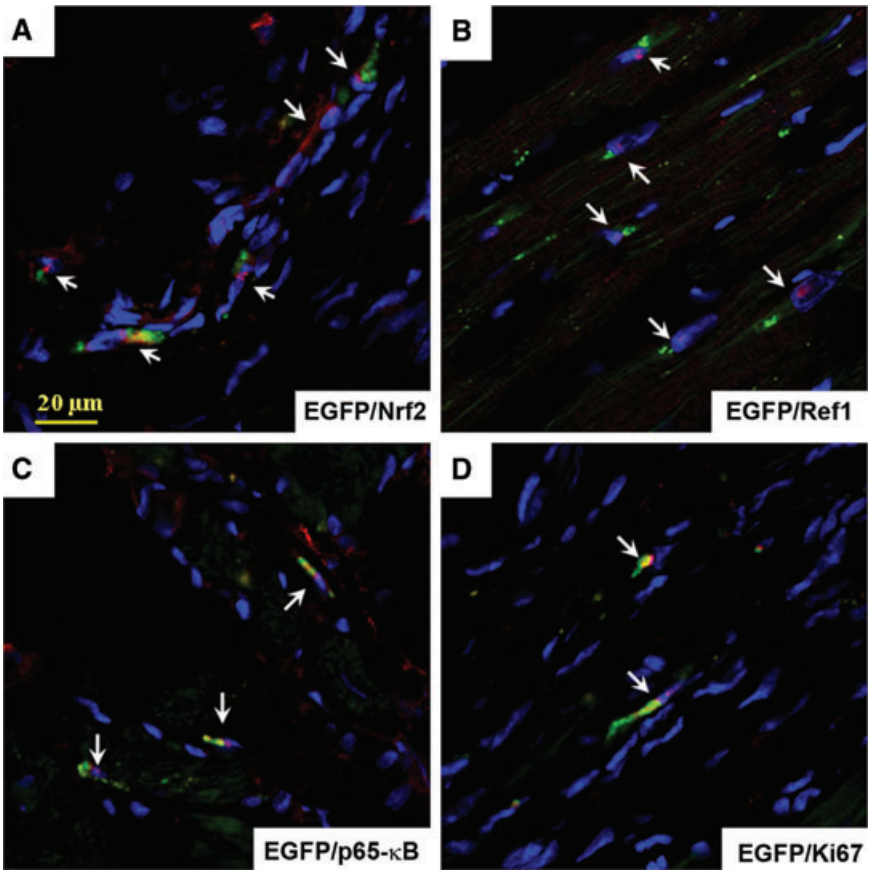


**Fig. 1** Immunofluorescence assessment of redox signalling and proliferative activity of EGFP-positive cardiac stem cells at day 7 after implantation. (**A**–**C**) Projections of nuclear factors Nrf2 (in red), Ref-1 (in red), and p65-κB (in red) respectively in the rat heart specimens from animals subjected to LAD occlusion and implanted with resveratrol-treated cell. EGFP cells appear in green. Localizations of Nrf2, Ref-1, p65-κB (in red) in the nuclei (in blue) are indicated with white arrows. (**D**) Projection of Ki67 in the rat heart specimens from animals subjected to LAD occlusion and implanted with resveratrol-treated cell. Localization of Ki67 (in red) is indicated with white arrows.

obtained using Zeiss LSM 510 confocal laser scanning microscope. The green channel settings for imaging of EGFP-labelled stem cells were excitation = 488 nm and emission = 509 nm. The signals from ALEXA-Fluoro 594 (red channel), ALEXA-Fluoro 647 (purple channel), GFP (green channel) and Hoechst 33342 (blue channel) were collected separately. However, to obtain optimal colour contrast of images, the data collected in the red and purple channels are shown in red and presented in separate figure panels. The background fluorescence was determined for Nrf2, Ki67 and p65 from images specimens labelled with the secondary antibodies only. Processing and analysis of digital images, including, localization of immunostained Ki67, Nrf2 and p65-κB in individual nuclei (region of interest), were conducted using SimplePCI High Performance Imaging software (Compix Inc., Hamamatsu Co., www.cimaging.net) and ImageJ software (http://rsb.info.nih.gov). The index of spatial correlation ($r$) of proteins with nuclei was determined by multiple pixel analysis for pairwise signal interaction of red and blue channels.

## Statistical analysis

All values were expressed as mean ± S.E.M. Analysis of variance test followed by Bonferoni's correction was first carried out to test for any differences between the mean values of all groups. If differences were established, the values of the treated groups were compared with those of the control group by a modified t-test. The results were considered significant if $P < 0.05$.

## Results

### Assessment of redox signalling and proliferative activity of cardiac stem cells implanted to myocardium

The first set of experiments was conducted to verify the presence of effective homing and survival of the resveratrol-treated EGFP-labelled cells implanted to the hearts subjected to the LAD occlusion. With this goal we employed fluorescence confocal imaging for analysis of microsections of cardiac tissues harvested at day 3 (not shown) and day 7 following surgery and implantation. High number of the EGFP-expressing cells were observed in the myocardium at day 7 after LAD occlusion (Fig. 1A–D in green).

We hypothesized that adaptive enhancement of the resveratrol-treated implanted cells was because of improvement of their resistance to stress factors including oxidative stress. To assess the activity of redox-sensitive signalling pathways and respective proliferative activity of the implanted cells, we analysed the nuclear localization of Nrf2, Ref-1, p65 subunit of NFκB (nuclear factor κB) using confocal immunofluorescence microscopy. As

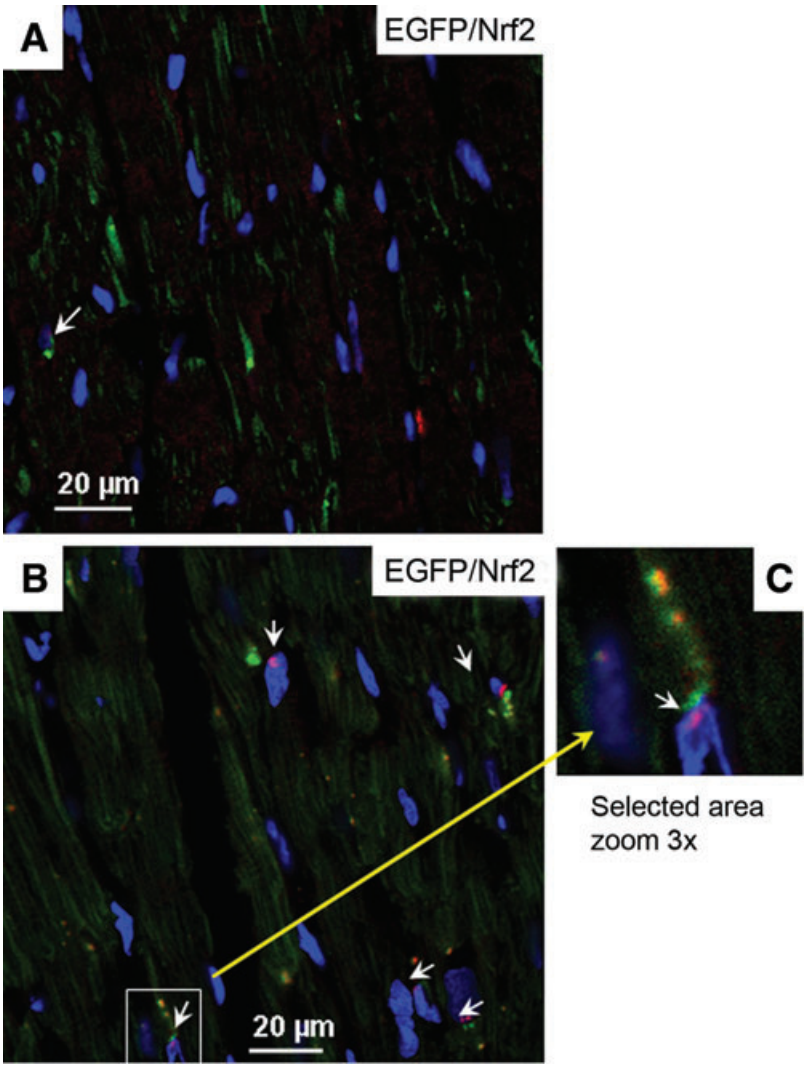


**Fig. 2** Immunofluorescence assessment of the combined response of Nrf2 and Ki67 in cardiac tissue after 4 months following implantation of cardiac stem cells. (**A**, **B**) Projections of Nrf2 in the rat heart specimens from control (*i.e.* implantation of resveratrol-non-treated cells) and the LAD occlusion implanted with resveratrol-treated cell groups, respectively. (**C**) 3× zoom of the selected area indicated in (**B**). Stem cells (in green) were labelled with EGFP as indicated in Materials and Methods. Localization of Nrf2 (in red) in the nuclei (in blue) is indicated with white arrows. (**D**, **E**) Projections of Ki67 in the rat heart specimens from control and the LAD occlusion implanted with resveratrol-treated cell groups, respectively. (**F**) 3× zoom of the selected area indicated in (**E**). Localization of Ki67 (in red) in the nuclei (in blue) is indicated with white arrows. The experimental conditions for the specimen processing, data acquiring, and image presenting are described in Materials and Methods.

demonstrated previously, this technique provides a great deal of reliable data compared to results from immunoblot assessment of the nuclear fraction of the same proteins [12].

The data presented in Figure 1 demonstrate that the EGFP-positive cells have increased nuclear level of Nrf2 (panel A, in red, indicated with arrows). Regulation of redox signalling by Nrf2 could also be regulated by Ref-1, which similarly to Nrf2 can bind to antioxidant response elements and interacts with NFκB—essential for protection against cardiac infarction. Nuclear localization of Ref-1 and p65-κB in EGFP-positive implanted cells is shown in Figure 1B and C (in red, indicated with arrows), respectively. That effect was accompanied by substantial elevation of Ki67 expression in EGFP-positive cells as compared to the host cells (panel D, in red, indicated with arrows), where Ki67 expression was used as an index of cell proliferative activity.

In the second set of experiments we analysed the effect of the treatment of the EGFP-labelled cells with resveratrol on their engraftment and therapeutic efficacy after 4 months following the LAD occlusion and implantation. High number of the EGFP-expressing cells were consistently present in the myocardium when the cells were pre-treated with resveratrol as compared to control (Figs 2, 4, 5, in green). Moreover, we observed the resveratrol-induced prolonged alterations on the redox status in these cells that was determined by assessment of responses of nuclear factors Nrf2, Ref-1 and NFκB. The data presented in Figure 2A–C show that the nuclear localization of Nrf2 was significantly increased in the group of LAD occlusion followed by implant with resveratrol-treated cardiac stem cells (compared to the control group implanted with resveratrol-non-treated cells). The indexes of spatial correlation '*r*' of Nrf2 immunoreactivity with nuclei for the projections presented in Figure 2A and B were 0.014 and 0.16, respectively. The EGFP-positive cells in the LAD occlusion specimens were characterized by a pronounced expression of Ki67 protein (Fig. 2E and F) compared to the control (Fig. 2D).

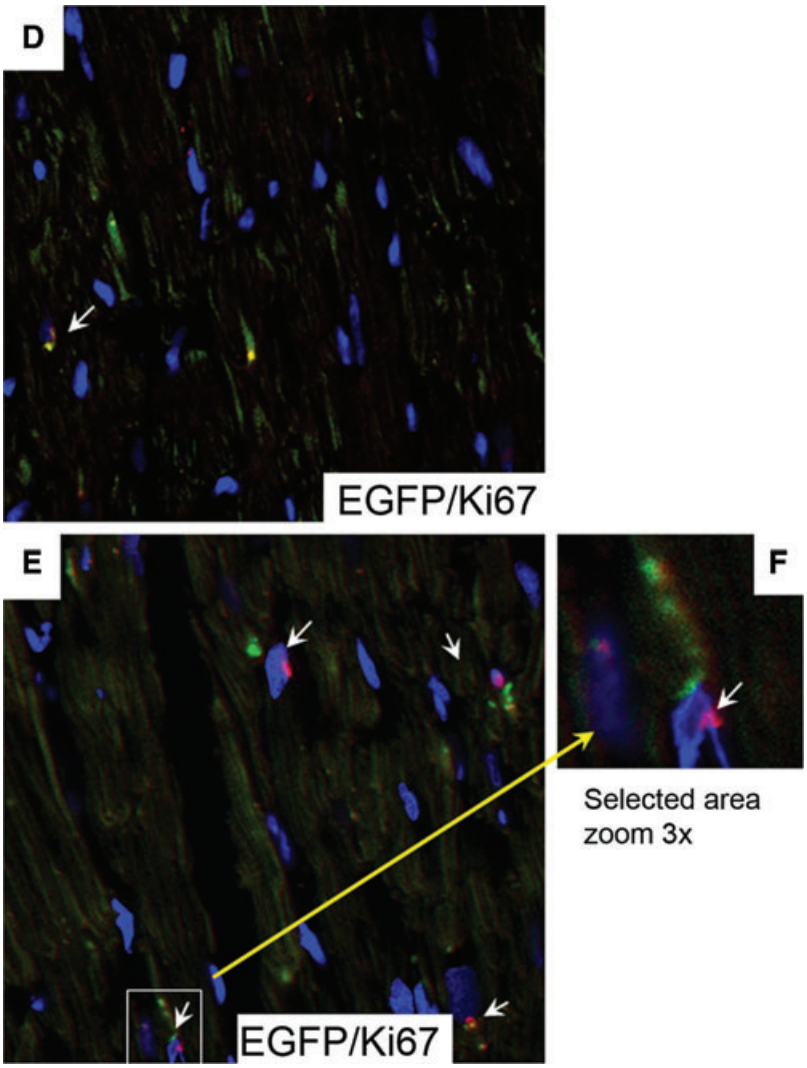


**Fig. 2** Continued

Nuclear localization of Ref-1 in EGFP-positive implanted cells is shown in Figure 3. We have found that the resveratrol-treatment procedure results in a prolonged increase in the Ref-1/nuclear interaction in hearts subjected to infarction. Thus, the indexes of spatial correlation '*r*' of Ref-1 immunoreactivity with nuclei for the projections presented in Figure 3D (control) and H (LAD occlusion) were 0.07 and 0.52, respectively. Similar effect was demonstrated for the p65 subunit of κB (Fig. 4A–C) that was accompanied by substantial elevation of Ki67 expression in EGFP-positive cells (Fig. 4D–F).

We have found that these proliferating EGFP-labelled cells show also immunoreactivity to the stroma-derived factor 1 (SDF-1), a marker of the mesenchymal cardiac precursors (Fig. 5A–D), suggesting a beneficial effect of pre-treatment with resveratrol on the engraftment of cardiac stem cells implanted to the myocardium subjected to LAD occlusion. Efficacy of engraftment of the implanted cells was documented by increase in immunoreactivity of myosin (Fig. 5E–H) and presence of immunoreactivity of c-Kit in the infarcted myocardium (Fig. 6). Based on these observations we suggest that the engrafted cardiac stem cells can differentiate into mature cardiac cells and/or promote regeneration and recovery of injured myocardium.

SDF-1 secretion from the injured heart is stimulated by ischaemia and it enhances the recruitment of haematopoietic, bone marrow derived progenitor cells to ischaemic myocardium. Our confocal microscopic images (Fig. 6) show that SDF-1 is closely associated with EGFP and c-kit 4 months after LAD occlusion in resveratrol-treated myocardium. These results suggest that SDF-1 plays an important role in the recruitment of adult cardiac stem cells to the ischaemic myocardium.

## M-mode echocardiography

The echocardiography data echoed the results of cardiac regeneration (Fig. 7). M-mode echo cardiogram of rats recorded at

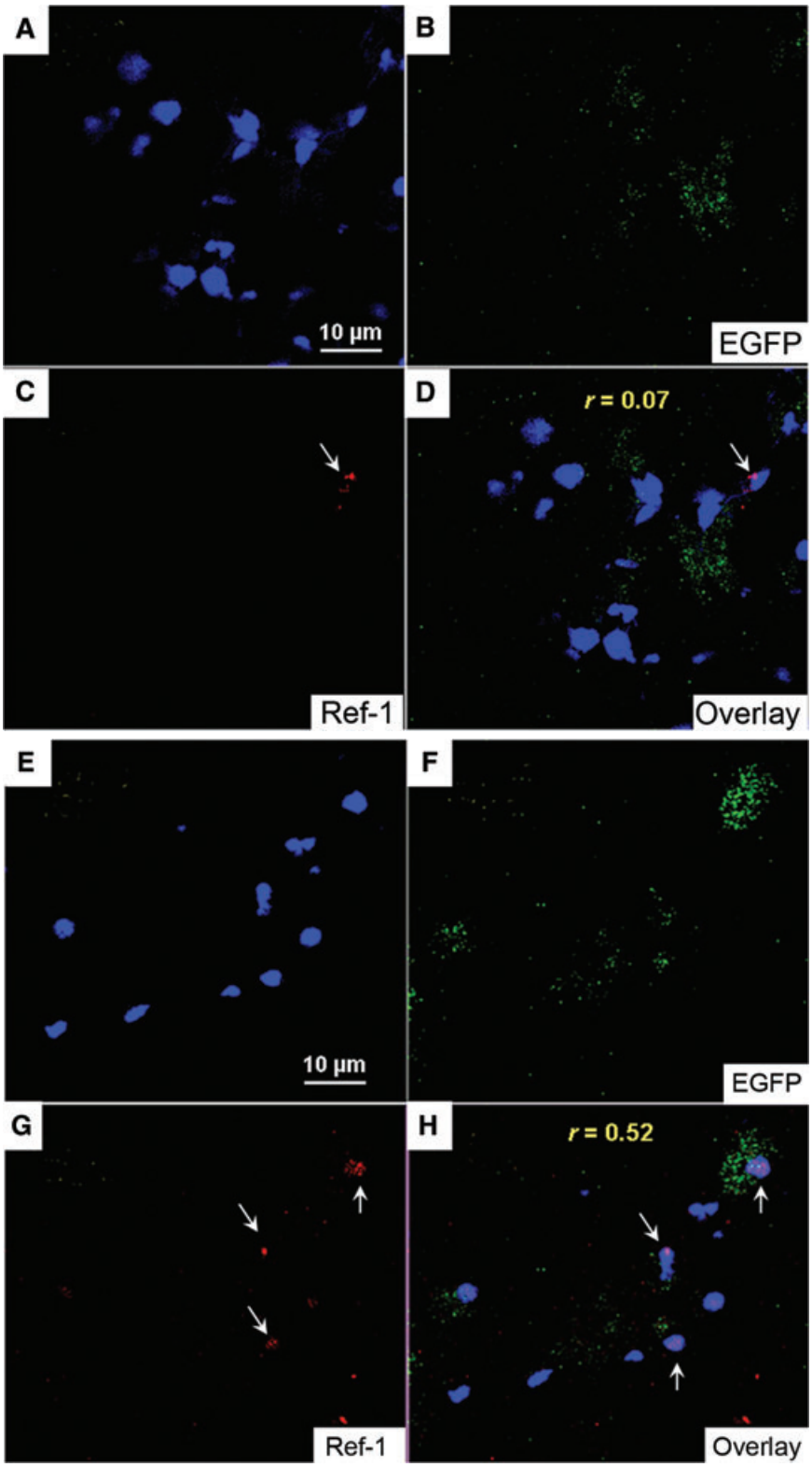


**Fig. 3** Immunofluorescence assessment of Ref-1 response in cardiac tissue after 4 months following implantation of cardiac stem cells. (**A**–**D**) Control and (**E**–**H**) the LAD occlusion implanted with resveratrol-treated cell groups. Projections of Ref-1 and nuclear localization of Ref-1 in cardiac cells are shown in (**C**), (**G**) and (**D**), (**H**), respectively. Stem cells were labelled with EGFP as indicated in Materials and Methods. Localization of Ref-1 (in red) in the nuclei (in blue) is indicated with white arrows.

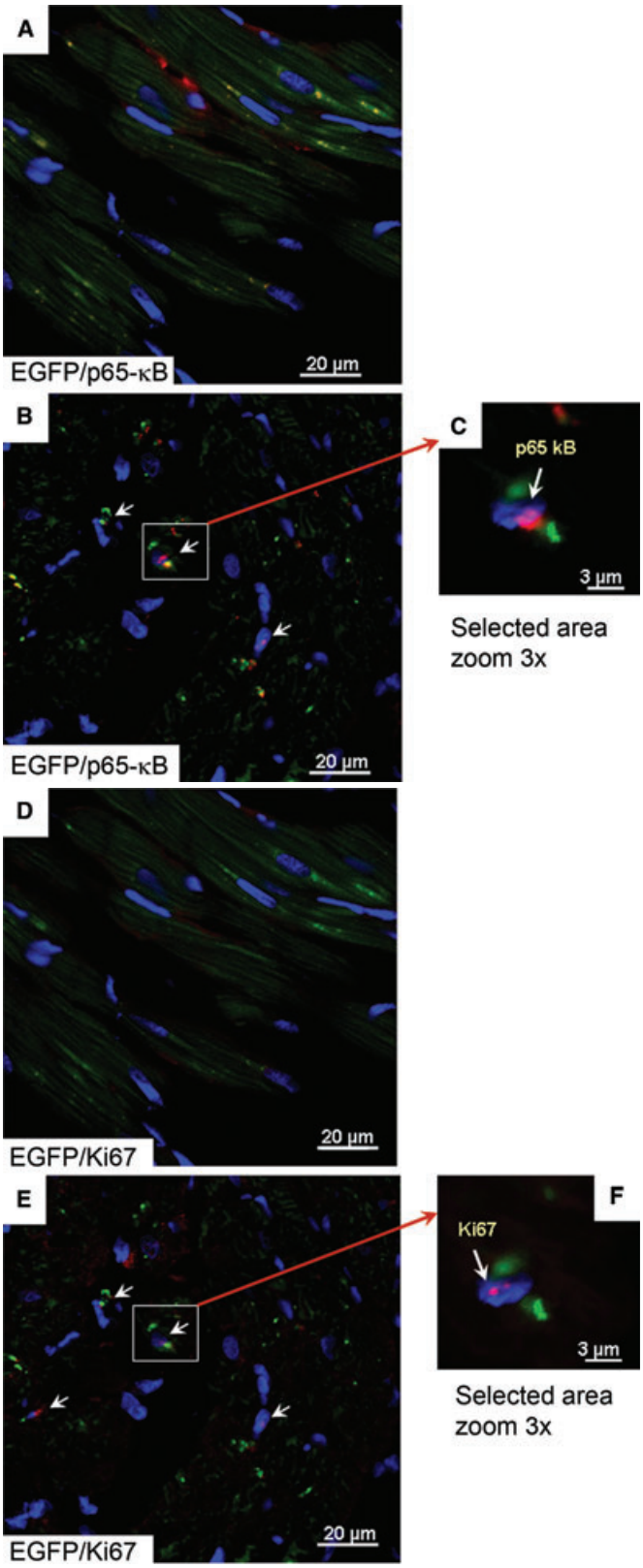


**Fig. 4** Immunofluorescence assessment of the combined response of NFκB and Ki67 in cardiac tissue after 4 months following implantation of cardiac stem cells. (**A**, **B**) Projections of p65- κB in the rat heart specimens from control and the LAD occlusion implanted with resveratrol-treated cell groups respectively. (**C**) 3× zoom of the selected area indicated in (**B**). Stem cells were labelled with EGFP as indicated in Materials and Methods. Localization of p65- κB (in red) in the nuclei (in blue) is indicated with white arrows. (**D**, **E**) Projections of Ki67 in the rat heart specimens from control and the LAD occlusion implanted with resveratrol-treated cell groups, respectively. (**F**) 3× zoom of the selected area indicated in (**E**). Localization of Ki67 (in red) in the nuclei (in blue) is indicated with white arrows.

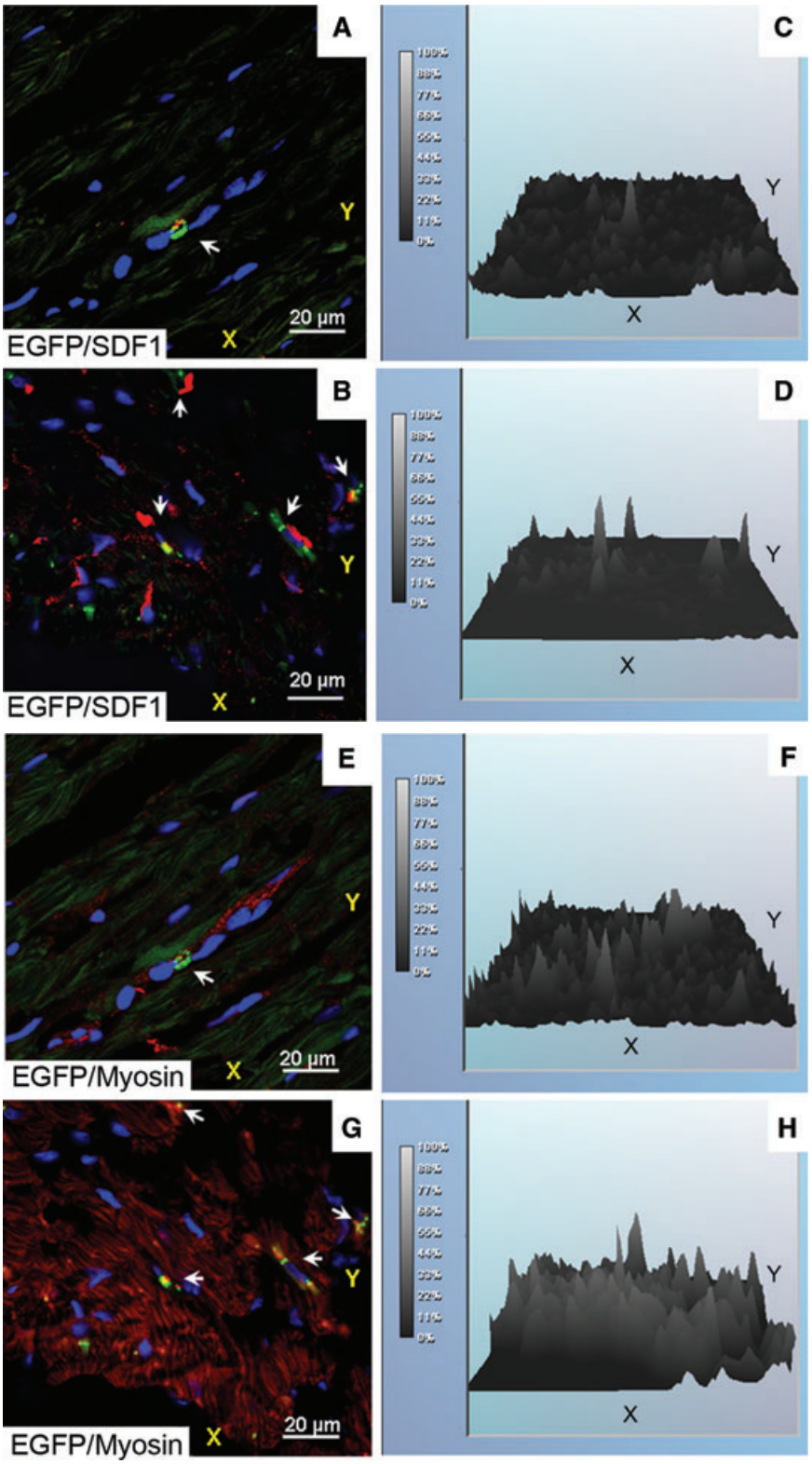


**Fig. 5** Immunofluorescence assessment of the combined effect of cardiac stem cells on the expression of SDF-1 and myosin in myocardium after 4 months following implantation. (**A**, **B**) Projections of SDF-1 in the rat heart specimens from control and LAD-treated groups, respectively. (**C**, **D**) Relative intensities of immunofluorescence of SDF-1 shown in (**A**) and (**B**), respectively. Stem cells were labelled with EGFP (in green) as indicated in Materials and Methods. Fluorescent signals from SDF-1 and nuclei were collected in the red and blue channels, respectively. Localization of SDF-1 in EGFP-labelled cells is indicated with white arrows. (**E**, **F**) Projections of myosin (in red) in the rat heart specimens from control and LAD-treated groups, respectively. (**G**, **H**) Relative intensities of immunofluorescence of myosin shown in (**E**) and (**F**), respectively.

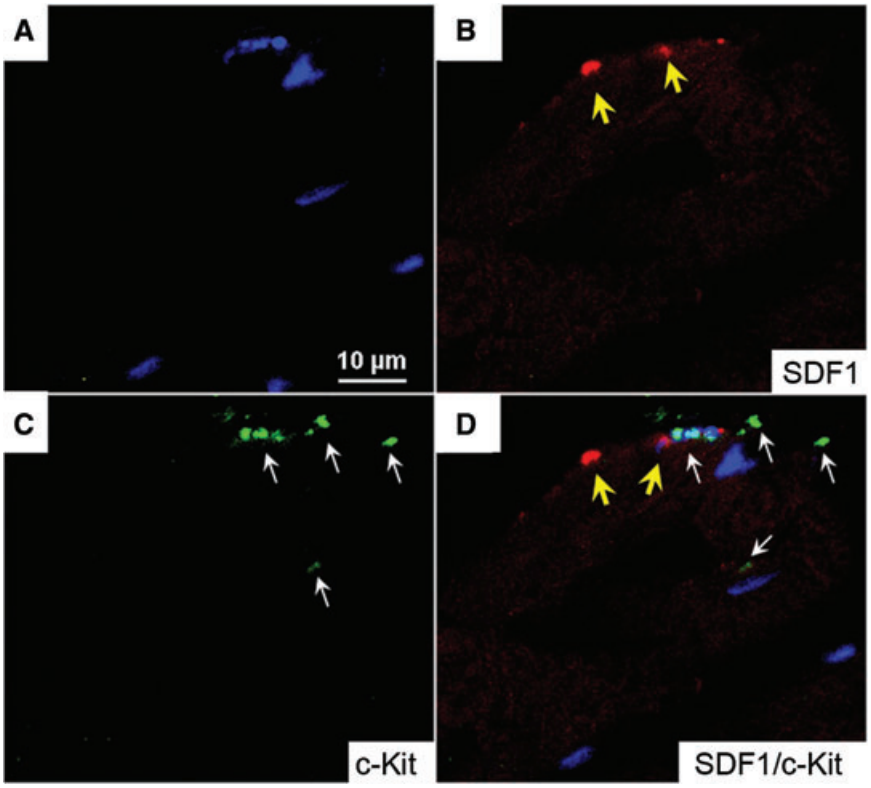


**Fig. 6** Immunofluorescence assessment of c-Kit and SDF-1 in cardiac tissue after 4 months following LAD occlusion and implantation of resveratrol-treated cardiac stem cells. (**A**) Nuclei counterstaining, (**B**) projection of SDF-1, (**C**) projections of c-Kit and (**D**) overlay of (**A**–**C**). Localization of SDF-1 (in red) and c-Kit (in green) is indicated with arrows.

30 days and 4 months after survival surgery reveal that resveratrol treatment enhanced the cardiac stem cell-mediated improvement in cardiac functional parameters such as LVID in systole, LVID in diastole, fractional shortening (FS), ejection fraction (EF) and cardiac output (CO).

## Discussion

The results of this study suggest that resveratrol-modified cardiac stem cells survived up to a period of 120 days and underwent active proliferation and differentiation. It appeared that these cells were able to regenerate infarcted myocardium resulting in improved cardiac function as revealed with M-mode echocardiography. These effects appear to be mediated through the ability of resveratrol to enhance the redox potential in the target organ.

The major hitch of the success of stem cell therapy is the limited survival of the cells after the therapy. In most cases, the stem cells do not survive beyond hours after the implantation because of the adverse environment of the injured tissue. When human mesenchymal stem cells injected into areas of rat peri-infarct and normal myocardium were compared after 28 days, 90% of the labelled cells were found in the normal myocardium, whereas only 18% of the cells were detectable in the infarcted myocardium [3]. It is believed that ischaemia creates an inhospitable environment by inducing local expression of inflammatory cytokines that promote cell apoptosis [4]. A number of approaches have been used to prolong the number and survival of stem cells after the therapy. For example, survival of rat mesenchymal stem cells injected into peri-infarct myocardium is enhanced by 20% by incubating them with SDF-1$\alpha$, an activator of the antiapoptotic Akt pathway [13]. In another study, transfection of the cells with Bcl-2 before injection to the infarcted heart, induced 1.2-fold increase in cell survival, 15% higher capillary density in infarct border zone and 17% smaller infarct size than control hearts [14]. As mentioned earlier, in a recent study, a group of rats were fed resveratrol, and when cardiac stem cells were injected into the infarcted myocardium, the cells survived up to a period of 30 days, and performed proliferation and differentiation leading to the regeneration of infarcted myocardium [10]. The results of this study indicated that resveratrol modified the redox environment in the infarcted heart, which was instrumental for the increased survival of the stem cells.

In this study, we directly incubated the cardiac stem cells with resveratrol, and shown that resveratrol can alter the redox environment of the cells as suggested by the assessment of responses of Ref-1, Nrf2 and NF$\kappa$B transcriptional factors. The results of the confocal microscopy indicate that improvement of the engraftment of implanted cells during 4 months of stem cell therapy is correlated with a prolonged induction of Nrf2 and Ref-1. Nrf2 and Ref-1 are the redox proteins that are abundantly present in mammalian cells

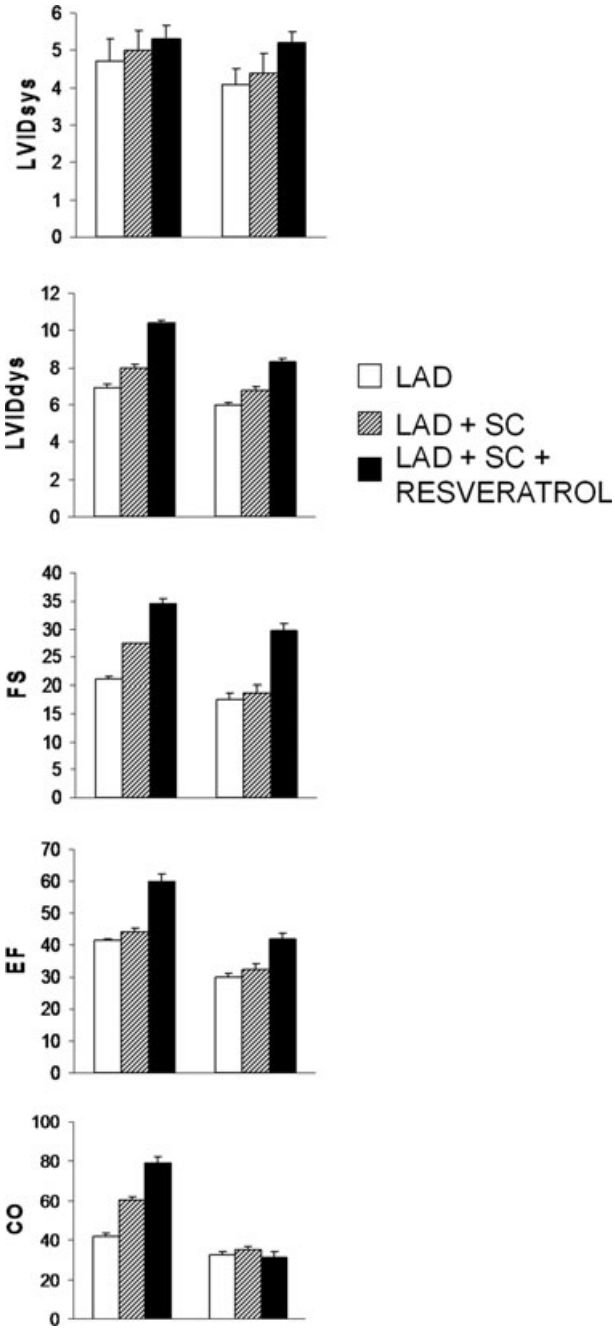


**Fig. 7** Cardiac functional parameters determined after 2 and 4 months of cell therapy determined by M-mode echocardiography. The results are mean ± S.E.M. of three different animals. LVIDsys: left ventricular internal diameter in systole; LVIDdys: left ventricular internal diameter in diastole; FS: fractional shortening; EF: ejection fraction; CO: cardiac output.

including the heart. They participate in the redox-regulated signal transduction processes and manipulate redox sensitive transcription factors such as NFκB [12, 15–17]. Increased DNA binding of NFκB is an essential element for the cardioprotection achieved by preconditioning as inhibition of NFκB abolished cardioprotection achieved by preconditioning [18]. In a previous study, we showed that preconditioning triggers the translocation of Ref-1 into the nucleus, where it becomes associated with the activated NFκB [12]. At the same time nuclear translocation of Ref-1 is associated with a reduction of oxidative stress in the heart.

It is universally believed that adverse tissue environment around the injured tissue such as infarcted heart is among other factors, increased amount of oxidative stress, which is responsible for the reduced viability and function of the stem cells. Accordingly, any intervention to reduce oxidative stress in the target tissue can improve the performance of cell-based therapy. As mentioned earlier, we could prolong the life and function of the cardiac stem cells after implantation into the infarcted heart by reducing the tissue environment with resveratrol. There we took a different approach, that is by maintaining a reducing environment in the body including the heart by feeding resveratrol. Resveratrol was shown to alter the oxidative environment into a reducing environment [18]. In contrast, in this study, the intracellular environment of the stem cells was successfully modified as demonstrated by the association of Nrf2, Ref-1 and NFκB with the stem cells. Although Ref-1 is a redox protein regulating redox-sensitive transcription factors, Nrf2 is a master gene of the endogenous defense system that binds to antioxidant response element. Normally, Nrf2 is retained in the cytosol with its natural inhibitor Keap-1 and redox activation of Nrf2–Keap-1 results in the dissociation of Nrf2 from Keap-1 leading to its stabilization followed by nuclear translocation [12, 15–17].

Resveratrol as a grape skin and red wine derived polyphenolic antioxidant has been found to protect almost all the vital organs including the heart [19]. This polyphenolic phytoalexin can reduce diverse cardiovascular problems including hypertension, atherosclerosis, cardiomyopathy, heart failure, diabetes, obesity and a variety of ischaemic heart diseases [19–23]. Resveratrol has been found to convert ischaemia-induced death signal into a survival signal by manipulating a number of genes and transcription factors. It can even modify the early stress processes by induction of autophagy [24]. Its ability to prolong the survival and functionality of stem cells certainly supports its numerous actions by which it promotes cell survival.

## Conflict of interest

The authors confirm that there are no conflicts of interest.

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