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Mechanisms of the protective effects of BMSCs promoted by TMDR with heparinized bFGF-incorporated stent in pig model of acute myocardial ischemia

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Abstract

This study investigates the mechanism by which transmyocardial drilling revascularization combined with heparinized basic fibroblast growth factor incorporated degradable stent implantation (TMDRSI) enhanced effects of bone marrow mesenchymal stem cells (BMSCs) transplantation against acute ischemic myocardial injury. After the mid-third of left anterior descending artery was ligated, miniswine were divided into none-treatment group (control, $n = 6$), BMSCs implantation group (C, $n = 6$), TMDRSI group (TS, $n = 6$) and TMDRSI and BMSCs implantation group (TSC, $n = 6$). Two channels of 3.5 mm diameter were established by a self-made drill in the ischemic region, into which a stent was implanted for the TS and TSC groups. Autologous BMSCs were transplanted into the ischemic region in C group or around the channels in TSC group. Expression of von Willebrand factor, vascular endothelial growth factor, interleukin-1β, transforming growth factor-β_3, cell proliferation and apoptosis, histological and morphological analyses, myocardial remodelling and cardiac function were evaluated at different time-points. Six weeks after the operation, the above indices were significantly improved in TSC group compared with others ($P < 0.05$), though C and TS groups also showed better results than the control group ($P < 0.05$). The new method was shown to have activated paracrine pathway of transplanted BMSCs, increased survival and differentiation of such cells, and enhanced effects of BMSCs transplantation on myocardial remodelling, which may provide a new strategy for cell therapies against acute ischemic myocardial injury.

Keywords: coronary artery disease • fibroblast growth factor 2 • stem cell transplantation • stent • ventricular remodelling

Introduction

Although bone marrow mesenchymal stem cells (BMSCs) implantation has emerged as a promising treatment for ischemic heart disease for their potential effects to improve myocardial function and reverse ventricular remodelling [1–4], experimental and clinical studies also yielded some negative results [5–7]. It was speculated that the effect of cell transplantation may be largely impacted by the hostile microenvironment of the ischemic region [8, 9].

In our previous report [10], we have described a novel method for myocardial revascularization, *i.e.* transmyocardial drilling revascularization (TMDR) combined with heparinized basic fibroblast growth factor (bFGF)-incorporated degradable tubular stent implantation (TMDRSI). Our preliminary results showed that, for a miniswine model of acute myocardial ischemia, this new method, combined with $Flk1^+CD34^-CD31^-$ BMSCs transplantation,

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enhanced angiogenesis and myocardial perfusion, and promoted cardiac function more effectively than single approaches [11]. To explore the underlying mechanism, the present study was set to assess the following parameters, which included differentiation and paracrine function of transplanted cells, cell proliferation and apoptosis, and myocardial remodelling.

Material and methods

Miniswine experiment was approved by Tianjin Administrative Committee for Laboratory Animals. Animal care and experimental procedures were carried out strictly in compliance with the Guide for the care and use of laboratory animals published by the National Institutes of Health in 1996.

Preparation of autologous Flk1$^+$CD34$^-$CD31$^-$ BMSCs, immunophenotyping analysis and *in vitro* labelling

Autologous BMSCs were isolated as described by our previous report [11]. Briefly, bone marrow aspirated from the anterior iliac crest was diluted with heparinized phosphate-buffered saline (PBS). Ficoll density gradient centrifugation (1500 rpm for 10 min. at 20°C) was used to separate BMSCs from other cells. The residual cells were incubated in a 95% humidified incubator at 37°C with a complete medium containing 58% DMEM/F12 (Gibco Life Technologies, Paisley, UK), 40% molecular cellular and developmental biology (MCDB)-201 (Sigma-Aldrich, St. Louis, MO, USA), 100 μg/ml penicillin, 2% foetal bovine serum, 100 U/ml streptomycin sulphate (Gibco Life Technologies) and 5% CO_2. After 48 hrs, suspended cells were discarded and the adherent layer was washed with fresh complete medium every 3 days, and continually cultured till about 90% confluence (~1 week). Cells were harvested by trypsinization (0.25% trypsin) before transplantation.

BMSCs were washed twice with PBS containing 0.5% bovine serum albumin (Sigma-Aldrich) and then stained with anti-human CD34, Flk1 (Santa Cruz Biotechnology, Santa Cruz, CA, USA), CD31 and CD45 (BD-PharMingen Biotechnology, San Diego, CA, USA) at 10–20 ng/ml for 30 min. at 4°C. The immunophenotype was analysed with fluorescence activated cell sorting [9, 11]. Before implantation, BMSCs were labelled with a cross-linkable membrane dye 1′-dioctadecyl-3,3,3′,3′-tetramethylindocarbocyanineperchlorate (CM-DiI, 3 μM, Invitrogen Corporation, Carlsbad, CA, USA).

Surgical procedure

Miniswine (25–30 kg) were anesthetized as described previously [10]. Briefly, the mid-third of left anterior descending artery was ligated for establishing the model of acute myocardial ischemia. Thereafter, the animals were randomly assigned to four groups (six each): Control group (no treatment), C group (cell transplantation only), TS group (TMDRSI only) and TSC group (TMDRSI + cell implantation).

After the model was successfully established, two transmyocardial tunnels (pitch of 10 mm) were created at the centre of ischemic area of the left ventricle from epicardium with a self-made drill equipped with a hollow drill bit of 3.5 mm in diameter at 13,000 rpm (TS and TSC groups). Immediately after the channels were created, a stent was manually implanted. Epicardial purse-string stitches placed in advance were ligated to control bleeding and fix the stent. Subsequently, autologous BMSCs (2×10^7) in 100 μl saline were injected with a sterile microinjection at five sites within the ischemic region (C group) or around the channels (TSC group). The needle was advanced 5 mm into the myocardium, and the cells were injected 1.5 mm away from the centre of the channel. Antibiotics were administrated intramuscularly for 3 days after the operation.

Preparation of the stent has been described in our previous report [10]. In this study, each stent contained 15 μg of recombinant human bFGF (molecular mass 17.4 kD, purity > 97%, R&D Systems Inc., Minneapolis, MN, USA) and 25 mg heparin (150 U/mg, Dingguo Biotechnology, Beijing, China).

Six weeks after the operation, the animals were killed with an overdose of potassium chloride. Tissues around the channels were harvested for RT-PCR and histological analysis.

Table 1 Primer sequences and RT-PCR conditions

Gene	Sequences	Product size (bp)	Tm (°C)	Cycles
GAPDH	5′-gacccttcattgacctccac-3′	336	50	30
	5′-cacgacatactcagcaccag-3′			
vWF	5′-cttggtcaaagacaacgggt-3′	445	60	30
	5′-tgagtctgttctgcaggtgg-3′			
VEGF	5′-atgaactttctgctctcttgggtgc-3′	529	50	30
	5′-gtcacatctgcaagtacgttcgtttt-3′			
IL-1β	5′-atggccatagtacctgaacccgcc-3′	704	52	30
	5′-accacttctctcttcaagtcccctg-3′			
TGF-β$_3$	5′-ggctcacgcatgaagatgcacttgc-3′	462	52	30
	5′-tagcgctgcttggctatgtgctcatc-3′			

GAPDH: glyceraldehyde-3-phosphate dehydrogenase; vWF: von Willebrand factor; VEGF: vascular endothelial growth factor; IL-1β: interleukin-1β; TGF-β$_3$: transforming growth factor-β$_3$.

RT-PCR analysis

The expressions of von Willebrand factor (vWF), vascular endothelial growth factor (VEGF), interleukin-1β (IL-1β), transforming growth factor-β$_3$ (TGF-β$_3$) were determined, with glyceraldehyde-3-phosphate dehydrogenase (GAPDH) used as the internal control. Primer sequence and RT-PCR reaction condition are showed in Table 1. An UVIpro gel documentation system (UVIpro, UVI, Cambridge, UK) was used for semi-quantization analysis of PCR product.

Histological analysis

Thin myocardium sections (5 μm) were counterstained with Masson trichrome to delineate the myofilament structure. For each group, five non-overlapping fields at 100× magnification in transverse sections were

randomly captured with a video camera and stored in TIFF format. Myocardial density (MD) was quantified with an Image Pro Plus (IPP) 6.0 software package (IPP, Media Cybernetics, MD, Silver Spring, USA). Positively stained areas were padded with a single colour and converted to pixels through optical density (OD) calibration.

Ki-67 staining was performed for determination of cell proliferation. Cell apoptosis was detected by a terminal deoxynucleotidyl transferase-mediated dUTP nick-end labelling (TUNEL) method.

Identification of transplanted cells

Cells labelled with CM-DiI (red fluorescence) *in vitro* were examined under a fluorescence microscope. Immunofluorescence was carried out with anti-human factor vWF antibody, anti-human smooth muscle actin antibody and anti-human cardiac Troponin T (cTnT) antibody (Abcam Ltd., Cambridge, UK) to identify the grafted cells and their types. Red fluorescence-positive sections were further processed by incubating with fluorescein isothiocyanate conjugated secondary antisera in frozen myocardial sections. All antibodies have cross-reactivity with miniswine. After rinsing with PBS, sections were observed and photographed under both fluorescence and light microscopes.

MRI scan

All studies were performed on a 1.5 T magnetic resonance imaging (MRI) scanner with a dedicated cardiac four-element phased-array receiver coil (Signa CV/i; GE Medical Systems, Milwaukee, WI, USA). Repeated instrumented breath-holds and gating to the electrocardiogram were applied to minimize the influence of cardiac and respiratory motion on data acquisition. Cine-MRI was performed with a steady-state free precession technique (Fiesta; GE Medical Systems) with the following imaging parameters: 20 temporal phases per slice; field of view 35 $\times$ 26 mm; slice thickness 8 mm; repetition time 3.6 msec.; time to echo 1.5 msec.; flip angle 45°; bandwidth 83 kHz; number of averages 0.75. Left ventricular end diastolic volume (EDV), end systolic volume (ESV), ejection fraction (EF) and local left ventricular wall thickness where stents or BMSCs were implanted were determined with a MR analytical software system (MASS version 5.1).

Statistical analysis

All offline measurements were made by investigators blinded to the treatment. Results were presented as mean $\pm$ SD. Statistical analysis was performed with a SPSS 13.0 software package (SPSS Inc, Chicago, IL, USA). Paired Student t-test was used for self-comparison. ANOVA with Bonferroni *post hoc* correction was used to compare experimental measurements in each group. A value of $P < 0.05$ was regarded as statistically significant.

Results

Of the 28 miniswine studied, 4 were excluded from further experiments due to intractable ventricular fibrillation after ligation of the left anterior descending artery, whereas 24 were ultimately determined for the subsequent procedures. No severe events of malignant arrhythmias, embolization, bleeding or haemodynamic abnormality had occurred during the stent or cell implantation procedures.

Morphology and immunophenotypes of BMSCs

After being cultured for 7 days, the cells had appeared as colonies of large flat cells or spindle-like cells. Fluorescence-activated cell sorting showed that such cells did not express haematopoietic markers CD34, CD45 or endothelial marker CD31, while the proportion of $Flk1^+$ cells was about 60%.

Enhanced expression of vWF, VEGF, IL-1β and TGF-β_3 in TSC group

Six weeks after treatment, semi-quantitative analysis of PCR product showed that the expression levels of vWF, VEGF, IL-1β and TGF-β_3 mRNA in TSC group were significantly increased compared with control, C and TS groups, though those in C and TS groups also up-regulated relative to the control group ($P < 0.05$). (Fig. 1)

Increased cell proliferation in TSC group

Representative photographs of Ki-67 stained sections of different groups are shown in Figure 2A to D. The count of $Ki\text{-}67^+$ cells revealed that cell proliferation has significantly increased in C group (15 $\pm$ 2 cell/hpf) and TS group (23 $\pm$ 2 cell/hpf) compared with the control group (9 $\pm$ 1 cell/hpf, $P < 0.001$), which was more significant in TSC group (40 $\pm$ 2 cell/hpf, $P < 0.001$) even at comparison with C and TS groups (Fig. 2E).

Inhibited cell apoptosis in TSC group

As shown in Figure 3, the amount of TUNEL-staining positive cells showed that cell apoptosis was significantly inhibited in C group (29 $\pm$ 4 cell/hpf, $P = 0.021$) and TS group (17 $\pm$ 2 cell/hpf, $P < 0.001$) compared with the control (35 $\pm$ 3 cell/hpf), which again was more significant in TSC group (10 $\pm$ 3 cell/hpf, $P < 0.01$) even at comparison with C and TS groups (Fig. 3E).

Enhancement of myocardial viability in TSC group

Representative photographs of Masson trichrome stained section of different groups are shown in Figure 4. Qualitative analysis of MD demonstrated significant increases in myocardial viability in C group (OD = 57,034 $\pm$ 5183 pixels/hpf, $P < 0.001$), TS group (OD = 94,800 $\pm$ 14,076 pixels/hpf, $P < 0.001$) and TSC group (OD = 148,002 $\pm$ 9556 pixels/hpf, $P < 0.001$) as compared with control group (OD = 16,384 $\pm$ 2172 pixels/hpf). Importantly,

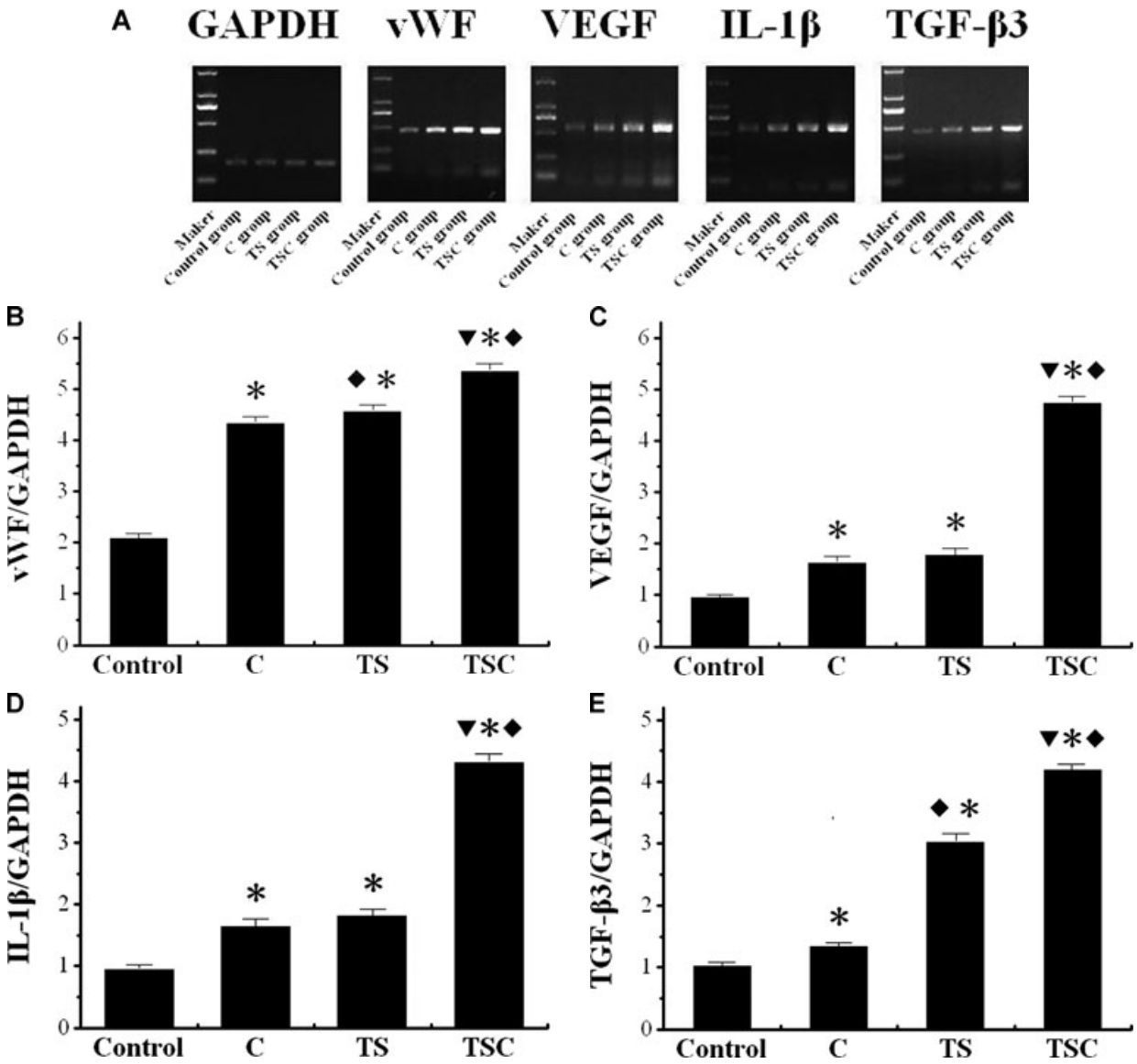

Fig. 1 Expressions of GAPDH, vWF, VEGF, IL-1β and TGF-β_3 mRNA. (**A**) shows representative photos of electrophoresis. Qualitative analysis was performed by BandScan 5.0 software. The ratios of vWF, VEGF, IL-1β and TGF-β_3 to GAPDH were compared in (**B**–**E**), respectively. $^{*}P < 0.05$ *versus* control group. $^{\blacklozenge}P < 0.05$ *versus* C group. $^{\blacktriangledown}P < 0.001$ *versus* TS group. GAPDH: glyceraldehyde-3-phosphate dehydrogenase; vWF: von Willebrand factor; VEGF: vascular endothelial growth factor; IL-1β: interleukin-1β; TGF-β_3: transforming growth factor-β_3.

there was a further increase in TSC group compared with C and TS groups ($P < 0.001$, Fig. 4E).

Identification of the transplanted cells

We tracked the engraftment of transplanted undifferentiated cells 6 weeks after the operation. Frozen sections within the core of the stent showed that DiI-labelled cells distributed in all regions and in particular around the stents in TSC group, whereas most transplanted cells were found in the peri-infarct regions in C group. Additionally, quantitative analysis of DiI revealed the enhanced survival of transplanted cells in TSC group (OD = 10,297 ± 658 pixels/hpf) compared with C group (OD = 3762 ± 213 pixels/hpf, $P < 0.001$, Fig. 5K). Immunofluorescence of cross-sections from TSC group showed that DiI-labelled cells not only expressed endothelial cell-specific marker vWF (Fig. 5A to C), but also smooth muscle cell-specific actin (Fig. 5D to F) and muscle-specific marker cTnT (Fig. 5G to J). In C group, however, only differentiation into endothelial cells was found. In contrast,

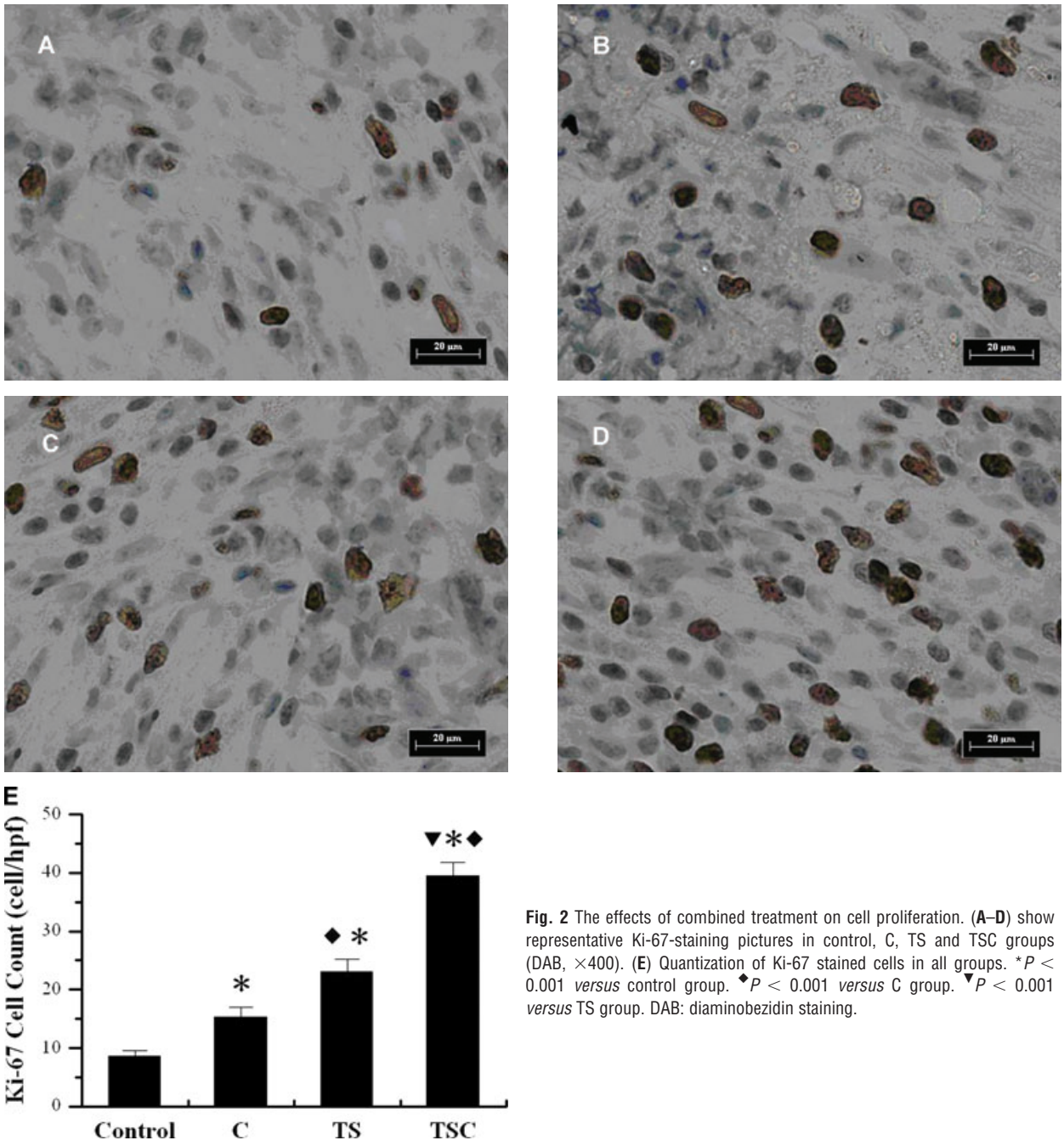

Fig. 2 The effects of combined treatment on cell proliferation. (**A**–**D**) show representative Ki-67-staining pictures in control, C, TS and TSC groups (DAB, ×400). (**E**) Quantization of Ki-67 stained cells in all groups. $^{*}P < 0.001$ *versus* control group. $^{\blacklozenge}P < 0.001$ *versus* C group. $^{\blacktriangledown}P < 0.001$ *versus* TS group. DAB: diaminobezidin staining.

sections from control and TS groups showed no evidence of red fluorescence.

Attenuated myocardial remodelling and improved left ventricular function in TSC group

No differences in EDV, ESV and local left ventricular wall thickness were found among the four groups prior to the treatment. Six weeks after treatment, no difference was found in local left ventricular wall thickness between C group (2.4 ± 0.1 mm, $P = 1$) and control group (2.3 ± 0.1 mm). However, a significant increase was detected in TS group (3.3 ± 0.2 mm, $P < 0.001$) when compared with C and control groups, and there was a further increase in TSC group (4.3 ± 0.2 mm, $P < 0.001$) compared with TS groups (Fig. 6B). EDV significantly decreased in TSC group (134 ± 8 ml) as compared with control group (167 ± 9 ml, $P < 0.001$),

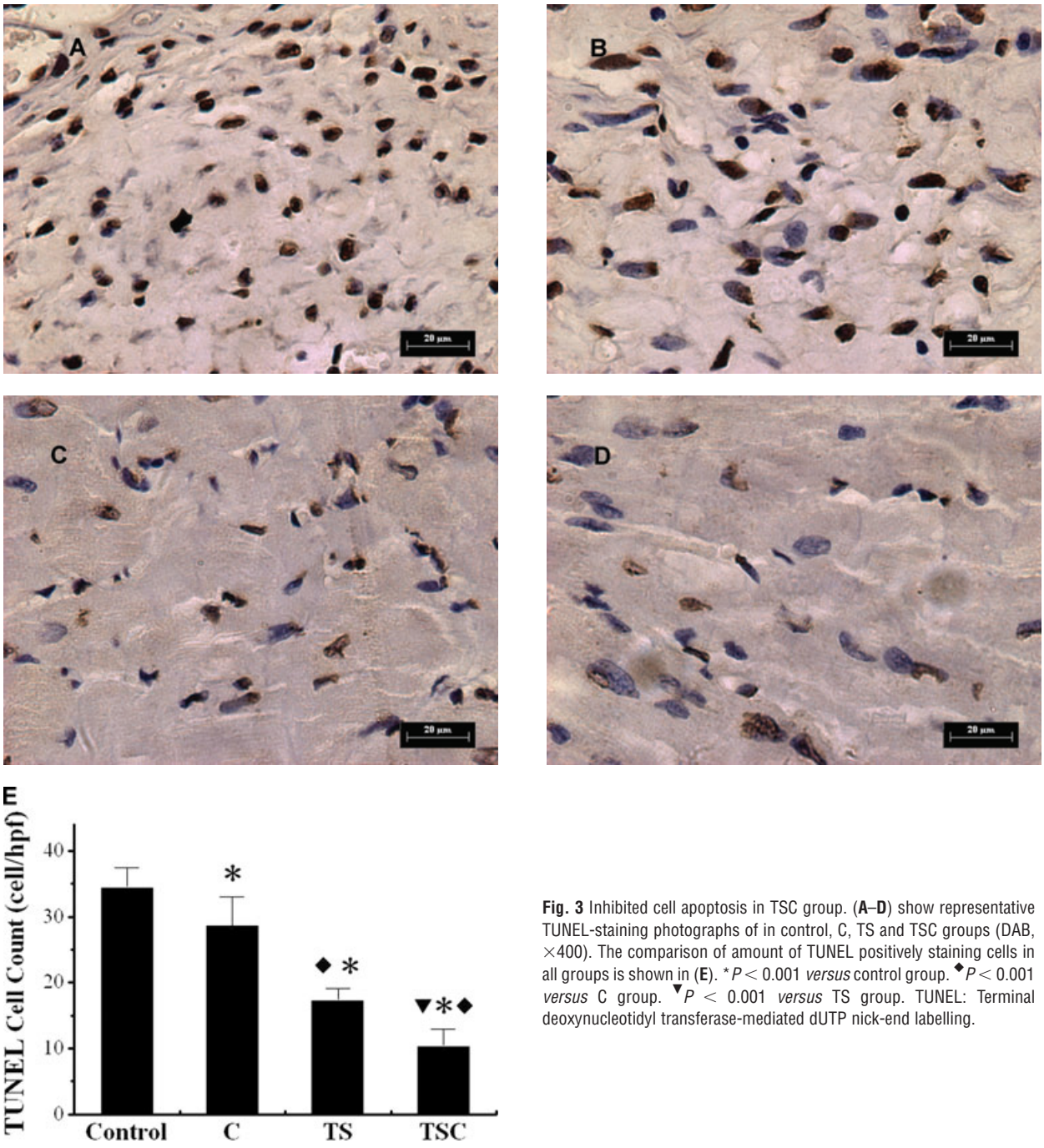

Fig. 3 Inhibited cell apoptosis in TSC group. (**A**–**D**) show representative TUNEL-staining photographs of in control, C, TS and TSC groups (DAB, ×400). The comparison of amount of TUNEL positively staining cells in all groups is shown in (**E**). $^{*}P < 0.001$ *versus* control group. $^{\blacklozenge}P < 0.001$ *versus* C group. $^{\blacktriangledown}P < 0.001$ *versus* TS group. TUNEL: Terminal deoxynucleotidyl transferase-mediated dUTP nick-end labelling.

C group (153 ± 5 ml, $P = 0.001$) and TS group (147 ± 7 ml, $P = 0.034$), although there was smaller EDV in C group ($P = 0.031$) and TS group ($P = 0.001$) than in the control group. Notably, there had been remarkable increases in all groups at self-comparison with pre-treatment (control group: 129 ± 7 ml, $P < 0.001$; C group: 128 ± 9 ml, $P < 0.001$; TS group: 129 ± 8 ml, $P < 0.001$; TSC group: 126 ± 10 ml, $P = 0.006$, Fig. 6C). Similar results were obtained for ESV. (Fig. 6D) Although EF was significantly decreased in all groups compared with pre-treatment (64 ± 2%, $P < 0.05$), there was a significant increase in TSC group (59 ± 2%)

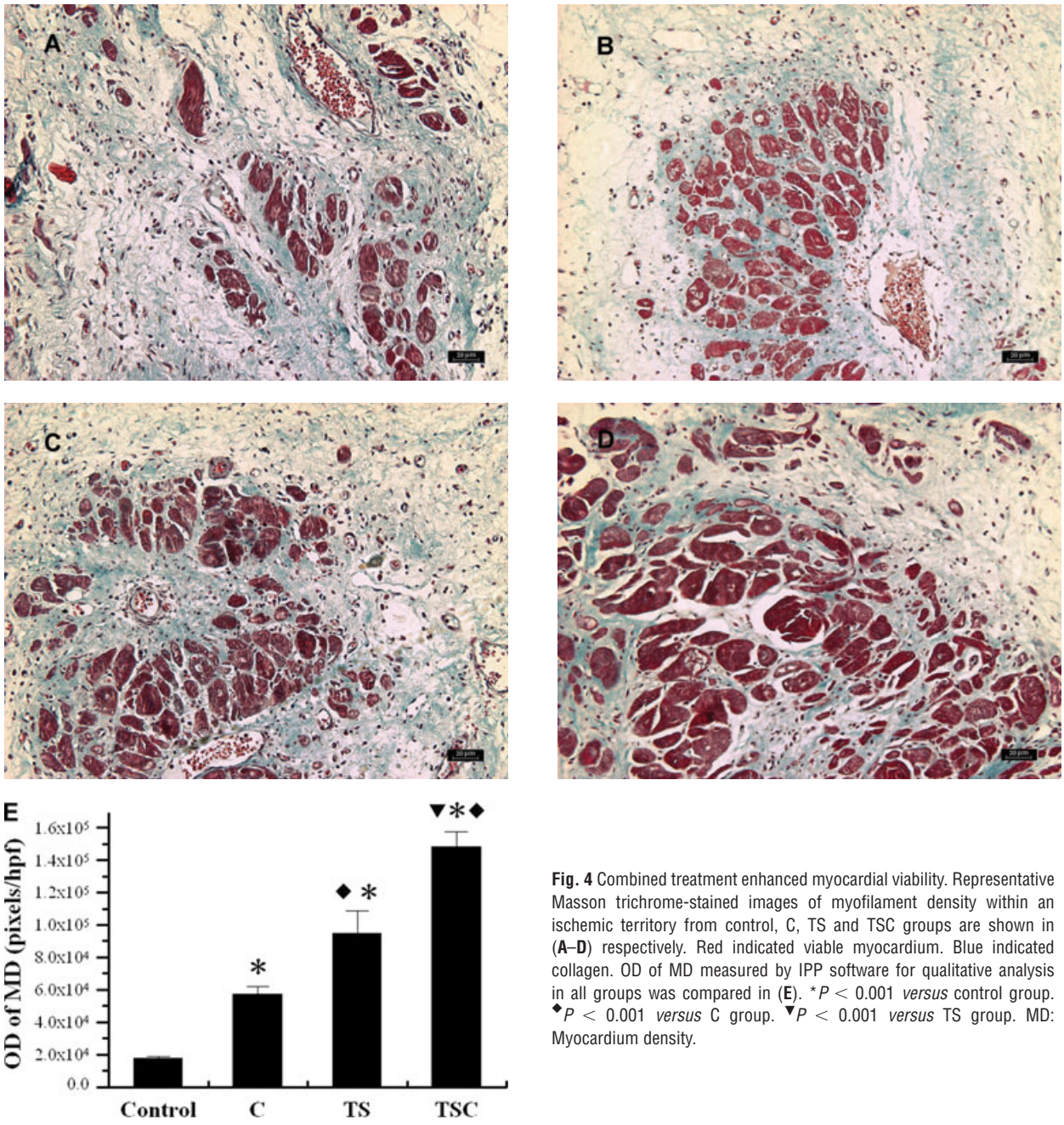

Fig. 4 Combined treatment enhanced myocardial viability. Representative Masson trichrome-stained images of myofilament density within an ischemic territory from control, C, TS and TSC groups are shown in (**A**–**D**) respectively. Red indicated viable myocardium. Blue indicated collagen. OD of MD measured by IPP software for qualitative analysis in all groups was compared in (**E**). $^{*}P < 0.001$ *versus* control group. $^{\blacklozenge}P < 0.001$ *versus* C group. $^{\blacktriangledown}P < 0.001$ *versus* TS group. MD: Myocardium density.

compared with control group (52 ± 2%, $P < 0.001$), C group (53 ± 2%, $P < 0.001$) and TS group (54 ± 3%, $P = 0.006$, Fig. 6E).

Discussion

Our study has demonstrated for the first time that TMDRSI can enhance the survival and differentiation of transplanted BMSCs, promoted paracrine function of such cells on repair of acute myocardial ischemia, and therefore can enable BMSCs transplantation to further attenuate myocardial remodelling at least for the subacute period.

Although BMSCs transplantation has provided a promising therapeutic option for patients with myocardial ischemia [9], no consensus was reached as to whether such therapy may bring protective effects at clinical trials. Some authors [1–3] reported

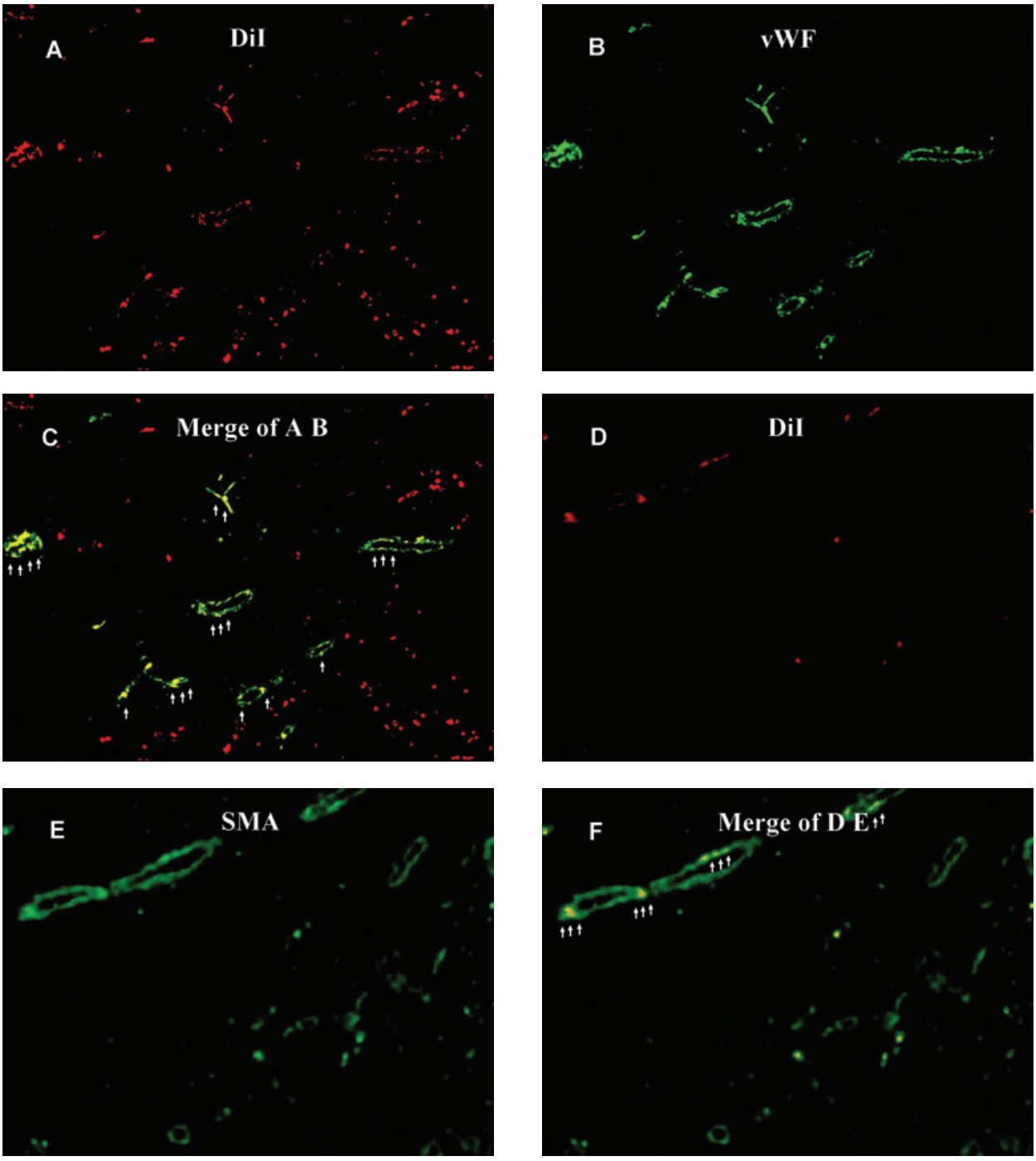

Fig. 5 Distribution and differentiation of transplanted cells. (**A**), (**D**) and (**G**) showed red fluorescence-positive cells distributing around the stent in the TSC group (100× magnification). (**B**) is the photograph of vWF staining. (**C**) is the merged picture of (**A**) and (**B**), revealing that survived transplanted cells were factor vWF^{+} (arrow). (**E**) shows stained smooth muscle actin. (**F**) is the merged picture of (**D**) and (**E**), suggesting differentiation into smooth muscle cells from transplanted BMSCs (arrow). (**H**) and (**I**) are cTNT and DAPI stained picture. (**J**) is the merged picture of (**G**), (**H**) and (**I**), indicating differentiation from transplanted BMSCs into cardiomyocytes and a structural integration between resident and neonatal myocytes (arrow). (**K**) shows comparison of quantization of DiI between C and TSC groups, with an increased survival of transplanted cells in TSC group compared with C group. $^{*}P < 0.05$ *versus* C group. cTNT: cardiac Troponin T.

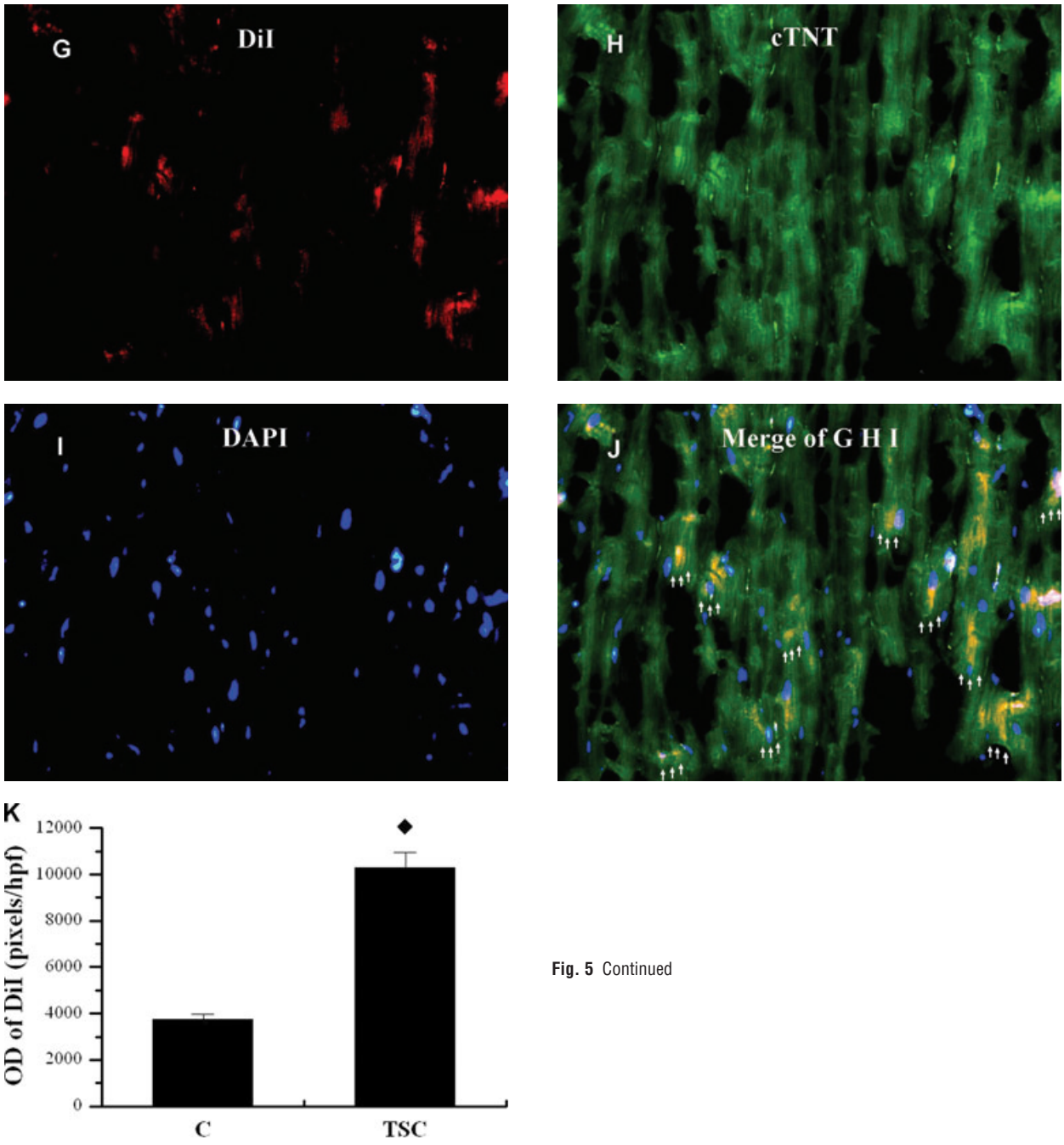

Fig. 5 Continued

that intracoronary transfer of autologous BMSCs improved cardiac function in patients with acute myocardial infarction. On the other hand, others have failed to prove the benefit after intracoronary injection of BMSCs [6, 7]. In present study, although we found that single BMSCs transplantation could slightly inhibit left ventricular remodelling after acute myocardial infarction, it was suggested that this minimal benefit should not be enough for repair of cardiac ischemic injury, which may limit its application for ischemic myocardial disease [8, 9].

Recently, we developed a new method of TMDRSI for patients with end-stage coronary artery disease who are not eligible for surgical intervention and other conventional treatments [10]. Moreover, our experiments have demonstrated that TMDRSI combined with BMSCs transplantation could provide more effective

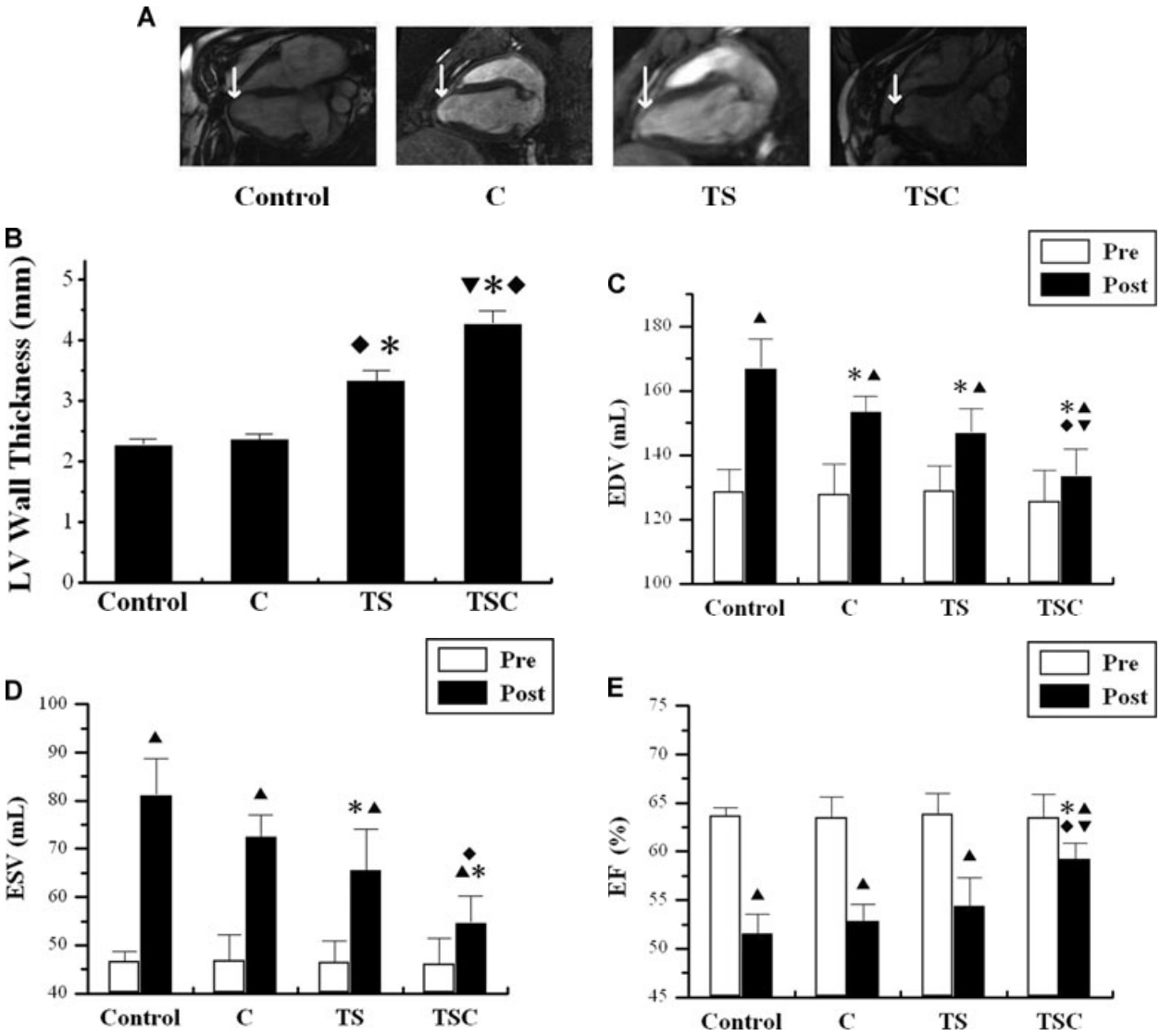

Fig. 6 MRI images of left ventricle. (**A**) shows long-axis MRI images of the left ventricle in all groups. Thickness of local left ventricular wall where the stents or BMSCs were implanted (arrow), LVEDV, LVESV and LVEF were determined and compared among all groups in (**B**), (**C**), (**D**) and (**E**), respectively. $^{\blacktriangle}P < 0.05$ by self-comparison between pre-treatment (pre) and post-treatment (post). $^{*}P < 0.05$ *versus* control group. $^{\blacklozenge}P < 0.05$ *versus* C group. $^{\blacktriangledown}P < 0.05$ v*ersus* TS group. EDV: end diastolic volume; ESV: end systolic volume; EF: ejection function; LVEDV: left ventricular end diastolic volume; LVESV: left ventricular end systolic volume; LVEF: left ventricular ejection function.

protection than any single method for left ventricular function against myocardial ischemia [11]. In the present study, the mechanism of this protective effect was further delineated.

BMSCs may mediate cardiac repair following ischemic injury by direct and indirect ways. It has been shown that BMSCs can differentiate into cardiomyocytes, endothelial cells and smooth muscle cells [12–14]. Recently, Rota *et al.* [13] found junctional and adhesion complexes between transplanted BMSCs and between transplanted BMSCs and adjacent myocytes in mice with myocardial infarction, which confirmed structural integration between resident and neonatal myocytes from BMSCs, and also demonstrated that the regenerated myocardium from BMSCs were functionally competent both electrically and mechanically. In our experiment, it was detected that transplanted cells were tightly juxtaposed with host endothelial cells and myocytes. As demonstrated by immunofluorescence, differentiation from BMSCs to cardiomyocytes and endothelial cells was significantly enhanced by TMDRSI, although immunohistological co-staining as a means of defining cell type has some limitations as there could be potential false positives.

Additionally, it has been demonstrated that BMSCs secreted angiogenic and anti-apoptotic factors such as vascular VEGF, IL-1β and TGF-β_3, which stimulate proliferation of endothelial cells and smooth muscle cells, and thereby can promote *in vitro* or *in vivo* arteriogenesis through paracrine pathways [9, 15–17]. Our results have shown an up-regulation of angiogenic and anti-apoptotic factors 6 weeks after therapy in TSC group, indicating that the paracrine pathway of BMSCs was activated by TMDRSI. Although enhanced cell proliferation and inhibited cell apoptosis could not be identified for implanted BMSCs, it was demonstrated that they were related to the paracrine pathway of BMSCs.

The heparin-incorporated stent implanted in transmural channel had kept the channel patent, promoted vascular formation and improved myocardial perfusion for tissue around the channels more effectively than traditional transmyocardial revascularization [10, 11]. The stent is degradable and can fulfil controlled release of bFGF, which may further promote angiogenesis and myocardial perfusion [18]. Hence, TMDRSI may provide adequate perfusion for the survival of BMSCs, allowing such cells to repair myocardial ischemic injury. As discovered, bFGF can promote proliferation and differentiation of BMSCs by activation of the ERK1/2 or the PKB signalling pathways [19, 20].

Although TMDRSI was inclined to provide more benefits than BMSCs implantation only, the additive effects of combined therapy were rather clear, suggesting that activated BMSCs may play a key role against cardiac ischemic injury by differentiating into functional endothelial cells and cardiomyocytes or paracrine pathway. Therefore, it is indicated that TMDRSI or other assistant methods that can activate BMSCs are necessary for transplantation of BMSCs, which may become a new trend of stem cell therapy.

Despite encouraging results, there are still questions and considerations that need to be further addressed. For instance, it has not been directly documented whether there are functional gap junctional coupling and intercellular electrical coupling between cardiomyocytes from BMSCs and adjacent myocytes. In addition, our study was performed on animal models, and the differences between human and pig hearts has not been taken into account.

In summary, TMDRSI may improve protection of BMSCs transplantation against cardiac ischemic injury through activating the paracrine pathway of transplanted BMSCs and enhancing survival and differentiation of such cells. Our method therefore may provide a new strategy for cell therapy of acute myocardial ischemia.

Acknowledgements

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