J. Cell. Mol. Med. Vol 16, No 12, 2012 pp. 3105-3115

BDNF-mediated migration of cardiac microvascular endothelial cells is impaired during ageing

Liang Cao [1, 2, 3, 4], Liang Zhang [1, 2, 3, 4], Siyun Chen [1, 2, 3], Ziqiang Yuan [5], Shaokun Liu [1, 2, 3], Xiaotao Shen [1, 2, 3, 4], Xin Zheng [1, 2, 3, 4], Xufeng Qi [1, 2, 3, 4], Kenneth K.H. Lee [1, 2, 6], John Yeuk-Hon Chan [1, 2, 3, 4], Dongqing Cai [1, 2, 3, 4, *]

[1] Key Laboratory for Regenerative Medicine, Ministry of Education, Ji Nan University, Guangzhou, China
[2] Joint Laboratory for Regenerative Medicine, Chinese University of Hong Kong-Jinan University, Guangzhou, China
[3] International Base of Collaboration for Science and Technology (JNU), The Ministry of Science and Technology & Guangdong Province, Guangzhou, China
[4] Department of Developmental & Regenerative Biology, Ji Nan University, Guangzhou, China
[5] Department of Molecular Genetics, Albert Einstein College of Medicine, New York, NY, USA
[6] Stem cell and Regeneration TRP, School of Biomedical Sciences, Chinese University of Hong Kong, Hong Kong, Hong Kong

Received: June 25, 2012; Accepted: August 16, 2012

Abstract

This study indicates that brain-derived neurotrophic factor (BDNF) can promote young cardiac microvascular endothelial cells (CMECs) to migrate *via* the activation of the BDNF-TrkB-FL-PI3K/Akt pathway, which may benefit angiogenesis after myocardial infarction (MI). However, the ageing of CMECs led to changes in the expression of receptor Trk isoforms in that among the three isoforms (TrkB-FL, TrkB-T1 and TrkB-T2), only one of its truncated isoforms, TrkB-T1, continued to be expressed, which leads to the dysfunction of its ligand, a decrease in the migration of CMECs and increased injury in ageing hearts. This shift in receptor isoforms in aged CMECs, together with changes in the ageing microenvironment, might predispose ageing hearts to decreased angiogenic potential and increased cardiac pathology.

Keywords: ageing • BDNF • cardiac microvascular endothelial cells • migration • TrkB

Introduction

Coronary artery disease-induced myocardial infarction (MI) is one of the leading causes of morbidity and mortality in elderly individuals. A therapeutic angiogenesis strategy has high potential for the regeneration of the infarcted myocardium [1–3]. Recent studies have shown that brain-derived neurotrophic factor (BDNF) and its receptor, TrkB, are expressed first during late gestation and then persistently at high levels into adulthood in the endothelial cells lining the arteries and capillaries of the heart. $BDNF^{-/-}$ mice exhibit the impaired survival of TrkB-expressing endothelial cells in intramyocardial arteries and capillaries in the late gestational and early postnatal periods. Conversely, BDNF overexpression in the mid-gestational mouse heart results in an increase in capillary density [4]. Compared with bFGF alone, exogenous delivery of bFGF and BDNF in ischaemic myocardium was able to improve the angiogenesis and left ventricular function of the ischaemic myocardium [5]. BDNF expression is up-regulated by neural signals from the young heart after MI and then protects the myocardium against ischaemic injury [6]. We previously reported that the number of truncated TrkB-positive cells and BDNF protein levels are higher in the aged cardiac microvasculature. BDNF levels in aged ischaemic cardiac microvasculature are higher compared with levels in young non-ischaemic, young ischaemic and old non-ischaemic hearts. The age-related increase in truncated TrkB in cardiac microvascular endothelial cells (CMECs) is apparently linked to an age-associated increase in inflammatory response and a significant increase in myocardial injury following coronary artery occlusion [7]. We and others have reported that the BDNF-TrkB pathway plays an important role in the development of cardiac vasculature, cardiac protection, angiogenesis of the myocardium and myocardial injury seen in the aged heart. In this study, we further determined if BDNF was able to promote the migration of CMECs,

*Correspondence to: Dongqing CAI,
Key Laboratory for Regenerative Medicine, Ministry of Education,
Ji Nan University, Guangzhou 510632, China.
E-mail: tdongbme@jnu.edu.cn

doi: 10.1111/j.1582-4934.2012.01621.x

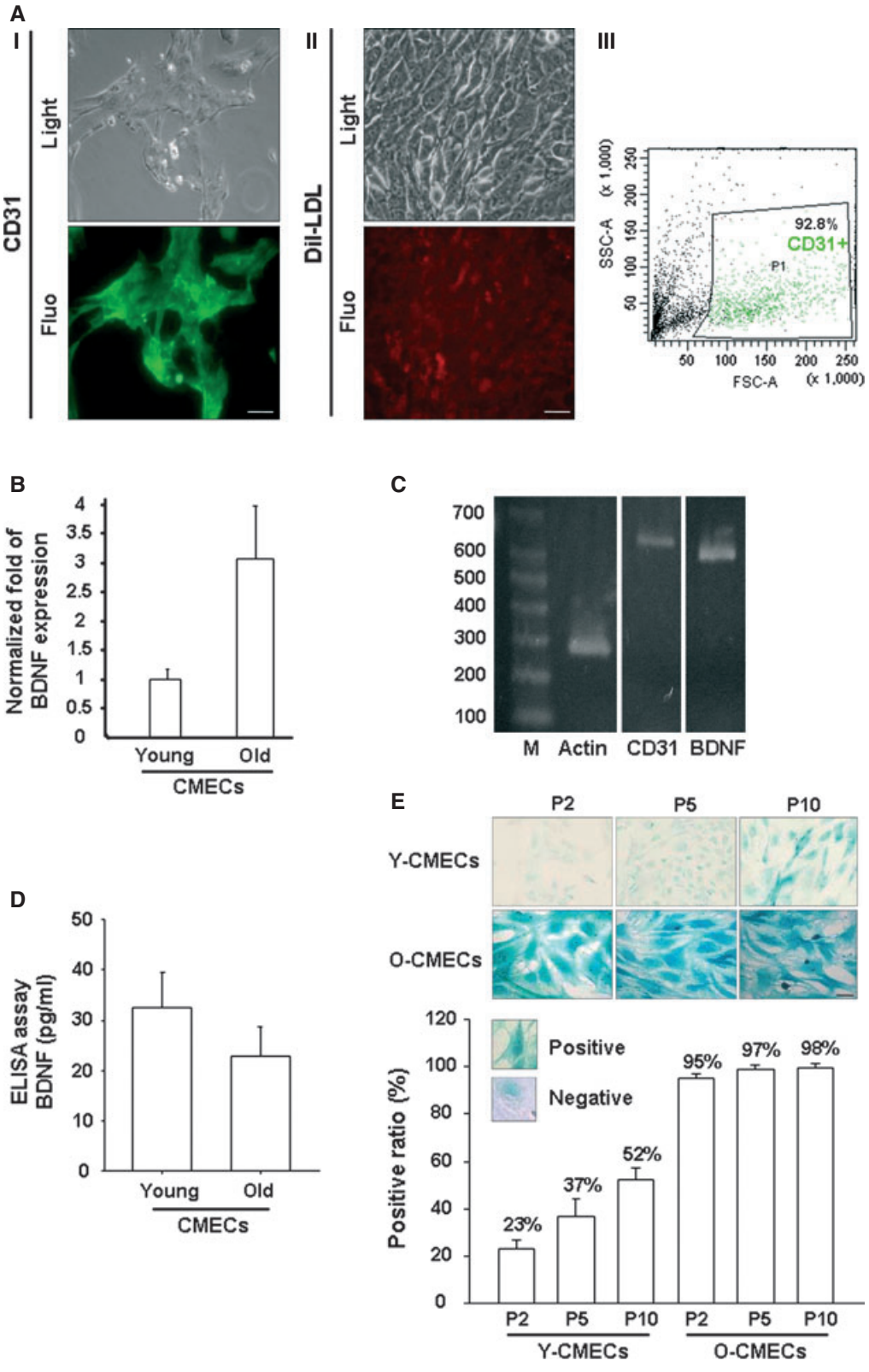


Fig. 1 Characterization of the isolated CMECs. (**A-I**) Immunofluorescent staining for CD31 indicated that more than 95% of the isolated CMECs were CD31+. (**A-II**) The fluorescent low-density lipoprotein (Dil-LDL) uptake assay revealed that more than 95% of the isolated CMECs was Dil-LDL positive. (**A-III**) The flow cytometry analysis confirmed that 92.8% of the isolated CMECs were CD31+. (**B**) The RT-PCR demonstrated that the isolated CMECs expressed CD31 and BDNF. (**C**) The real-time PCR showed that young and old CMECs both expressed BDNF. However, the expression level in old CMECs was higher than that in young CMECs. $*P < 0.05$ (old CMECs *versus* young CMECs) (**D**) SA-β-gal staining shows that numbers of SA-β-gal-positive CMECs from old rats (at Passage-2, Passage-5 and Passage-10) were significantly higher than those from young rats at the same passages. All of the experiments ($n = 3$) above were performed in triplicate. CMECs: Cardiac microvascular endothelial cells. Bars are 50 μm. $*P < 0.05$ (compared with Y-CMECs-P2). P2, Passage-2.

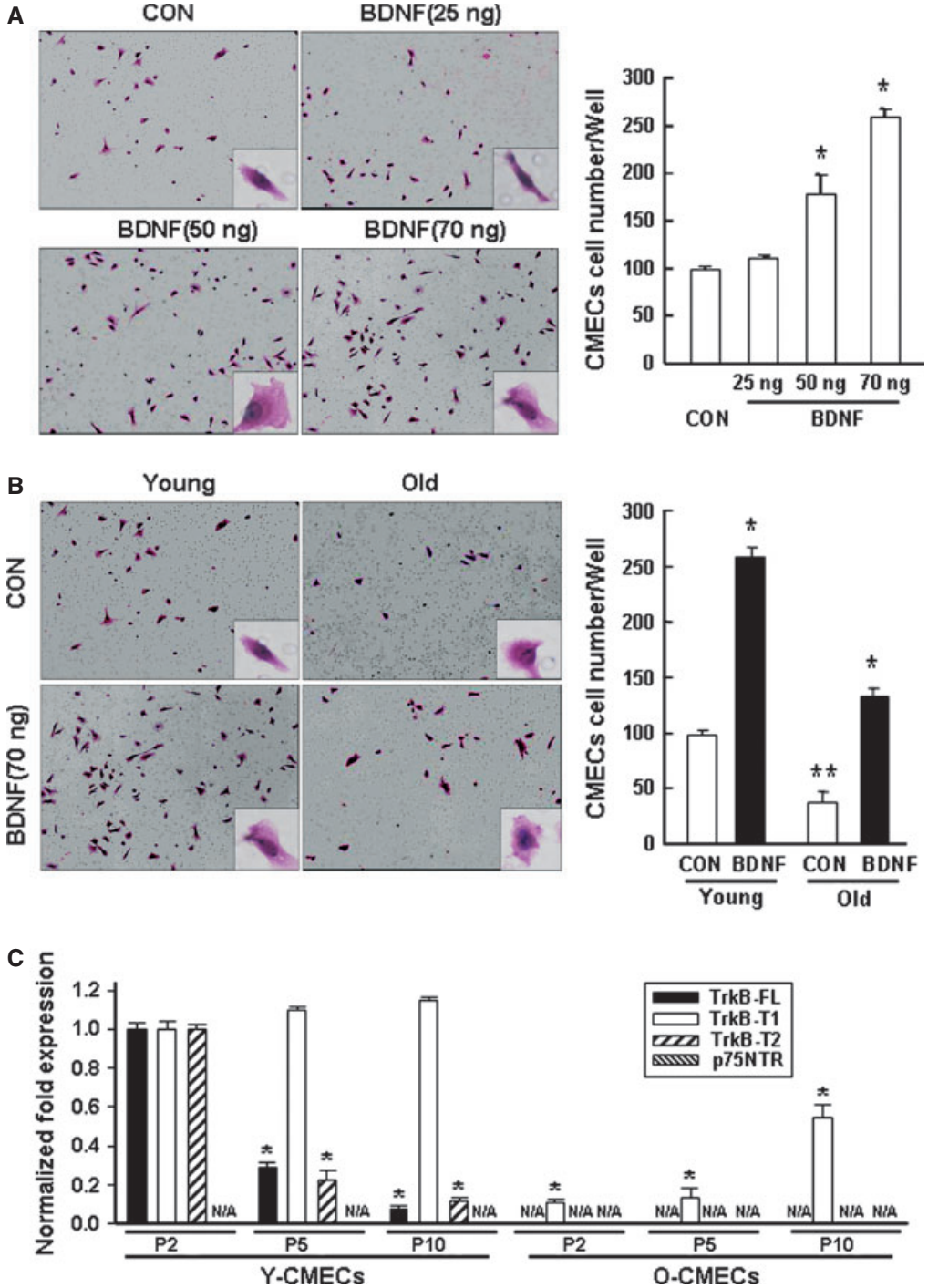


Fig. 2 BDNF-mediated migration of CMECs declined during ageing and age-related changes in TrkB isoforms. (**A**) The transwell motility assay showed that BDNF enhances CMEC migration and that the effect of BDNF on migration was dose dependent (*$P < 0.05$, BDNF *versus* control). (**B**) The assay also showed the age-related decline of CMEC migration. In addition, the migratory response of old BDNF-treated CMECs was reduced when compared with that of young CMECs. Two-way ANOVA: $F(1, 8) = 465.566$, $P < 0.05$ for age, $F(1, 8) = 879.060$, $P < 0.05$ for BDNF treatment; and $F(1, 8) = 60.241$, $P < 0.05$ for age × BDNF treatment. *$P < 0.05$ (young BDNF *versus* young control or old BDNF *versus* old control). **$P < 0.05$ (old control *versus* young control). (**C**) qPCR quantification analysis of young CMECs indicated that the cells expressed all three of the isoforms of the BDNF receptor (TrkB-FL, TrkB-T1 and TrkB-T2) but not $p75^{NTR}$. In old CMECs, only TrkB-T1 was expressed *($P < 0.05$ compared with Y-CMECs-P2). All of the experiments ($n = 3$) above were performed in triplicate. P2: Passage-2.

angiogenesis in cardiac ischaemia and the activation pathway. In addition, we examined the potential age-related decrease and impairment of BDNF, its receptor and the related angiogenic potential of CMECs and aged hearts.

Materials and methods

Animals

In this study, we used 6- and 20-month-old female Sprague–Dawley rats. Animal care, surgery and handling procedures for this study were performed in accordance with the rules of The Ministry of Science and Technology of the People's Republic of China [(2006)398] and were approved by the Ji Nan University Animal Care Committee. The detailed methodology and statistical analysis are included in the Supplementary material.

Results

Phenotype and β-gal staining for isolated CMECs

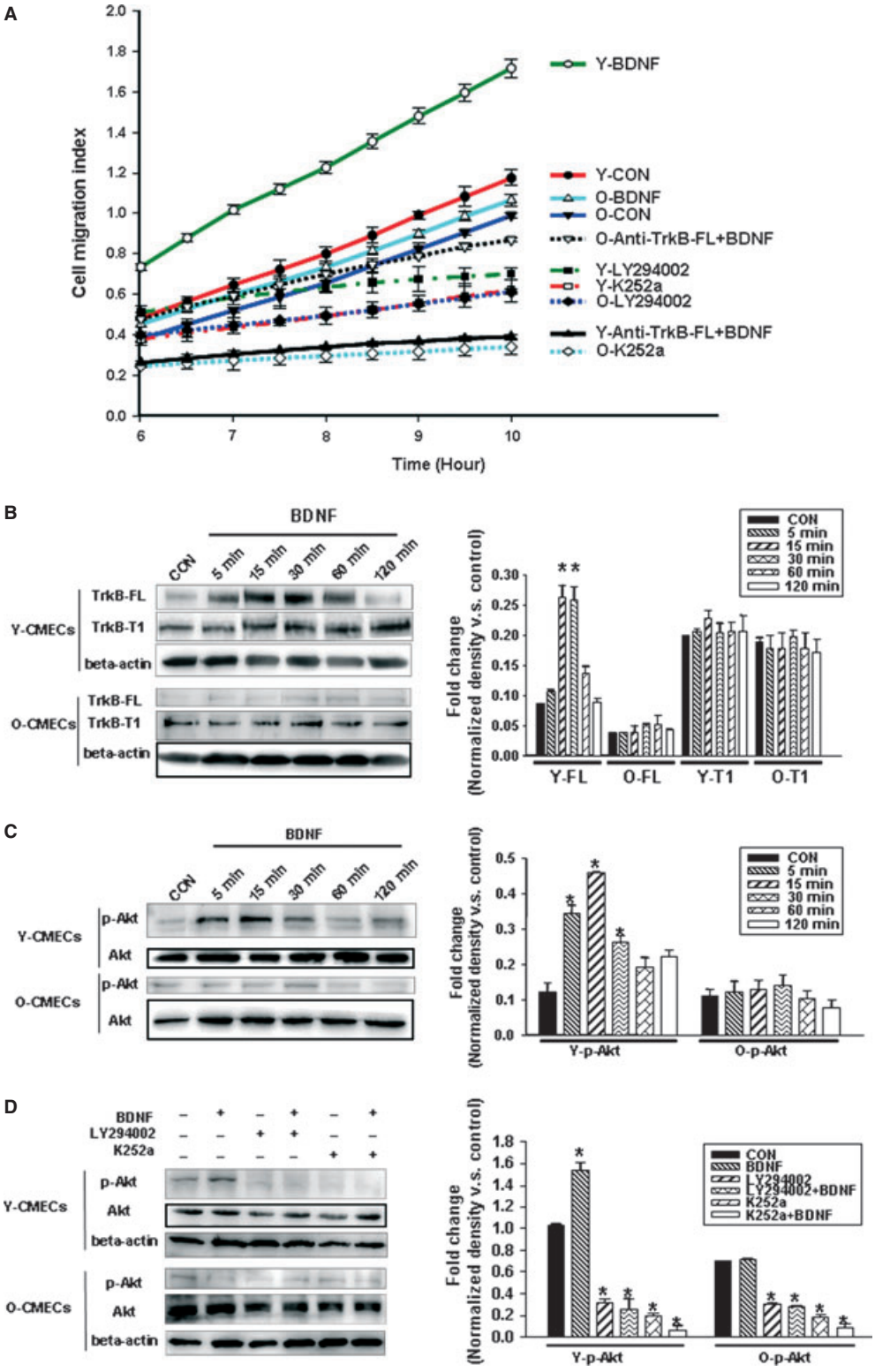
Immunofluorescent staining for CD31 indicated that more than 95% of the isolated CMECs were CD31 positive (Fig. 1A-I). The fluorescent low-density lipoprotein (Dil-LDL) uptake assay revealed that more than 95% of the isolated CMECs were Dil-LDL positive (Fig. 1A-I). Flow cytometry analysis further confirmed that 92.8% of the isolated CMECs were CD31 positive (Fig. 1A-III). RT-PCR revealed that the isolated CMECs expressed CD31 and BDNF (Fig. 1B). Real-time PCR also indicated that the young and old CMECs expressed BDNF. In addition, expression of BDNF in old CMECs was higher than that in young CMECs (Fig. 1C). The SA-*β-gal* assay revealed that the replicative senescence percentages of positive CMECs from young rats (at Passage-2, Passage-5 and Passage-10) were 23%, 37% and 52%, respectively, whereas the replicative senescence percentages for CMECs from old rats (at Passage-2, Passage-5 and Passage-10) were 95%, 97% and 98% respectively (Fig. 1D). All of these results confirmed that the isolated and cultured CMECs used in this study maintained the normal endothelial phenotype and expressed BDNF. In addition, replication senescence existed in the CMECs.

BDNF promotes the migration of CMECs *via* activation of the BDNF-TrkB-FL-PI3K/Akt pathway

Promoting the migration of endothelial cells is one of the critical events for angiogenesis. The transwell motility assay was applied to evaluate the possible effect of BDNF on the migration of CMECs. The results of the transwell motility assay revealed that BDNF promoted the migration of young CMECs. The extent of the migration of the CMECs occurred in a BDNF dose-dependent manner. Specifically, the extents of migration were 258 ± 9 cells/well in 70 ng/ml BDNF, 178 ± 20 cells/well in 50 ng/ml BDNF and 109 ± 2 cells/well in 25 ng/ml BDNF. In the control, the extent of migration was 98 ± 4 cells/well ($P < 0.05$; Fig. 2A and B). Because the maximal effect of BDNF was observed at 70 ng/ml, this concentration was used in subsequent studies. In addition to the transwell motility assay, we also performed real-time measurements of the migration of CMECs using an xCelligence RTCA-DP instrument. BDNF treatment promoted the migration of young CMECs after a 6-hr treatment compared with the control (Fig. 3A).

The pathway of BDNF-mediated CMEC migration was further investigated. There are three isoforms of the TrkB receptor in mammalian cells. The full-length isoform (TrkB-FL) is a tyrosine kinase receptor that transduces the BDNF signal [8–10]. The two truncated isoforms, TrkB-T1 and TrkB-T2, possess the same extracellular domain, transmembrane domain and the first 12 intracellular amino acids in sequence as does TrkB-FL; however, the C-terminal sequences are isoform specific: T1 has 11 amino acids and T2 has 9 amino acids [8]. The results of the real-time PCR revealed that young CMECs expressed all of the subtypes of the TrkB receptors (TrkB-FL, TrkB-T1 and TrkB-T2) at Passage-2, but FL and T2 were down-regulated at Passage-5 and Passage-10 (Fig. 2C). In addition, our Western blot results showed that young CMECs expressed TrkB-FL and TrkB-T1. BDNF treatment up-regulated the expression of TrkB-FL, but not TrkB-T1, when young CMECs were treated for 5 min. (Fig. 3B). BDNF increased the phosphorylation of Akt in young CMECs at approximately 5 min. after treatment and reached maximal levels after 15 min. of treatment, maintaining the increased level approximately

Fig. 3 BDNF-mediated CMEC migration *via* the TrkB-FL-PI3K/Akt pathway. (**A**) The real-time migration assay showed that BDNF promotes the migration of both young and old CMECs. However, this BDNF-mediated migratory response was determined to be dysfunctional in old CMECs compared with that in young CMECs. LY294002 (inhibitor of PI3K/Akt) and K252a (tyrosine kinase inhibitor) inhibited the migration of young and old CMECs. In addition, the migration indices of LY294002- and K252A-treated young and old CMECs were significantly lower compared with those of the parallel young and old control groups and the young and old BDNF-treated groups respectively. Furthermore, the migration indices for the individual groups of old CMECs were lower than those of the corresponding young groups. The BDNF-mediated migration indices for young and old CMECs pre-incubated with anti-TrkB-FL were lower than those of the corresponding young and old BDNF-treated and control groups respectively. (**B**) The Western blot revealed that BDNF increased the expression of TrkB-FL in young CMECs at approximately 5 min. after treatment but not in old CMECs. (**C**) BDNF increased Akt phosphorylation 5 min. after treatment. However, BDNF failed to increase Akt phosphorylation in old CMECs. (**D**) LY294002 inhibited the BDNF-mediated phosphorylation of Akt. Treatment with K252a also inhibited Akt phosphorylation. No parallel effects were identified in old CMECs. All of the experiments ($n = 3$) above were performed in triplicate. *$P < 0.05$ (BDNF *versus* control or inhibitor *versus* control).



2 hrs after treatment (Fig. 3C). However, LY294002, an inhibitor of PI3K/Akt, inhibited the BDNF-mediated phosphorylation of Akt. Treatment with K252a, a kinase inhibitor that is known to selectively block tyrosine kinase isoforms of the Trk family [11–13], also inhibited the BDNF-mediated phosphorylation of Akt (Fig. 3D). In addition, our migration assay indicated that LY294002 and K252a inhibited the migration of the young CMECs (Fig. 3A). The above results revealed that the BDNF-mediated increase in Akt phosphorylation was associated with an increase in the migration of young CMECs. In addition, the TrkB-FL receptor (a tyrosine kinase receptor) was mainly involved in the BDNF-induced migration of young CMECs.

To confirm that the BDNF-mediated migration was associated with ligand–receptor binding, blocking assays using the TrkB-FL antibody and an shRNA knockdown assay were performed. The results of the blocking assay showed that the BDNF-mediated migration index for young CMECs pre-incubated with anti-TrkB-FL was lower than that for either the control or the BDNF-treated group (Fig. 3A). In addition, transfection with TrkB-shRNA into young CMECs decreased the expression of the BDNF receptors (TrkB-FL, TrkB-T1 and TrkB-T2; Fig. 4A-I) and also decreased BDNF-mediated CMEC migration (Fig. 4B). Our above findings indicated that the BDNF-mediated migration of CMECs was due to the activation of the BDNF-TrKB-FL-PI3K/Akt pathway.

Because $p75^{NTR}$ is able to bind all neurotrophins [14, 15], it may bind BDNF and then transduce the signal to regulate CMECs. To exclude the effect of $p75^{NTR}$ in this experiment, real-time PCR evaluating $p75^{NTR}$ expression in CMECs was conducted. Our real-time PCR results showed that $p75^{NTR}$ was not expressed in either isolated young or old CMECs (Fig. 2C).

Age-related decrease in TrkB-FL expression impairs BDNF-mediated CMEC migration

The possible age-related dysfunction in BDNF-mediated migration for CMECs was further evaluated by using a transwell motility assay. The capacity to migrate in response to BDNF declined in old CMECs. Analysis of the data by two-way ANOVA indicated that the differences were significant. In the controls, the numbers of CMECs that migrated were 98 ± 4 cells/well and 38 ± 4 cells/well for the young and old groups respectively ($P < 0.05$). In addition, BDNF increased the number of migrating CMECs in the young and old groups compared with their parallel controls. The introduction of BDNF increased CMEC migration in both the young and old groups: the young group had 258 ± 9 cells/well (young control, 98 ± 4/well) and the old group had 132 ± 8 cells/well (old control, 38 ± 4/well) (control *versus* BDNF group, $P < 0.05$). The CMEC migration in the old BDNF-treated group (132 ± 8 cells/well) was significantly higher than that of the old control group (38 ± 4 cells/well, $P < 0.05$). However, these measurements were significantly lower than those of the young BDNF-treated group (258 ± 9 cells/well, $P < 0.05$, Fig. 2B). The real-time migration assay also revealed that the migration index for the old group was lower than those of the young control and the young BDNF-treated group. In addition, the migration index for the old BDNF-treated group was higher than that of the old control group (Fig. 3A).

The expression profiles of TrkB-FL, TrkB-T1 and TrkB-T2 were further investigated using quantitative PCR. The TrkB-FL and TrKB-T2 expression levels were undetectable in old CMECs at Passage-2, Passage-5 and Passage-10, whereas both young and old CMECs expressed TrkB-T1 (Fig. 2C). In addition, both young and old CMECs expressed BDNF (Fig. 1D).

The age-related decrease in TrkB-FL expression further suggests that dysfunction of the BDNF-TrkB-PI3K/Akt pathway might occur in the migration of old CMECs. Western blot results revealed that TrkB-FL was not expressed (or was expressed at a very low level) in old CMECs compared with young CMECs. In contrast, TrkB-T1 expression was maintained in old CMECs (Fig. 3B). However, BDNF was not able to increase Akt phosphorylation in old CMECs, similar to LY294002, BDNF+LY294002, K252a or BDNF +K252a (Fig. 3C and D). The real-time migration assay also indicated that the migration indices of LY294002- and K252A-treated old CMECs were significantly lower compared with those of the old controls and the old BDNF-treated groups respectively. In addition, the migration indices of the individual groups of old CMECs were lower than those of the parallel young groups (Fig. 3A). The results of the blocking assay showed that the BDNF-mediated migration index of the old CMECs pre-incubated with anti-TrkB-FL was lower than those of the BDNF-treated and the control groups (Fig. 3A).

In addition, transfection with TrkB-shRNA in the old CMECs significantly decreased the expression of TrkB-T1 (Fig. 4A-II). The extents of migration were 7 ± 2 cells/well in the TrkB-shRNA-transfected old CMECs group, 14 ± 5 cells/well in the TrkB-shRNA-transfected, BDNF (70 ng/ml)-treated old CMECs group, 41 ± 7 cells/well in the old CMECs control group and 65 ± 6 cells/well in the BDNF (70 ng/ml)-treated old CMECs group. The extent of migration of the TrkB-shRNA-transfected BDNF (70 ng/ml)-treated old CMECs group was significantly lower than those of the old CMECs control group and the BDNF (70 ng/ml)-treated old CMECs group. However, the extent of migration was significantly higher than that of the TrkB-shRNA-transfected old CMECs group ($P < 0.05$, Fig. 4B).

In vivo angiogenesis potential of BDNF in the heart

The possible *in vivo* angiogenic effect of BDNF in the myocardium was further investigated. We injected 500 ng of BDNF into the left ventricular myocardia of non-ischaemic young and old hearts. The vessel density at the injection site was analysed 2 days after injection using immunostaining for vWF, an endothelial cell marker. The vessel densities in BDNF-injected young and old hearts were similar to the parallel controls. The density of the BDNF-treated young heart was 59 ± 17/mm^2 (control: 54 ± 4/mm^2), and the density of the BDNF-treated old heart was 58 ± 6/mm^2 (control: 59 ± 16/mm^2). Analysis of the data by two-way ANOVA revealed no significant differences (Fig. 5A).

Furthermore, the possible angiogenic effect of BDNF during an ischaemic situation was investigated using a left anterior descending coronary artery (LAD) ligation. As in our pilot study, we found that an

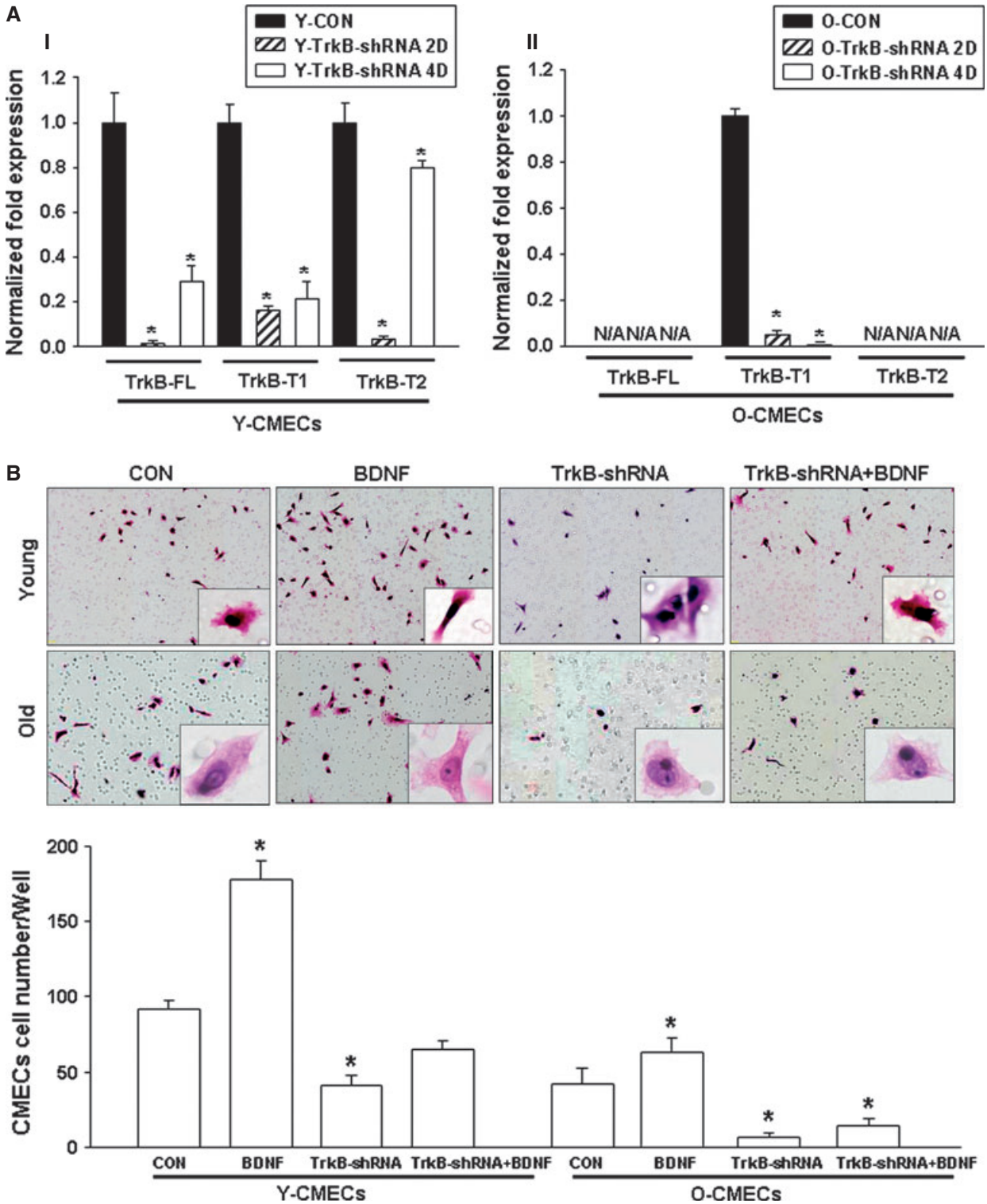


Fig. 4 shRNA knockdown of TrkB receptors inhibited BDNF-mediated CMEC migration. (**A**) and (**B**) TrkB-shRNA transfection repressed BDNF receptor (TrkB-FL, TrkB-T1 and TrkB-T2) expression and BDNF-mediated young and old CMEC migration. All of the experiments ($n = 3$) above were performed in triplicate. $*P < 0.05$ (shRNA *versus* control or BDNF *versus* control).

injected dose of more than 100 ng of BDNF resulted in a mortality rate of approximately 60% in the LAD-ligated old rats. Therefore, 70 ng of BDNF, which increased the migration of CMECs *in vitro*, was applied in this study. Immunostaining for vWF was again applied to determine the vessels at the infarcted zone and the border zone 14 days after the LAD ligation. Analysis of data by two-way ANOVA indicated significant differences between the vessels at the infarcted zone and the border zone. The vessel density of the infarcted zone in the BDNF-injected young heart ($95 \pm 12/mm^2$) was higher than that of the control ($63 \pm 14/mm^2$; $P < 0.05$). In addition, the vessel density of the infarcted zone in the BDNF-injected old heart ($65 \pm 5/mm^2$) was slightly higher than that of the control, but the difference was not statistically significant ($56 \pm 2/mm^2$; $P > 0.05$). However, the vessel density of the infarcted zone in the BDNF-injected old heart ($65 \pm 5/mm^2$) was lower than that of the BDNF-injected young heart ($95 \pm 13/mm^2$; $P < 0.05$; Fig. 5B). The density of the BDNF-treated young heart in the border zone ($64 \pm 5/mm^2$) was also higher than that of the control ($38 \pm 3/mm^2$; $P < 0.05$). The density of the BDNF-treated old heart in the border zone ($47 \pm 2/mm^2$) was similar to that of the control ($45 \pm 2/mm^2$; $P > 0.05$). The vessel density of the border zone in the BDNF-injected old heart ($47 \pm 2/mm^2$) was lower than that of the BDNF-injected young heart ($64 \pm 5/mm^2$; $P < 0.05$; Fig. 5B).

To evaluate the possible therapeutic effect of BDNF in ischaemic rat hearts, the infarct sizes were simultaneously compared. Analysis of the data by two-way ANOVA indicated significant differences in the infarcted sizes. The infarction size (%LV) of the BDNF-treated old group (0.40 ± 0.05) was higher than that of the old control group (0.32 ± 0.06; $P < 0.05$). However, the infarction size (%LV) of the BDNF-treated young group (0.34 ± 0.06) was similar to that of the young control group (0.33 ± 0.07; $P > 0.05$; Fig. 5C).

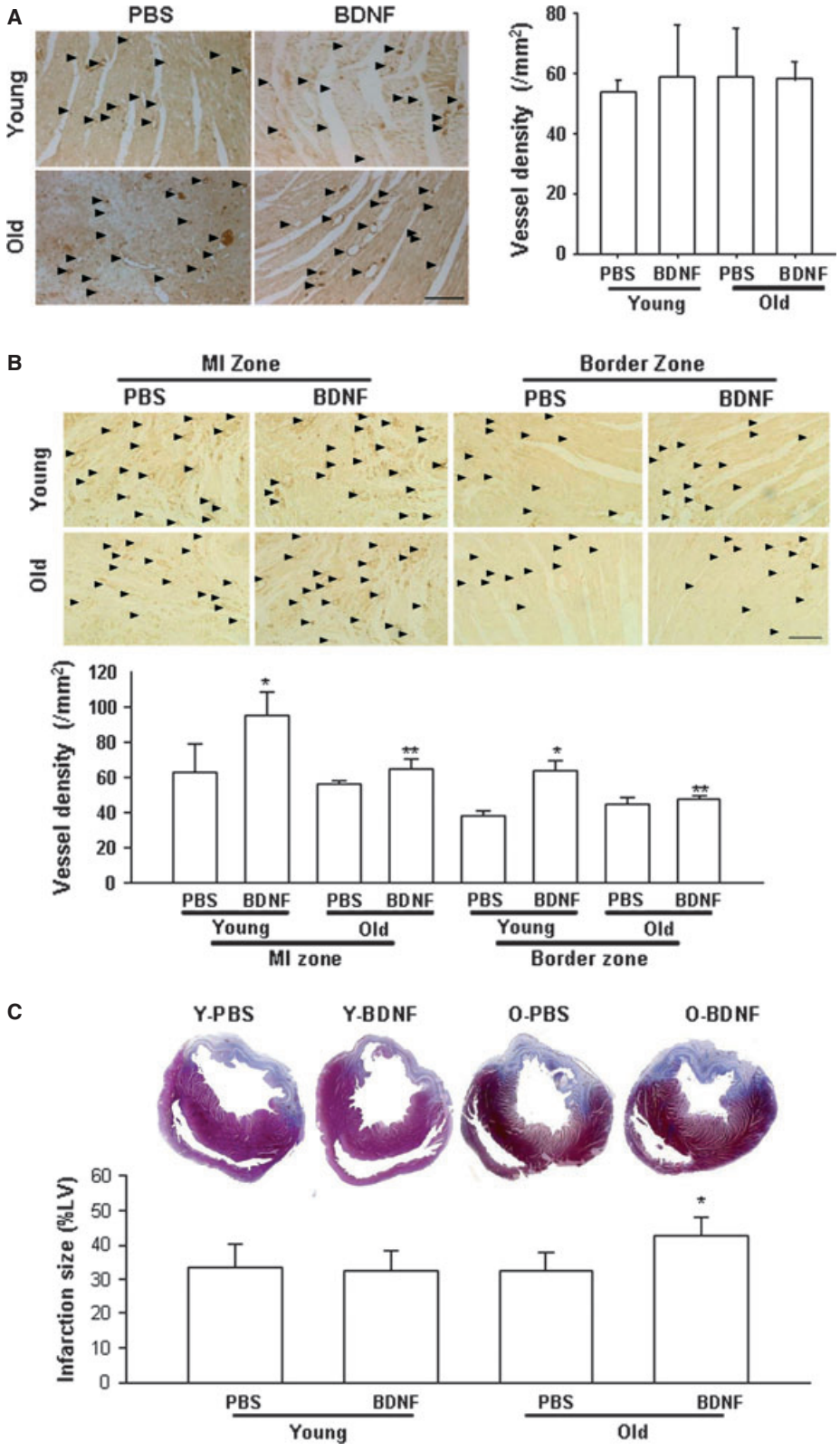
Discussion

In this study, CMECs were used to investigate the effects of BDNF on cell migration. Our RT-PCR results indicated that young CMECs expressed BDNF and its three receptors TrkB-FL, TrkB-T1 and TrkB-T2. The results suggested that BDNF plays an important function in CMECs, possibly using an autocrine/paracrine modality. The results of the transwell and real-time cell migration assays revealed that BDNF promoted CMEC migration in a dose-dependent manner, suggesting that BDNF acted as a chemotaxis factor for the CMECs to promote their migration.

Our results also showed that BDNF up-regulated TrkB-FL expression in young CMECs as early as 5 min. after treatment, which was accompanied by a corresponding increase in the phosphorylation of Akt. The increased Akt phosphorylation was at a maximum level at 30 min. after treatment and remained at an increased level for approximately 2 hrs. However, BDNF did not affect TrkB-T1 expression. Because TrkB-FL, but not TrkB-T1 or TrkB-T2, contains the tyrosine kinase domain [8–10], it is suggested that the BDNF-mediated migration of young CMECs is mainly *via* binding to TrkB-FL, activating the associated tyrosine kinase and then activating the PI3K/Akt signalling pathway by increasing Akt phosphorylation. Indeed, our shRNA knockdown assay also confirmed that transfection of TrkB-shRNA into young CMECs decreased the expression levels of all of the isoforms of the BDNF receptor and decreased the BDNF-mediated migration of CMECs. Furthermore, blocking TrkB-FL with its antibody was found to decrease BDNF-mediated CMECs migration. In addition, LY294002, an inhibitor of PI3K/Akt, and K252a, a selective inhibitor of Trk receptors [11–13], inhibited the BDNF-mediated phosphorylation of Akt. Both LY294002 and K252a inhibited the migration of young CMECs, which clearly indicates that the BDNF-mediated migration of young CMECs was due to the activation of the BDNF-TrKB-FL-PI3K/Akt pathway.

Although BDNF was still able to promote the migration of old CMECs, the initiation of the migration of old CMECs was not as effective as that of young CMECs. Because 98% of the old CMECs in this study were positive for SA-β-*gal* staining, it is possible that the senescence of CMECs impaired the BDNF-mediated migration. The TrkB receptor expression profiles showed that old CMECs only expressed TrkB-T1 but not TrkB-FL and TrkB-T2. Thus, the BDNF-mediated migration was dysfunctional in old CMECs because TrkB-FL was not expressed, and BDNF failed to induce the phosphorylation of Akt in old CMECs. Thus, the age-related decrease in TrkB-FL expression and

Fig. 5 Exogenous BDNF increases angiogenesis in the infarcted zones of MI in young hearts. (**A**) Immunostaining for vWF at day 2 after injection revealed that the vessel densities in BDNF-injected young and old hearts were similar to those in their parallel controls. Two-way ANOVA: $F(1, 25) = 0.112$, $P > 0.05$ for age, $F(1, 25) = 0.255$, $P > 0.05$ for BDNF treatment and $F(1, 25) = 0.468$, $P > 0.05$ for the age $\times$ BDNF treatment. (**B**) Immunostaining for vWF at day 14 after the LAD-ligated heart injection revealed that the vessel density of the infarcted zone in the BDNF-injected young heart was higher than that of the control. The vessel density of the BDNF-treated young heart in the border zone was also higher than that of the control ($P < 0.05$). In addition, the vessel density of the infarcted zone in the BDNF-injected old heart was slightly higher than that of the control, but the difference was not statistically significant ($P > 0.05$). The vessel density in the border zone of the BDNF-treated old heart was similar to that of the control. The vessel density of the infarcted zone in the BDNF-injected old heart was lower than that of the BDNF-injected young heart, and the vessel density of the border zone in the BDNF-injected old heart was lower than that of the BDNF-injected young heart. Two-way ANOVA: $F(1, 14) = 9.797$, $P < 0.05$ for age, $F(1, 14) = 11.536$, $P < 0.05$ for BDNF treatment and $F(1, 14) = 3.602$, $P > 0.05$ for the age $\times$ BDNF treatment in the MI zone; and $F(1, 13) = 6.344$, $P < 0.05$ for age, $F(1, 13) = 56.828$, $P < 0.05$ for BDNF treatment and $F(1, 13) = 38.022$, $P < 0.05$ for the age $\times$ BDNF treatment in the border zone $^*P < 0.05$ (BDNF *versus* PBS), $^{**}P < 0.05$ (old BDNF *versus* young BDNF). Bars are 100 μm. All of the experiments above were performed in five animals. (**C**) The infarction size (%LV) of the BDNF-treated young group was similar to that of the young control group ($P > 0.05$). However, the infarction size (%LV) of the BDNF-treated old group was higher than that of old control group ($P < 0.05$). Two-way ANOVA: $F(1, 25) = 3.877$, $P > 0.05$ for age, $F(1, 25) = 3.825$, $P > 0.05$ for BDNF treatment and $F(1, 25) = 5.668$, $P < 0.05$ for the age $\times$ BDNF treatment. LV: left ventricle. $^*P < 0.05$ (BDNF *versus* PBS). All of the experiments above were performed in five animals.



the consequential impairment of BDNF-TrkB-FL-PI3K/Akt signalling are the reasons for the dysfunction of BDNF-mediated migration in old CMECs.

Although only TrkB-T1 was detected in old CMECs, BDNF was still able to promote CMEC migration, but at a significantly lesser extent than the migration of young CMECs, which suggested that TrkB-T1 had some activity in promoting migration. In addition, the expression of TrkB-T1 was significantly lower when old CMECs were transfected with TrkB-shRNA; in parallel, the migration capacity was significantly lower when compared with young and old controls, young and old BDNF-treated groups and young and old BDNF+TrkB-shRNA-transfected groups. These results implied that old CMECs might still be able to transduce downstream signalling through the BDNF-TrkB-T1 pathway to mediate migration using an as yet unknown mechanism. However, our quantitative PCR assay confirmed that neither young nor old CMECs used in this study expressed $p75^{NTR}$, a non-specific receptor for all neurotrophic factors. Therefore, we could also exclude the idea that the BDNF-mediated migration of old CMECs was attributed to transduction *via* non-specific binding with $p75^{NTR}$. Indeed, recent studies revealed that TrkB-T1 was able to regulate the morphology of neurons and glial cells *via* BDNF-mediated signalling of the Rho GDP dissociation inhibitor I and the actin cytoskeleton [16–18], which indicates that TrkB-T1 has a functional cytoplasmic domain. In addition, the BDNF-TrkB-FL (possibly with TrKB-T1 or TrKB-T2) used by young CMECs might be more effective than the BDNF-TrkB-T1 pathway alone used by old CMECs. Therefore, the possible downstream effectors and the pathway related to BDNF-TrkB-T1-mediated migration in old CMECs need to be investigated further in the future. This ageing-associated change is apparently important in the dysfunction of cardiac angiogenesis seen in aged hearts.

In addition, we discovered that exogenous delivery of BDNF into young ischaemic hearts significantly increased the number of blood vessels present in the ischaemic regions. However, this delivery had no effect on non-ischaemic young and old hearts, suggesting that even though CMECs normally express BDNF at basal levels in the young heart, BDNF does not individually initiate angiogenesis. BDNF-mediated angiogenic potential might cross-talk with the focal microenvironment of inflammation. Indeed, previous reports documented that the BDNF pathway was associated with vascular inflammation [19] and peripheral inflammation [20]. Previously, we demonstrated that BDNF could mediate the enhancement of inflammation and injury in the aged heart under ischaemic situations. In addition, our *in vivo* studies revealed that the dose of BDNF (70 ng) that promotes angiogenesis in an infarcted zone failed to decrease the infarct sizes of MI in young hearts. The inability to reduce the size of the infarct area by the delivery of BDNF was likely not due to the dose of BDNF used because we previously found that using five times the present dose of BDNF did not decrease the infarct size in the young heart [7]. These findings suggest that in the adult, BDNF might initiate a multitude of effects *in vivo*, such as recruiting $TrkB^+$ endothelial cells [21], focal inflammation [7] and promoting the migration of CMECs, as found in this study, which all have important effects in cardiac angiogenesis. However, BDNF alone might not be enough to achieve the cardiac protection necessary to decrease the infarct size of MI.

We also found that the delivery into the old heart of the same amount of BDNF as that found in the young heart led to an increase in the infarct size of MI. These results suggested that senescence-associated down-regulation of TrkB-FL in CMECs not only contributed to the dysfunction of the BDNF-mediated migration of CMECs, which diminished the angiogenic potential of BDNF in aged hearts, but also shifted the BDNF-transducing pathway from BDNF-TrkB-FL in young hearts to the BDNF-TrkB-T1 in old hearts. This change would induce more myocardial injuries in aged hearts, such as an increase in the infarct size, super-inflammation and apoptosis, which were observed in this study and our previous study [7, 22, 23]. Indeed, other recent studies also indicated that BDNF in coronary circulation was increased in unstable angina patients [24]. In addition, the serum BDNF in patients with acute myocardial infarction was increased compared with individuals with stable angina pectoris [25]. Our findings provide a possible functional link for the shifting of the BDNF-TrkB pathway to the poor regenerative capacity observed in old hearts. Therefore, further studies on the BDNF-TrkB-T1 pathway in old CMECs and aged hearts will be beneficial to fully elucidate the molecular mechanisms of age-related dysfunction in the angiogenesis and regeneration capacity of old hearts.

Acknowledgements

This work was supported by the 863 grant (2007AA02Z105), NNSFC (30770886, 30570369, 30340038, 30973158, 81170324), GDKG-NSF (04105826), GDG-STD (2004B30601007), ICGGD (2009B050900007) and FRFCU (21609408).

Conflicts of interest

The authors confirm that there are no conflicts of interest.

References

1. **Segers VF, Lee RT.** Protein therapeutics for cardiac regeneration after myocardial infarction. *J Cardiovasc Transl Res*. 2010; 3: 469–77.
2. **Giacca M, Zacchigna S.** VEGF gene therapy: therapeutic angiogenesis in the clinic and beyond. *Gene Ther*. 2012; 19: 622–9.
3. **Tao Z, Chen B, Tan X, *et al.*** Coexpression of VEGF and angiopoietin-1 promotes angiogenesis and cardiomyocyte proliferation reduces apoptosis in porcine myocardial infarction (MI) heart. *Proc Natl Acad Sci USA*. 2011; 108: 2064–9.
4. **Donovan MJ, Lin MI, Wiegn P, *et al.*** Brain derived neurotrophic factor is an endothelial cell survival factor required for intramyocardial vessel stabilization. *Development*. 2000; 127: 4531–40.

5. **Liu Y, Sun L, Huan Y, *et al.*** Application of bFGF and BDNF to improve angiogenesis and cardiac function. *J Surg Res*. 2006; 136: 85–91.
6. **Okada S, Yokoyama M, Toko H, *et al.*** Brain-derived neurotrophic factor protects against cardiac dysfunction after myocardial infarction via a central nervous system-mediated pathway. *Arterioscler Thromb Vasc Biol*. 2012; 32: 1902–9.
7. **Cai D, Holm JM, Duignan IJ, *et al.*** BDNF-mediated enhancement of inflammation and injury in the aging heart. *Physiol Genomics*. 2006; 24: 191–7.
8. **Barbacid M.** The Trk family of neurotrophin receptors. *J Neurobiol*. 1994; 25: 1386–403.
9. **Kaplan DR, Miller FD.** Neurotrophin signal transduction in the nervous system. *Curr Opin Neurobiol*. 2000; 10: 381–91.
10. **Fenner BM.** Truncated TrkB: beyond a dominant negative receptor. *Cytokine Growth Factor Rev*. 2012; 23: 15–24.
11. **Berg MM, Sternberg DW, Parada LF, *et al.*** K-252a inhibits nerve growth factor-induced trk proto-oncogene tyrosine phosphorylation and kinase activity. *J Biol Chem*. 1992; 267: 13–6.
12. **Nye SH, Squinto SP, Glass DJ, *et al.*** K-252a and staurosporine selectively block autophosphorylation of neurotrophin receptors and neurotrophin-mediated responses. *Mol Biol Cell*. 1992; 3: 677–86.
13. **Meuchel LW, Thompson MA, Cassivi SD, *et al.*** Neurotrophins induce nitric oxide generation in human pulmonary artery endothelial cells. *Cardiovasc Res*. 2011; 91: 668–76.
14. **Numakawa T, Suzuki S, Kumamaru E, *et al.*** BDNF function and intracellular signaling in neurons. *Histol Histopathol*. 2010; 25: 237–58.
15. **Hempstead BL.** The many faces of p75NTR. *Curr Opin Neurobiol*. 2002; 12: 260–7.
16. **Hartmann M, Brigadski T, Erdmann KS, *et al.*** Truncated TrkB receptor-induced outgrowth of dendritic filopodia involves the p75 neurotrophin receptor. *J Cell Sci*. 2004; 117: 5803–14.
17. **Ohira K, Homma KJ, Hirai H, *et al.*** TrkB-T1 regulates the RhoA signaling and actin cytoskeleton in glioma cells. *Biochem Biophys Res Commun*. 2006; 342: 867–74.
18. **Ohira K, Kumanogoh H, Sahara Y, *et al.*** A truncated tropomyosin-related kinase B receptor, T1, regulates glial cell morphology via Rho GDP dissociation inhibitor 1. *J Neurosci*. 2005; 25: 1343–53.
19. **Donovan MJ, Miranda RC, Kraemer R, *et al.*** Neurotrophin and neurotrophin receptors in vascular smooth muscle cells. Regulation of expression in response to injury. *Am J Pathol*. 1995; 147: 309–24.
20. **Lee SL, Kim JK, Kim DS, *et al.*** Expression of mRNAs encoding full-length and truncated TrkB receptors in rat dorsal root ganglia and spinal cord following peripheral inflammation. *NeuroReport*. 1999; 10: 2847–51.
21. **Kermani P, Rafii D, Jin DK, *et al.*** Neurotrophins promote revascularization by local recruitment of TrkB+ endothelial cells and systemic mobilization of hematopoietic progenitors. *J Clin Invest*. 2005; 115: 653–63.
22. **Cai D, Xaymardan M, Holm JM, *et al.*** Age-associated impairment in TNF-alpha cardioprotection from myocardial infarction. *Am J Physiol Heart Circ Physiol*. 2003; 285: H463–9.
23. **Edelberg JM, Wong A, Holm JM, *et al.*** Phage display identification of age-associated TNFalpha-mediated cardiac oxidative induction. *Physiol Genomics*. 2004; 18: 255–60.
24. **Ejiri J, Inoue N, Kobayashi S, *et al.*** Possible role of brain-derived neurotrophic factor in the pathogenesis of coronary artery disease. *Circulation*. 2005; 112: 2114–20.
25. **Lorgis L, Amoureux S, de Maistre E, *et al.*** Serum brain-derived neurotrophic factor and platelet activation evaluated by soluble P-selectin and soluble CD-40-ligand in patients with acute myocardial infarction. *Fundam Clin Pharmacol*. 2010; 24: 525–30.