Comparison of Various Niches for Endothelial Progenitor Cell Therapy on Ischemic Myocardial Repair

Coexistence of Host Collateralization and Akt-Mediated Angiogenesis Produces a Superior Microenvironment

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Objective—Comparative studies are lacking that show the effects of different microenvironments on the activity of engrafted stem cells after myocardial infarction (MI). Here, we analyzed the temporal and spatial variations of angiogenesis, collateralization, and the expression of Akt-related signals after MI to test whether the effects of endothelial progenitor cells (EPCs) were different.

Methods and Results—After the induction of MI, pigs were selected that did not develop a collateral coronary circulation (R0) or developed a significant collateral coronary circulation (R2). Both sets were allocated randomly to 4 groups: phosphate-buffered saline (intramyocardial injection of phosphate-buffered saline), EPC transplantation, LY294002 (intramyocardial injection of an Akt inhibitor), and EPCs plus LY294002. Infarcted porcine hearts at different time points and under different collateralized conditions exhibited a variety of vascular microenvironments. At 14 days post-MI, angiogenesis and the expression of Akt-mediated angiogenic cytokines predominated in R2 porcine hearts. When grafted into this microenvironment, EPCs induced the greatest effects in impeding the development of heart failure, preserving left ventricular function and dimensions, and inhibiting infarct expansion. LY294002 significantly reduced these effects.

Conclusion—These findings suggest that the microenvironment that coexists with collateralization and Akt-mediated angiogenesis appears to be more beneficial to cardiac repair induced by EPC therapy than other niches after MI. (Arterioscler Thromb Vasc Biol. 2012;32:910-923.)

Key Words: angiogenesis ■ cell physiology ■ growth factors ■ ischemia ■ vascularbiology

Endothelial progenitor cells (EPCs) have shown regenerative potential in myocardial ischemic animal models with respect to cardiomyocytes and new blood vessel formation. With the recent surge of interest in EPC-based cardiac regenerative therapies for patients with myocardial infarction (MI), the identification of clinically applicable strategies is of paramount importance. Nevertheless, the implementation of this novel approach in a clinical setting requires the understanding of a number of key aspects that remain poorly understood. Among the main issues, the optimal timing for therapy and the most appropriate stem cell microenvironment are particularly important to maximize cell transplantation efficiency. For example, in the REPAIR-AMI trial, patients treated for up to 4 days after MI showed no benefit, whereas later treatment (days 4–8) provided an enhanced improvement of ejection fraction during follow-up. The initial pilot study by Erbs et al demonstrated that EPC transplantation (Tx) improves left ventricular ejection fraction (LVEF) and remodeling to a greater degree in patients with acute MI than in patients with old MI. Although it is not entirely clear which variables might influence outcome, where ischemic/necrotic tissue may provide an entirely different environment (or “niche”) to dictate cell fate, it is evident from various studies that transplanted stem cell function and numbers are limited by inflammation, insufficient vascular supply that results from ischemia, and loss of angiogenic factors after MI. Sustained coronary occlusion may lead to the progressive extension of infarcted areas and to fibrous development, and the regenerative response induced by cell therapy is limited in its ability to salvage a deteriorating myocardium.
However, when stem cells were modified with powerful angiogenic factors, including vascular endothelial growth factor (VEGF), angiopoietin-1 (Ang-1), or basic fibroblast growth factor (bFGF), and then transplanted into the ischemic rat heart, a better prognosis was achieved because of improved angiogenesis and restored global cardiac function. These results indicate that the expression of angiogenic factors is a key method of enhancing stem cell therapy. One problem that remains is whether the persistent expression of foreign proteins could lead to malignant transformation or late cell failure. The strategies for exogenous gene or protein treatments are limited. Exploration of the host endogenous cytoprotection or angiogenesis mechanisms to improve the effect of stem cell therapy would be more favorable.

Phosphatidylinositol 3-kinase (PI3K)/Akt signaling has been reported to play a major role in cell survival, cell proliferation, and angiogenesis in response to hypoxia. Akt signaling via parallel targeting with VEGF, bFGF, or Ang-1 plays an indispensable role in the induction of angiogenesis. However, whether the change in the expression level of Akt signaling-related proteins after MI is implicated in the neovascularization induced by stem cell therapy remains unknown.

To investigate these questions, we used a porcine model of MI and selected animals that either had no collateral coronary circulation (CCC) (R0) or had an abundant CCC (≥R2) at different postinfarct time points after MI. The present study was designed to gain insights into the temporal progression and spatial variations in the expression changes of inflammatory factors, angiogenic cytokines, collateral circulation, and angiogenesis in different ischemic microenvironments. We then compared the effects of these microenvironments on myocardial repair induced by autologous EPC therapy and determined whether Akt signals are involved in the underlying mechanisms.

Materials and Methods
An expanded Materials and Methods section containing details regarding animal allocation, animal model, study design, biopsy, blood high-sensitivity C-reactive protein measurement, myeloperoxidase detection, manipulation of EPCs, coronary angiography, echocardiography, histology, immunofluorescence, quantitative real-time reverse transcription–polymerase chain reaction (qRT-PCR), immunoblotting, and statistics is available in the online-only Data Supplement.

Animal Model, Study Design, and Biopsy Techniques
MI of miniswine (weight: 20–25 kg) was induced by ligation of the left anterior descending coronary artery approximately halfway between the origin of the left anterior descending artery and the apex. The infarcted miniswine with ejection fractions <45% at 1, 14, or 28 days after MI and with Rentrop scores of 0 (R0) or ≥2 (R2) were selected. These pigs then underwent a cardiac biopsy.

Blood High-Sensitivity C-Reactive Protein Measurement
Measurements of high-sensitivity C-reactive protein were performed on the COBAS Integra (Roche Diagnostics Ltd) with the C-reactive protein–latex assay.

Myeloperoxidase Detection
Myeloperoxidase was quantitated spectrophotometrically according to the manufacturer’s instructions.

Preparation, Labeling, and Transplantation of EPCs
Preparation, labeling, and transplantation of EPCs followed, as described previously. Coronary Angiography, Left Ventriculography, and Echocardiography
Rentrop grades of collateral circulation were assessed and counting of visible collateral vessels was performed by coronary angiography. Cardiac functions were evaluated by echocardiography.

Histology and Immunofluorescence
The size of the infarct was obtained by calculating the percentage of the infarct area against the whole LVEF area with the use of a digital image program (Scion ImageJ). The biopsy tissues and the peri-infarct regions from the autopsy specimens were embedded in paraffin or frozen for cryostat sectioning and were then stained by hematoxylin and eosin or immunofluorescence assays.

qRT-PCR and Immunoblotting
Peri-infarct myocardial tissues from the biopsied or autopsied tissues were harvested and pulverized to extract RNA or protein for qRT-PCR and immunoblotting.

Online Data Supplement Tables and Figures
Table I in the online-only Data Supplement describes the primers for qRT-PCR. Table II in the online-only Data Supplement shows the cardiac geometry calculated by echocardiography. Figure I in the online-only Data Supplement presents the experimental flow of the control and treatment groups. Figure II in the online-only Data Supplement shows the representative macroscopic and transverse myocardial sections stained by triphenyl tetrazolium chloride, and Figure III demonstrates the regulation of Akt-mediated neovascularization and cell proliferation induced by host vascular niches and stem cells after MI.

Results
A flow chart of the study protocol is shown in Figure I in the online-only Data Supplement. Of the 113 experimental pigs used in these studies, 5 normal animals were randomly chosen to be euthanized for the measurement of baseline levels of Akt-mediated cytokines and growth factors in cardiac tissue. Overall, 108 pigs underwent surgery. A total of 60 pigs with LVEF <45% and with collateral vessels of R0 or R2 evaluated by the Rentrop score underwent myocardial biopsy. After biopsy, 5 R0 or R2 pigs at 1, 14, and 28 days post-MI received a local intramyocardial injection of EPCs. Meanwhile, we performed the same surgery to induce an MI model in a further 105 pigs. Ten pigs with R0 at 1, 14, and 28 days post-MI were randomly divided into receiving either a local intramyocardial injection of phosphate-buffered saline (PBS) or LY294002; an additional 10 pigs with R2 were randomly assigned to receive either an injection of PBS or LY294002 with EPCs. To investigate the effects of LY294002 on the Akt-mediated pathway in the repair of ischemic myocardium, we chose 10 pigs with R0 or R2 at 14 days post-MI. EPCs and LY294002 were coinjected in R0 pigs, and LY294002 alone was injected into the R2 pigs. Thereafter, all animals were followed up for 1 month. No deaths occurred in this cohort of pigs during cell transplantation. Thirteen pigs died during follow-up. A total of 87 pigs...
survived to undergo serial functional studies by the end of this part of the study (Figure I in the online-only Data Supplement). No marked difference in mortality was found between any of the groups.

Serial Changes of Inflammation and Angiogenesis in the Infarcted Pigs

We initially examined the temporal changes in the inflammatory reaction and blood vessel density in the host heart with the use of a porcine model of MI. Hematoxylin and eosin staining showed that the strongest inflammatory reactions were observed at 1 day post-MI (Figure 1A), especially in the R0 pigs. This observation was confirmed by the occurrence of the highest myeloperoxidase level and high-sensitivity C-reactive protein at this time point, which was a quantitative index for the inflammatory reaction (Figure 1B and 1C). After 14 days, no significant inflammation was observed in any of the infarcted hearts. Blood vessel density was assessed by anti-factor VIII staining. Capillaries were identified as a single layer of factor VIII-positive cells with a flattened morphology, whereas arterioles were recognized as that of the factor VIII vessels with a luminal diameter of 10 to 100 μm (Figure 1D) and as staining positive for α-smooth muscle actin with a visible lumen (Figure 1E, arrows). A significant increase in the mean arteriolar and capillary densities was observed in the R2 animals and peaked on day 14 post-MI (Figure 1F and 1G).

Temporal Expression of Inflammatory and Akt-Mediated Factors After MI

Real-time reverse transcription–PCR was performed to quantify the mRNA expression of cardiac inflammatory and Akt-mediated factors in the peri-infarct border biopsy tissues. Compared with the baseline levels (day 0), the highest expression levels of interleukin-1 (IL-1), monocyte chemotactic protein-1 (MCP-1), and tumor necrosis factor α (TNFα) occurred within 1 day post-MI in both models; these gradually declined thereafter and were similar in both groups after 7 days post-MI (Figure 2A). In the R2 model, the gene expression of IL-1, MCP-1, and TNFα within 3 days was lower than in the R0 model (P<0.05). However, the angiogenic cytokines (Ang-1, bFGF, and VEGF) showed opposite changes: bFGF, VEGF, and Ang-1 were increased by 7-fold, 6-fold, and 11-fold, respectively, at 1 day post-MI and peaked at 14 days post-MI with 11-fold, 7-fold, and 14-fold differences, respectively, in the R2 group compared with the baseline level; the increase of these expression levels was much less in the R0 group compared with the R2 group (Figure 2A). There was an 8-fold difference in the expression of Akt at 14 days post-MI in the R2 model compared with the baseline levels, but this increase was relatively low in the R0 model with peaks that were different by 5-fold (Figure 2A). At day 28 post-MI, these angiogenic factors showed no significant differences between the R0 and R2 groups. To verify the correlation between the mRNA and protein levels, we measured the protein expression of 1 representative factor, Akt, by Western blotting. Immunoblots showed that the level of Akt was consistently higher in the R2 group compared with the R0 group over 14 days, and the protein levels correlated well with mRNA levels (Figure 2B). Similar to Akt expression, both representative Western blots with anti-Ser473 and anti-Thr408 antibodies and the bar graph of both Ser473 and Thr408 levels showed the greatest protein and phosphorylation levels in the pigs with R2 14 days after MI (Figure 2B).

To determine the relationship between proangiogenic mediators and vascular endothelial cells (ECs), we performed double staining with proangiogenic cytokines and the EC marker factor VIII. Immunofluorescence showed that MCP-1 and TNFα were rarely expressed by the vascular ECs, and the ECs in some blood vessels expressed IL-1. bFGF, Ang-1, VEGF, and Akt was more abundantly expressed in the R2 group than in the R0 group. bFGF, Ang-1, and VEGF were expressed mainly in the cytoplasm of the vascular ECs. Akt was expressed mainly in the cytoplasm of ECs and in cardiomyocytes in regions with a rich vasculature (Figure 2C, arrows). These expression patterns were seen with the greatest significance in the R2 day 14 animals. Therefore, we concluded that vascular ECs in the ischemic myocardial niche played a main role in intrinsic angiogenesis by secreting proangiogenic cytokines. These data indicate that the inflammatory reaction was maximal on day 1, especially in the infarcted hearts with R0; however, collateralization, angiogenesis, and Akt-mediated angiogenic cytokines were at their highest levels at 14 days and reached the greatest extent in the infarcted hearts with R2. In addition, the expression levels of Akt in the R2 group at 14 days post-MI correlated positively with the mRNA expression of VEGF, bFGF, and Ang-1, and the vessel density (r=0.953, 0.898, 0.925, and 0.949, respectively; P<0.05 for all comparisons).

Therefore, we chose 1, 14, and 28 days post-MI as the time points of cell therapy to investigate the effect of various niches in infarcted swine model on myocardial repair induced by EPC therapy, or with the injection of PBS, LY294002 (an Akt inhibitor), or EPCs plus LY294002.

EPC Characterization

After 21 to 28 days of culture, late outgrowth EPCs were used as the donor cells. Before transplantation, a small portion of the cells were chosen at random to identify EPC characteristics. Two aspects have predominantly used to identify EPCs: (1) cell surface markers that indicate both cellular naïveté and endothelial origin; and (2) functional phenotypes that imply the presence of endothelial precursors. The late outgrowth EPCs formed in vitro colonies with a typical cobblestone morphology, with an angiogenic capability and strong expression of the blood EC marker factor VIII, but they did not express the cardiomyocytic marker myosin heavy chain (Figure 3A). In line with the literature reported by Le Ricousse-Roussanne et al, Fadini et al, and Zhang et al, these EPCs expressed the cellular naïveté marker, CD133 (82.95±1.05%) and the hematopoietic progenitor cell marker CD117 (86.20±1.38%); and were negative for the panleukocyte marker CD45 (Figure 3B). The late outgrowth EPCs had significantly higher secretion levels of angiogenic cytokines, including VEGF, bFGF, and Ang-1, than those of inflammatory factors, including IL-1, MCP-1, and TNFα (Figure 3C).
Figure 1. Serial changes of the inflammatory response and angiogenesis in the peri-infarct area of infarcted miniswine hearts. A and D, Representative images from hematoxylin and eosin staining and immunofluorescent staining with factor VIII to measure blood vessel density from serial cardiac biopsy samples (day 1 [D1], D14, and D28) obtained from the animal hearts with R0 and R2, respectively. Images show the strongest inflammatory response at D1 post–myocardial infarction (MI) in the infarcted hearts with R0 (A). However, both the capillary density and the arteriolar density (D, white arrows) were seen to be the greatest in the peri-infarct area of pigs with R2 at D14 post-MI. E, Capillaries were identified as a single layer of factor VIII–positive cells (red), whereas arterioles were recognized as staining positive for α-smooth muscle actin (green) and as having a visible lumen (arrows). Images presented in E show the same trend as those in D: a significant increase in the mean arteriolar and capillary densities was observed in the R2 animals and peaked on D14 post-MI. Scale bars=50 μm. B, C, F, and G, Quantitative analysis of myeloperoxidase (MPO), high-sensitivity C-reactive protein (hs-CRP), arteriolar density, and capillary density at days 1, 3, 7, 14, 21, and 28 post-MI in the infarcted porcine hearts with R0 or R2. P<0.05: †vs the baseline (D0), ‡vs R0 at each time point, §vs D1, ¶vs D3, ¶¶vs D7, and ¶¶vs D14 post-MI in each group (n=5 in each group).
Figure 2. Serial expression changes of multiple cytokines in peri-infarct myocardium after endothelial progenitor cell (EPC) transplantation. A, Quantitative real-time reverse transcription–polymerase chain reaction was performed to measure the level of gene expression from the samples harvested from the peri-infarct myocardium of pigs at the baseline (pre–myocardial infarction [MI], day 0 [D0]) and 1, 3, 7, 14, 21, and 28 days after MI. Compared with the baseline, the infarcted hearts with R0 expressed the highest levels of the inflammatory cytokines at D1 post-MI, and the greatest degree of Akt-mediated angiogenic cytokines was seen in the hearts with R2 at D14 post-MI. B, Representative immunoblot electrophoresis and the subsequent quantification showed the protein levels of Akt, phospho-Akt (pAkt) Ser473, and pAkt Thr308, respectively. The ratio of phosphorylated Akt at Thr308 and Ser473/total Akt was calculated (n=5 per group). P<0.05: *vs the baseline (D0, before myocardial infarction); †vs R0 at each time point.
Host Collateralization Augments the Effects of EPC Therapy on Cardiac Function and Cardiac Geometry

At 30 days after Tx, the LVEFs of PBS-treated porcine hearts in both the R0 and R2 groups showed no significant change in comparison with their preinjection function. EPC treatment at 1 or 14 days post-MI in both models resulted in an improvement of LVEF and LV-fractional shortening compared with the LVEF and LV-fractional shortening of animals that received a PBS injection alone; the maximal improvement was observed in the R2 pigs treated by EPC at 14 days post-MI (Figure 4A). In contrast, in comparison with the respective baseline levels, the injection of PBS sustained the dilation of both the LV end systolic volume and the LV end diastolic volume. The injection of LY294002 alone exacerbated this remodeling process. However, Tx significantly ameliorated these indices, and the effect was found to be the greatest in the R2 pigs that received cell therapy at 14 days post-MI. Interestingly, the improvement in these indices that had resulted from Tx was abrogated by coinjection of LY294002. No marked difference in these indices was found in either group that received cell therapy at 28 days post-MI. Echocardiography showed that the systolic activity of the left ventricular (LV) wall at 30 days after treatment was stronger in the R2 group with Tx at 14 days post-MI than in the other groups (Figure 4B, arrows).

Consistent with the changes in LV cardiac function, compared with the values before cell therapy, porcine left ventricles showed progressive thinning of end diastolic interventricular septum thickness after MI, as well as thickening of the end diastolic posterior wall thickness and dilation of the LV-end-diastolic diameter. Injection of PBS alone aggravated these LV remodeling changes, and LY294002 treatment further worsened them (Table II in the online-only Data Supplement). For the EPC-treated groups, at 1 month after cell therapy, all LV remodeling indices were significantly better than those in the PBS groups (*P*<0.05). The greatest improvement was observed in the R2 pigs that had received cell therapy at 14 days post-MI (*P*<0.05), and LY294002 treatment abrogated this effect (*P*<0.05).

Akt Signaling in the Host Vascular Niche Promotes Neovascularization Induced by EPC Therapy

Before EPC therapy, coronary angiography demonstrated total occlusion of the left anterior descending artery.
Figure 5 A, black arrows), and the distal region from the ligature site was perfused via collateral vessels in the pigs with R2 but not in those with R0 CCC (Figure 5A, white arrowheads). At 1 month after cell therapy, although EPC therapy showed no significant increment of the Rentrop grades between the R0/H11001 Tx and R2/H11001 Tx groups compared with those before cell therapy (Figure 5C), visible collateral vessels (\( \text{H}11350 \frac{\text{H}9262}{\text{m}} \) in diameter\( \text{H}18 \) that branched from the distal portion of the left anterior descending artery in the direction of the infarct were marked in the EPC–treated animals, and the numbers of these collateral vessels were significantly increased in the R2/H11001 Tx pigs that received cell therapy at 14 days post-MI compared with the respective R0/H11001 Tx and PBS groups (4.0\( \text{H}11006 \) 0.7 versus 2.2\( \text{H}11006 \) 0.4 or 1.0\( \text{H}11006 \) 0.3, all \( P < 0.05 \); Figure 5A and 5D). Tissue sections were stained for anti–factor VIII antibody (to detect ECs). The numbers of arterioles and capillaries were greater in the R2 pigs that received cell therapy at 1 or 14 days post-MI than in the PBS groups (\( P < 0.05 \)) and were the greatest in the EPC-treated R2 pigs at 14 days post-MI in comparison with the R0/H11001 Tx and PBS groups (Figure 5B, 5E, and 5F). Double-staining with smooth muscle actin and endothelial factor VIII showed the same increase of the numbers of arterioles in the EPC-treated R2 pigs at 14 days post-MI (data not shown). Treatment with LY294002 appeared to prevent significant increases in vessel density.

**The Host Vascular Niche Enhances the Effects of Infarct Size Reduction of EPC Therapy**

As shown in the Table, compared with PBS treatment alone, EPC therapy resulted in a significant reduction in infarct size of the pigs that received EPC therapy at 1 or 14 days post-MI, and the effect was the greatest in the R2 group that received cell therapy at 14 days post-MI (18.5%; \( P < 0.05 \)). LY294002 abolished this effect of EPC therapy. The infarct size showed no significant differences between both the R0 and R2 models that received cell therapy at 28 days post-MI. There was no significant difference in arterial blood pressure, arterial \( P_{\text{O}_2} \), and heart rate between all groups 1 month after cell therapy. Triphenyl tetrachloride chloride staining showed that the infarct size 1 month after EPC therapy was given at 14 days post-MI was significantly smaller in the R2+Tx group than those in other groups, and some myocardial regeneration had occurred (Figure II in the online-only Data Supplement).
Figure 5. Coronary collateral circulation derived from coronary angiography and angiogenesis analysis. A and B, Images from the coronary angiography (A) and vascular density images assessed by immunofluorescence (B) in the R0+/EPC transplantation (Tx) and R2+/EPC groups that received cell therapy 1 day post-myocardial infarction (MI) and in the R0+/Tx and R2+/Tx groups that received stem cell therapy at 14 days, respectively. The black arrows and white arrowheads show the blocked left anterior descending coronary arteries and collateral circulation, respectively. Both the number of the capillary density and the arteriolar density (B, white arrows) were seen to be the greatest in the peri-infarct area of pigs with R2 at day 14 post-MI. Scale bars = 50 μm. C and D, Quantitative data of the changes in the Rentrop score and the numbers of visible collateral vessels (B, white arrowheads) in various groups 1 month after cell therapy, respectively. E and F, Capillary density and arteriolar density in various groups 1 month after cell therapy, respectively. P<0.05: *vs the respective group that received EPC therapy at 1 day post-MI; †vs the respective group receiving EPC therapy at 14 days post-MI; ‡vs R0+/phosphate-buffered saline (PBS); §vs R0+/Tx; ¶vs R2+/PBS; #vs R2+/Tx (n=4, 4, 3, 4, 5, and 4 in the R0+/PBS, R0+/Tx, R0+/LY294002 [LY], R2+/PBS, R2+/Tx, and R2+/Tx+LY groups receiving the treatment of PBS, Tx, or LY injection at 1 day post-MI, respectively; n=5, 5, 4, 5, 5, and 4 in the R0+/PBS, R0+/Tx, R0+/LY, R2+/PBS, R2+/Tx, and R2+/Tx+LY groups at 14 days, respectively; n=4, 4, 4, 5, 5, and 4 in the R0+/PBS, R0+/Tx, R0+/LY, R2+/PBS, R2+/Tx, and R2+/Tx+LY groups at 28 days, respectively).
Western blot analysis showed the same change of Akt protein expression in all the groups (Figure 6B).

Because maximal Akt activity is dependent on the phosphorylation status of both Thr308 and Ser473 residues, we serially measured the Akt phosphorylation with Ser473 and Thr408 antibodies. Western blotting revealed that cell therapy induced Akt phosphorylation at Thr-308 and Ser-473, and collateral vessels before cell therapy (R2) further strengthened this phosphorylation, whereas LY294002 injection markedly weakened the levels of phosphorylation. Phosphorylation of both Ser473 and Thr408 remained at low levels in both the R0+PBS and R2+PBS groups, and LY294002 injection alone further decreased the ratios of phosphorylated Ser473 and Thr408 to total Akt (Figure 6C and 6D). The inflammatory factors IL-1, MCP-1, and TNFα showed no significant difference between all the groups at 30 days post-MI (data not shown).

Immunofluorescence showed that Akt was detected in vascular ECs as well as ischemic lesions; Ang-1, bFGF, and VEGF were expressed mainly by the blood vessels and transplanted EPCs in the R2 pigs who had rich collateral vessels and received EPC therapy 14 days post-MI (Figure 6E, arrows), but the expression levels of these mediators were significantly decreased in the R0 pigs who had poor collateral vessels and received EPC therapy at 28 days post-MI (data not shown). Collectively, these findings suggest that the initial upregulation of Akt-mediated proangiogenic cytokine expression is induced by myocardial ischemia, and EPC therapy sustains this upregulation.

**The Akt Signaling–Induced Host Vascular Niche Accelerates the Propagation of Engrafted EPCs and Host ECs, and EPC Incorporation**

Figure 7A and 7E shows that rich microvessels with upregulation of Akt in the R2 groups before cell therapy caused a dose-dependent increase in the number of 4',6-diamidino-2-phenylindole (DAPI)–prelabeled EPCs in the ischemic zone, which became the most significant in the pigs that received cell therapy 1 day post-MI (data not shown). The greatest ratio between the number of double-stained Ki-67 and VIII cells double positively stained with DAPI and factor VIII showed no significant difference between all the groups at 30 days post-MI (Figure 6F, arrow). Collectively, these findings suggest that the initial upregulation of Akt-mediated proangiogenic cytokine expression is induced by myocardial ischemia, and EPC therapy sustains this upregulation.
Figure 6. Angiopoietin-1 (Ang-1), basic fibroblast growth factor (bFGF), vascular endothelial growth factor (VEGF), Akt, Ser473, and Thr308 expression in the infarcted porcine hearts after stem cell therapy. A and D, The quantitative analysis data of Ang-1, bFGF, VEGF, and Akt mRNA levels and the ratios of phosphorylated Ser473 or Thr408 to total Akt were measured by quantitative real-time reverse transcription–polymerase chain reaction and Western blot in the left ventricular peri-infarct areas at 30 days post-EPC transplantation (Tx), respectively. $P<0.05$: *vs the respective group receiving endothelial progenitor cell (EPC) therapy at 1 day post–myocardial infarction (MI), †vs the respective group receiving EPC therapy at 14 days post-MI, ‡vs R0/PBS, §vs R0/Tx, ¶vs R2/PBS, R2/Tx, and R2/Tx+LY groups receiving the treatment of PBS, Tx, LY, PBS, Tx, or Tx+LY injection at 1 day post-MI, respectively; n=5, 5, 4, 5, 5, and 4 in the R0/PBS, R0/Tx, R0/LY, R2/PBS, R2/Tx, and R2/Tx+LY groups receiving the treatment of PBS, Tx, LY, PBS, Tx, or Tx+LY injection at 14 days, respectively; n=4, 4, 4, 5, 5, and 4 in the R0/PBS, R0/Tx, R0/LY, R2/PBS, R2/Tx, and R2/Tx+LY groups receiving the treatment of PBS, Tx, LY, PBS, Tx, or Tx+LY injection at 28 days, respectively. B, Immunoblots showed that Akt was also upregulated at the protein level. Protein expression correlated well with mRNA expression. C, Representative Western blots of the Ser473 and Thr408 protein levels in the porcine hearts in individual groups 1 month post-Tx. Blots in both B and C represent at least 4 independent experiments. E, Immunofluorescence staining shows the local expression of Akt, VEGF, bFGF, and Ang-1 in the peri-infarct areas from the R2/Tx groups 1 month post-Tx. Blots in both B and C represent at least 4 independent experiments. E, Immunofluorescence staining shows the local expression of Akt, VEGF, bFGF, and Ang-1 in the peri-infarct areas from the R2/Tx groups 1 month post-Tx. Scale bars=50 μm. Akt, VEGF, bFGF, and Ang-1 stained red (cytoplasm stained red); the nuclei of transplanted EPCs were prelabeled with 4',6-diamidino-2-phenylindole (DAPI) and appeared blue after staining. Some of the DAPI-prelabeled EPCs were also stained with Akt, VEGF, bFGF or Ang-1 (arrows).
All of the increases in cell proliferation were blocked by LY294002.

**Discussion**

Several new and important findings have arisen from the present study. First, infarcted porcine hearts under different time points and collateralized conditions exhibited a variety of vascular microenvironments. At 14 days post-MI, angiogenesis and the expression of Akt-mediated angiogenic cytokines predominated in the porcine hearts with a rich collateral circulation. Second, when grafted into this microenvironment, EPCs caused the greatest effects on impeding the development of heart failure, which preserved LV function and dimensions and inhibited infarct expansion. Third, the Akt inhibitor LY294002 resulted in a significant suppression of VEGF, Ang-1, and bFGF expression and a reduction in the amount of angiogenesis, host EC proliferation, and EPC proliferation. This illuminated the potential mechanisms for the observed therapeutic synergistic effect of the host vascular niche and EPC therapy on ischemic myocardial repair. Together, the findings of this study imply that the vascular niche with the greatest level of Akt-mediated angiogenic cytokine expression, angiogenesis, and collateralization before cell therapy serves as a superior microenvironment for stem cells engrafted into ischemic hearts.

MI in humans and animal models results in characteristic histological and humoral changes in host niches, which consist of inflammation, angiogenesis, and soluble factor expression in the ischemic myolytic areas.1,6,20 These
changes are believed to represent an intrinsic adaptive response of the heart to ischemia. Similar values for the vascular densities and expression levels of angiogenic factors between the individual time points occurred in the pigs without CCC (R0 pigs), which was consistent with the findings of Vandervelde et al, who demonstrated that the expression of proangiogenic genes such as bFGF and VEGF changed only marginally post-MI. In the present study, we did not clarify the interaction between proangiogenic cytokines and individual cells in the ischemic niches, nor did we perform staining for activated forms of growth factor receptors in the vascular niches. These issues will be addressed in future studies. However, we indeed observed that 3 proangiogenic mediators, bFGF, Ang-1, and VEGF, were expressed mainly by the vascular ECs, and Akt protein was detected mainly by vascular EC and myocardial cells. These data suggest that these 2 cell types, particularly ECs, were key regulators for autologous angiogenesis in the ischemic niches. Furthermore, our study revealed that the R2 pig infarcted hearts with rich CCC showed a significant increase of the expression of bFGF, VEGF, and Ang-1, which was concomitant with a lower level of neutrophil attraction within 14 days post-MI; however, the inflammatory response was the strongest at the first day after MI in the infarcted hearts with R0. In parallel, Akt, a powerful modulator of angiogenesis, showed a similar pattern of progressive increases in expression in the R2 pig ischemic zone within 14 days post-MI. Moreover, the phosphorylation state of Akt at both Ser473 and Thr308 showed similar patterns of change in the total levels of cardiac Akt protein. These results imply that host endogenous angiogenesis, Akt-mediated angiogenic cytokines, and inflammation are composed of different microenvironments under a variety of settings induced by MI.

Exploration of the niches that require regeneration is indispensable for the optimization of stem cell therapy. To date, there have been no preclinical or clinical studies to investigate the impact of individual stages and blood supply conditions post-MI on the efficacy and safety of cell therapy in patients or large animals with acute MI. We were the first to choose 1, 14, and 28 days post-MI as the time points that represented the acute, subacute, and chronic stages post-MI, and to use CCC (R0) or CCC (R2) as the representative models of different vascular conditions. The degree of cardiac function improvement and infarct size reduction correlated with the amount of angiogenesis and cytokine expression that was observed before cell therapy; in both the R0 and R2 groups, cell therapy was given in higher levels of angiogenesis and Akt-mediated angiogenic factor expression, and this treatment enabled a greater reduction in infarct size, improvement in cardiac function, and alleviation in LV remodeling (as shown in Table II in the online-only Data Supplement). EPC therapy at 14 days post-MI led to the greatest magnitude of these outcomes in comparison with those at other time points in R2 pigs. Other mouse studies have shown similar changes, although MI led to a transient upregulation of angiogenic cytokines and continued at this level in the EPC group.1 In this study, there was no significant difference in LVEF and LVFS between different time points in the PBS groups, whereas cell therapy within 14 days post-MI can improve cardiac function via the upregulation of Akt pathway genes. In the Western blots of Figure 6B and 6C and the bar graph of Figure 6D, it seems that not only Akt expression but also Akt phosphorylation is increased rather than decreased regardless of any treatments involving PBS injection, EPC therapy, or LY294002 treatment after 1 and 14 days post-MI compared with after 28 days post-MI. This time difference may be of critical importance to the interpretation of the presented data. The expression of Akt, Ang-1, bFGF, and VEGF showed a similar difference, which suggests that Tx enhances the expression of Akt, Ang-1, bFGF and VEGF in a time-relative manner after MI. Moreover, these effects were found to be more significant in the R2+Tx groups, which had high intrinsic levels of Akt and its effector genes before cell therapy. This finding implied that the synergistic effects of cell therapy with the host intrinsic Akt-mediated factors could produce the most beneficial results. In contrast, EPC therapy at 1 day post-MI did not cause as much improvement in cardiac function and infarct size in the pigs with R0 as in the pigs with R2, as the former had a higher degree of inflammation. This implied that the higher expression of inflammatory factors at 1 day post-MI did not result in the further improvement of cardiac function and infarct size, with the exception of the effect of engrafted EPCs and intrinsic angiogenic cytokines. The mechanisms of how inflammation affected the effect of EPC therapy in ischemic tissue in the present study require further investigation. The increased expression levels of Akt and cytokines alone (eg, in R0+Tx groups) did not result in as high an enhancement of cardiac function induced by EPC therapy as the coexistence of host angiogenesis and Akt-mediated cytokines (eg, in R2+Tx groups). Taken together, only the synergistic action of cell therapy with a superior microenvironment enriched with collateralization, proangiogenic factors, and angiogenesis could lead to these optimal effects on the repair of infarcted myocardium.

Although a direct reason for these phenomena remains unclear after this present study, there might be some regulatory factors involved in this synergistic effect. Akt (protein kinase B), a serine/threonine kinase, is activated by many growth factors, such as Ang-1, bFGF, and VEGF.22–24 In this study, the local expression of VEGF, bFGF, and Ang-1 proteins in the peri-infarct areas was consistent with Akt expression. Thus, it can be seen that these growth factors, among others, induced by myocardial ischemia triggered Akt phosphorylation and activated Akt expression. High numbers of ECs in ischemic niches enhanced these effects. Hung et al found that the angiogenic effects of stem cells can activate the Akt pathway in a dose-dependent manner, which is in agreement with our study’s findings that EPC therapy further enhanced Akt expression and phosphorylation by secretion of Ang-1, bFGF, and VEGF (as shown in Figures 6A, 6E, and 3C). The findings of Babaei et al demonstrated that the angiogenic effects of Ang-1 are dependent on the PI3K/Akt pathway. As the PI3K pathway is activated during angiogenesis, VEGF- or bFGF-dependent angiogenesis requires the signaling mediated by the PI3K pathway.27 Our observations in this study also show the following: (1) the expression levels of Akt in the R2 group at 14 days post-MI correlated
positively with the mRNA expression of VEGF, bFGF, and Ang-1, and the vessel density; (2) Tx in an environment with higher expression levels of Akt and Akt phosphorylation led to a substantial increase in angiogenesis and cell proliferation, and a good CCC further augmented this increase; (3) LY294002 treatment significantly suppressed Akt activity by decreasing the ratios of phosphorylated Ser473 and Thr408 to total Akt for 1 month after EPC therapy; and (4) Akt inhibitor LY294002 decreased Ang-1, bFGF, and VEGF expression and abrogated angiogenesis, which was consistent with other studies that demonstrated that inhibition of PI3K-Akt pathway blocked Ang-1-induced sprouting activity in ECs.38 abolished bFGF-induced EC migration,29 and reduced constitutive and hypoxia-inducible levels of VEGF.30 This indicates that the Akt inhibitor significantly attenuates the expression of VEGF, Ang-1, and bFGF by preventing the phosphorylation of Akt in Ser473 and Thr308. Enoki et al.31 showed similar findings that the injection of LY294002 into rat hearts 1 month post-MI abrogated all of the beneficial effects of mesenchymal cell transplantation on increased neovascularization at both 3 days and 1 month after cell therapy. All of these data provide further evidence that angiogenesis and cell proliferation signals of the growth factors in ECs are mediated by both host vascular ECs and EPCs through the PI3K/Akt signal transduction pathway. Figure III in the online-only Data Supplement summarizes our current knowledge of how Akt-mediated angiogenesis and cell proliferation is regulated.

Although cotreatment with EPCs and LY294002 impaired post-MI recovery, the present study does not enable us to understand the underlying causative mechanisms: is the negative effect due to worsened vascular angiogenesis and a poor myocardial niche for EPC engraftment? Or are EPCs negatively affected by LY294002 treatment? Additional investigations need to pretest EPCs with LY294002 before transplantation to exclude the effect of LY294002 on EPCs. Moreover, activation of endogenous Akt is required for the full phosphorylation of Thr308 and Ser473. Phosphorylation of Ser473 by itself causes the partial activation of Akt, and full phosphorylation of Thr308 and Ser473. Phosphorylation of Ang-1, bFGF, and VEGF by preventing the phosphorylation of Akt in Ser473 and Thr308. Enoki et al.32 showed similar findings that the injection of LY294002 into rat hearts 1 month post-MI abrogated all of the beneficial effects of mesenchymal cell transplantation on increased neovascularization at both 3 days and 1 month after cell therapy. All of these data provide further evidence that angiogenesis and cell proliferation signals of the growth factors in ECs are mediated by both host vascular ECs and EPCs through the PI3K/Akt signal transduction pathway. Figure III in the online-only Data Supplement summarizes our current knowledge of how Akt-mediated angiogenesis and cell proliferation is regulated.

Although cotreatment with EPCs and LY294002 impaired post-MI recovery, the present study does not enable us to understand the underlying causative mechanisms: is the negative effect due to worsened vascular angiogenesis and a poor myocardial niche for EPC engraftment? Or are EPCs killed or otherwise impaired by LY294002? Additional investigations need to pretest EPCs with LY294002 before transplantation to exclude the effect of LY294002 on EPCs. Moreover, activation of endogenous Akt is required for the full phosphorylation of Thr308 and Ser473. Phosphorylation of Ser473 by itself causes the partial activation of Akt, and full phosphorylation of Thr308 and Ser473 acts synergistically to generate a high level of Akt activity.19 In this study, Akt expression was different between groups, which may be correlated with the observed difference in Akt activity (phosphorylation of Ser473 and Thr308). Further work is required to clarify the mechanism of how different phosphorylation patterns of Akt at Thr308 and Ser473 in various niches result in various expression levels of Akt and phospho-Akt induced by myocardial ischemia and cell therapy. Surprisingly, we observed that LY294002 decreased Akt mRNA expression. This may be related to the regulatory feedback mechanism described in Figure III in the online-only Data Supplement, and as follows: the PI3K/Akt pathway regulating gene transcription of VEGF, bFGF, and Ang-1 in turn triggers Akt phosphorylation and activates Akt expression. Thus, LY294002 at first inhibits PI3K/Akt phosphorylation and downregulates the expression of VEGF, bFGF, and Ang-1, which in turn decreases Akt mRNA expression. LY294002 injection may lead to impairment of engrafted EPCs and host ECs, causing a reduction of the mRNA levels of Akt expressed by these cells. The precise mechanism of how LY294002 decreases Akt mRNA expression requires further investigation.

In conclusion, our findings underscore the likelihood that Akt-mediated angiogenic cytokines induced by MI not only must be considered as a part of the native physiological mechanisms that enhance angiogenesis but also act as potential synergists with EPC therapy. In this manner, the niche that is enriched with angiogenesis and proangiogenic cytokines may serve as an optimal host microenvironment for transplanted stem cells post-MI, and this area of physiology represents a potential therapeutic target for the future treatment of ischemic diseases.

Acknowledgments

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Disclosures

None.

References


Comparison of Various Niches for Endothelial Progenitor Cell Therapy on Ischemic Myocardial Repair: Coexistence of Host Collateralization and Akt-Mediated Angiogenesis Produces a Superior Microenvironment

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Materials and Methods

Animal allocation

Miniswine (weight: 20–25 kg) were obtained from the Shanghai Animal Administration Center. This study was approved by the Animal Care and Use Committee of Fudan University and was in compliance with the “Guide for the Care and Use of Laboratory Animals” published by the National Academy Press.

Out of the 113 experimental pigs used in these studies, five normal animals were randomly chosen to be sacrificed for the measurement of baseline levels of Akt-mediated cytokines and growth factors in the cardiac tissue. Overall, 108 pigs underwent surgery. Eighty-one survived the operation to simulate the occurrence of a MI; a further 21 animals met the exclusion criteria for ventricular fibrillation, EF ≥45% (ejection fraction, as assessed by echocardiography), or a Rentrop score of R1, and were excluded from the study as a consequence. Therefore, a total of 60 pigs with R0 or R2 underwent myocardial biopsy (Fig. S1). After biopsy, five R0 or R2 pigs at 1 d, 14 d, and 28 d post-MI received a local intramyocardial (i.m.) injection of EPCs. In order to investigate the outcome of transplanted EPCs under various host niches, we performed the same surgery to induce an MI model in a further 105 pigs. Ten pigs with R0 at 1 d, 14 d, and 28 d post-MI were randomly divided into receiving either a local i.m. injection of PBS or LY294002; a further 10 pigs with R2 were randomly assigned to receive either an injection of PBS or LY294002 with EPCs. No deaths occurred in this cohort of pigs during cell transplantation. Twelve pigs died during follow-up. A total of 78 pigs survived to undergo serial functional studies by the end
of this part of the study (Fig. S I). No marked difference in mortality was found between any of the groups (Table S II).

**Anesthesia and monitoring**

The pigs were sedated with ketamine (15–20 mg/kg) and diazepam (1.5–2 mg/kg); anesthesia was maintained with thiopental (1–2 mg/kg/min, intravenously [i.v.]). An i.m. injection of atropine (30-50 µg/kg) was administered as a pre-anesthetic. In all experiments, the average baseline values of the mean aortic blood pressure (ABP), heart rate (HR), and arterial blood PO$_2$ (P$_{aO2}$) were 108 ± 3.6 mmHg, 89 ± 2.7 bpm, and 102 ± 3.7 mmHg, respectively. HR was monitored during electrocardiography (ECG) and coronary angiography (CAG), and ABP was measured throughout the coronary angiography follow-up period.

**Animal model, study design, and biopsy techniques**

For the surgical procedure, the animals were incubated and ventilated mechanically with a respirator. A lateral thoracic incision was performed over the apex of heart, and the pericardium was opened. A silk suture was placed around the left anterior descending (LAD) coronary artery approximately halfway between the origin of the LAD and the apex to allow for the ligation of the vessel. Arterial occlusion was confirmed by electrocardiography (ECG), echocardiography, and CAG 60 min after ligation. The infarcted miniswine with EF <45% were subjected to coronary angiography (CAG) at 1, 14 or 28 days after MI to grade the collateral filling of the occluded vessels in accordance with the Rentrop score, as previously described$^{[1]}$: 0 = absent; 1 = filling of side branches of the target occluded epicardial vessel without
visualization of the vessel itself; 2 = partial filling of the epicardial segment via a collateral circulation; 3 = complete filling of the epicardial segment of the occluded vessel. Animals were chosen at individual time-points with Rentrop scores of 0 (R0) or ≥2 (R2). Five biopsy specimens from the different peri-infarcted areas of each heart were then used to evaluate the expression of cardiac genes related to stem cells and the density of blood vessels within the peri-infarcted areas prior to stem cell therapy.

**Blood high-sensitivity C-reactive protein measurement**

Fasting blood samples were obtained from every pig just after CAG. Blood was drawn and centrifuged immediately, and serum was then placed into aliquots and stored at -80°C. Measurements of high-sensitivity C-reactive protein (hs-CRP) were performed on the COBAS Integra (Roche Diagnostics Limited, Mannheim, Germany) with the CRP-latex assay in both the high-sensitivity application (range, 0.2 to 12 mg/L; 7.6% at a level of 1.02 mg/L, 3.3% at 1.79 mg/L, and 1.3% at 4.36 mg/L) and the normal application (range, 2 to 160 mg/L; 2.4% at a level of 29.5 mg/L and 1.3% at a level of 113 mg/L).

**Myeloperoxidase detection**

Tissue samples from the peri-infarcted areas of miniswine myocardium were homogenized in saline (0.9 mL saline per 100 mg tissue). After centrifugation for 15 minutes at 3,000 g, the supernatant was collected and stored at -70°C until use. Myeloperoxidase (MPO) was quantitated spectrophotometrically by measuring the absorbance of MPO at 460 nm according to the instructions of the manufacturer. One unit of MPO was defined as the quantity of the MPO enzyme that hydrolyzes the
peroxide substrate at a rate of 1 mM/min at 25°C.

*Preparation, labeling, and transplantation of endothelial progenitor cells*

Prior to the preparation of the MI model, a circulating blood (CB) sample of approximately 80 mL was aspirated from the femoral artery of each pig. CB mononuclear cells were isolated by centrifugation on a Histopaque 1077 gradient (Sigma-Aldrich, St. Louis, USA) according to the manufacturer’s instructions and were plated at a density of 0.8-1.0×10^6 cells/cm² on fibronectin–coated Petri dishes (Sigma-Aldrich) with endothelial cell basal medium (EBM-2). Non-adherent cells were removed at day four, and cultures were then supplemented with new aliquots of media. After 7–10 days, monolayers of cobblestone-appearing cells appeared. We cultured cells for a further 21 to 28 day period and used the late outgrowth EPCs for the following analysis and therapy \[^2, 3\]. EPCs were collected and stained with 4',6-diamidino-2'-phenylindole (DAPI; Roche Diagnostics), as described previously \[^4\]. Cells were suspended in ice-cold phosphate-buffered saline (PBS) at a density of 1 x 10^7 cells/mL.

Immediately after biopsy, a median sternotomy was performed on all of the selected animals. A total of 5×10^7 EPCs in 5 ml of PBS were introduced by a direct i.m. injection into the peri-infarcted regions of the myocardium. A mean average of 11 (range: 9–12) injections of 0.25–0.5 mL of the solution that contained EPCs were performed in the peri-infarcted area in each animal. A further 10 pigs that were R0 or R2 at 1 d, 14 d, or 28 d post-MI randomly received an IM injection of 5 ml of PBS alone, LY294002 (100 mg/kg) in R0, or a co-injection of EPCs and LY294002 in R2,
respectively.

In order to investigate the effects of LY294002 on the Akt-mediated pathway in the repair of ischemic myocardium, we chose 10 pigs with R0 or R2 at 14 d post-MI. EPCs and LY294002 were co-injected in R0 pigs, and LY294002 alone was injected into the R2 pigs. Thereafter, all animals were followed-up for 1 month (Fig. S I).

**Characterization of endothelial progenitor cells**

In order to assess marker expression and functional characterization of EPCs, a portion of the cultured EPCs prior to transplantation were randomly allocated to either flow cytometry analysis (FACS) or immunofluorescence assays. In order to analyze the cell surface markers of EPCs, the following monoclonal antibodies (mAbs) conjugated to fluorochromes were used: anti-CD34-PE (MA1-19770; Thermo Scientific, Waltham, MA, USA), CD45-FITC (C2399-07L; United States Biological, Swampscott, MA, USA), CD14-FITC (20-783-74842; GenWay Biotech, San Diego, CA, USA), CD11b-PE (301307; BioLegend, San Diego, CA, USA), VEGFR2 (KDR, ab9530; Abcam, Cambridge, UK), and CD133 (AP2010b; Abgent, San Diego, CA, USA). For direct labeling, cells were incubated with 1 µg/ml of antibody for 30 min at room temperature. For indirect fluorescence, cells were first incubated with 1 µg/ml of the primary antibody and then with 1 µg/ml of the secondary antibody for 45 min at room temperature. Cells were analyzed on a FACScan™ using Cell Quest (BD Biosciences, San Jose, CA, USA) and WinMDI (Scripps Research Institute, La Jolla, CA, USA) software. At least 5,000 events were analyzed in each test. Mouse or rabbit IgG1, IgG2a, and IgG2b (Becton Dickinson, Mountain View, CA, USA) were used as
isotype controls.

Immunofluorescence was performed to investigate the expression of inflammatory and angiogenic cytokines in cultured EPCs. The cells were labeled with Hoechst-33342, and stained with the following antibodies in dilutions of 1:150 and 1:50: IL-1 (ab25102; Abcam), MCP-1 (ab21396; Abcam), TNF-α (T1816-1MG; Sigma-Aldrich, Islandia, NY), Ang-1 (ANG11-A; Alpha Diagnostic Intl., San Antonio, Texas, USA), bFGF (ab8880; Abcam), or VEGF (PC315-20UG; Calbiochem, San Diego, CA, USA). This was followed by incubation with a FITC- or TRITC-conjugated secondary antibody.

To detect the differentiation capacity of EPCs, the cultured cells pre-equipped with a coverslip (poly-lysine-treated) in 6-well plates were induced to differentiate into endothelial cells by the addition of bFGF and VEGF in DMEM/F12 medium\(^5\). After 4 d, the cells were fixed with acetone. The immunofluorescence staining of differentiated EPCs and undifferentiated EPCs was performed for detection of the von Willebrand factor (vWF, factor VIII) or myosin heavy chain (MHC). The cells were labeled with Hoechst-33342, and then stained with anti-factor VIII (MCA4677; AbD Serotec, Kidlington, UK) or MHC (ab15; Abcam) antibodies in a dilution of 1:150.

**Coronary angiography, left ventriculography, and echocardiography**

Coronary angiography, left ventriculography, and echocardiography were performed in sedated miniswine prior to MI, immediately after MI, pretransplantation, and 1 month after transplantation. We evaluated the extent of the collateral blood flow to the LAD semi-quantitatively by the graded Rentrop score.
Two-dimensional (2D) images were obtained at the midpapillary and apical levels with the use of echocardiography. Left ventricular end-diastolic and end-systolic volumes (LVEDV and LVESV) were measured using the biplane area-length method. Left ventricular (LV) volumes at the end-diastolic and end-systolic phases and the LVEF were calculated by the modified Simpson's method [6]. From the M-mode recordings, measurements of the LV end-diastolic diameter (LVEDD), end systolic diameter (LVESD), end diastolic inter-ventricular septum thickness (EDIVST), and LV end diastolic posterior wall thickness (EDPWT) were obtained. LV fractional shortening (LVFS, %) was calculated with the following formula: (LVEDD-LVESD)/LVEDD×100%.

**Histology and immunofluorescence**

The hearts of the miniswine were removed and weighed, and the LVs were divided into five or six transverse sections from the location of the ligation to the apex in a plane parallel to the atrioventricular groove. The slice with the maximum crosssectional area was chosen and immersed in 0.09 mol/L PBS (pH 7.4) that contained 1.0% triphenyl tetrazolium chloride (TTC, Sigma) for 20 min at 37°C in order to evaluate the infarcted area. The size of the infarct was obtained by calculating the percentage of the infarcted area against the whole LV area with the use of a digital image program (Scion ImageJ). The other LV sections were divided into three portions: the infarcted zone, which was defined as a myocardial region that was devoid of myocytes; the peri-infarct region, which was the area between 2 and 5 cm from the infarct; and the distant region, which was the area that was more than 5 cm
from the infarct.

The biopsy tissues and the peri-infarct regions resected from the autopsy specimens were embedded in paraffin or frozen for cryostat sectioning and were then stained by hematoxylin and eosin (H&E), or immunofluorescence with antibodies against the following proteins: IL-1, MCP-1, TNF-α, factor VIII, αSMA (alpha smooth muscle actin; ab7817, Abcam), Ang-1, bFGF, VEGF, MHC and Akt (ab35738l, Abcam). The number of vessels was expressed as the mean average number of factor VIII⁺ ECs per square millimeter. Capillaries were defined at factor VIII⁺ staining as vessels with diameters that were smaller than 10 µm with a single layer of endothelial cells. Arterioles were defined at immunofluorescence as vessels with a lumen diameter of 10-100 µm and a smooth muscle wall [7], and were further confirmed by immunofluorescent counterstaining with α-SMA to detect arterioles [8].

To observe the degree of cell proliferation in the peri-infarct area, the cryostat sections were stained with anti-Ki-67 antibody as mentioned above. For the quantification of capillary densities, arterioles, DAPI-positive cells, and proliferating cells, at least four randomly contiguous fields at both sides of the peri-infarct border zone in nonconsecutive tissue sections were examined. The number of positive cells in each high-power field was converted to cells per mm². Researchers who were unaware of the identity of the slides evaluated the density of arteries and capillaries or the number of cells in each field by counting vessels or cells in five randomly chosen unit areas (500 µm²) with the use of ocular micrometers (Olympus). The total number of vessels in 25 U areas (5 fields with 5 U areas in each field) were counted.
Interobserver variation was <5%. [9]

qRT-PCR and immunoblotting

Peri-infarct myocardial tissues from the biopsied or autopsied tissues were harvested and pulverized to extract RNA or protein. Total RNA was extracted with the use of RNA-Stat (Iso-Tex Diagnostics, Friendswood, TX, USA) according to the instructions of the manufacturer. The extracted RNA (500 ng) was subject to cDNA synthesis with Taqman Reverse Transcription Reagents (Applied Biosystems) to a final volume of 20 µl. All probes and primers were designed using Express Primer 3 software developed by the Whitehead Institute for Biomedical Research. The nucleotide sequences of selected genes were obtained from GenBank, and the primer information is shown in Table S1. The increase in fluorescence of 6-FAM (6-carboxyfluorescein) was automatically measured during PCR. Cycle thresholds (CT) for the individual reactions were determined using ABI Prism SDS 2.0 data processing software (Applied Biosystems). Relative mRNA transcript levels were quantified with the $2^{\Delta\Delta CT}$ method, using GAPDH (glyceraldehyde 3-phosphate dehydrogenase) as an internal control.

To confirm the protein expression of the genes of interest, Western blot assays were performed with modifications, as described previously.[4] In brief, protein extracts (100 µg per sample) were separated using SDS-PAGE (Bio-Rad Laboratories, Hercules, CA, USA) and electrotransferred onto PVDF membranes (GE Healthcare, Piscataway, NJ, USA). Samples were probed with Akt, pAkt Ser-473 (#9271; Cell Signaling), and pAkt Thr-308 (#5056; Cell Signaling) antibody. GAPDH (ab9482;
Abcam) served as a positive control. The target protein levels were determined as the ratios of the target protein/GAPDH using Image-Quant software. Since activity of Akt depends on phosphorylation of the protein, Akt activity were determined by measuring the phosphorylation level of their downstream signaling molecules on Western blot\cite{10,11}. The ratios of phosphorylated Akt at Thr-308 and Ser-473 against total Akt were calculated from porcine cardiac tissues after MI.

**Statistical analysis**

Results are expressed as mean averages ± standard error of the mean (SEM). The surgical team and investigators were blinded to the treatment and results until the study analysis was completed, the results were tabulated and the reports were released. We determined the statistical significance by an analysis of variance (ANOVA) for multiple comparisons. A value of p<0.05 was considered to be statistically significant.

**Results**

*LY294002 weakens the Akt-mediated pathway for ischemic myocardial repair*

In order to investigate the effects of LY294002 on the Akt-mediated pathway for the repair of ischemic myocardium, R0 pigs received a co-injection of EPCs and LY294002 14 d post-MI or an injection of LY294002 in R2 pigs. The LY294002 injection caused further a reduction of the LVEF (39.8% ± 1.4% in the R0+Tx+LY group and 30.3 ± 1.2% in the R2+LY group) and LVFS (22.4 ± 0.9% in the R0+Tx+LY group and 16.5 ± 0.7% in the R2+LY group), aggravation of the LVESV and LVEDV (LVESV: 25.7 ± 1.6 mL in the R0+Tx+LY group and 34.8 ± 1.0 mL in
the R2+LY group; LVEDV: 42.7 ± 2.1 mL in the R0+Tx+LY group and 49.9 ± 1.7 mL in the R2+LY group, respectively). They also had reduced levels of neovascularization (numbers of visible collateral vessels: 1.4 ± 0.5 in the R0+Tx+LY group and 0.5 ± 0.3 in the R2+LY group; number of arterioles: 105 ± 12/mm² in the R0+Tx+LY group and 79 ± 6/mm² in the R2+LY group; number of capillaries: 245 ± 27/mm² in the R0+Tx+LY group and 138 ± 16/mm² in the R2+LY group, respectively) in comparison with the R0+Tx and R2+PBS groups.

References


Table Legends

Table S I: The primers for real-time RT-PCR of porcine tissues.

Table S II: Cardiac geometry from echocardiography before and after cell therapy in the R0 and R2 animals. p<0.05: * vs. pre-Tx; † vs. the group that received EPC therapy at 1 d post-MI; ‡ vs. the group that received EPC therapy at 14 d post-MI; § vs. R0+PBS; ¶ vs. R0+LY; ‹ vs. R0+Tx; # vs. R2+PBS; ** vs. R2+LY; †† vs. R2+Tx. Tx, transplantation; EDIVST, end-diastolic interventricular septal thickness; EDWT, wall thickness; EDPWT, left ventricular end-diastolic posterior wall thickness; LVEDD, left ventricular end-diastolic diameter.

Figure Legends
Figure S I: Experimental flow of the control and treatment groups. Abbreviations: R0, R2, D1, D14, D28, Tx, LY, MI = without collateral vessels, with Rentrop score ≥2 collateral vessels, 1 d, 14 d, 28 d post-MI, EPC therapy, IM injection of LY294002, myocardial infarction, respectively.

Figure S II: Representative macroscopic (upper line) and transverse myocardial sections stained by TTC (lower line) of the R2+PBS, R0+Tx, R2+Tx, and R2+Tx+LY groups 1 month after EPC therapy was given 14 d post-MI. None of the infarcted myocardium was stained red by TTC; the pale region is the infarcted myocardium.

Figure S III: Regulation of Akt-mediated neovascularization and cell proliferation induced by host vascular niches and stem cells after myocardial infarction.
<table>
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<th>Gene</th>
<th>Description</th>
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Figure S I

[Diagram showing experimental workflow with various groups, time points, and follow-up procedures.]
Figure S II
Figure S III

Vascular niches/stem cells

Growth factors:
bFGF, Ang-1, VEGF

LY294002

PI3K phosphorylation of Akt (Thr308+ Ser473)

Akt

Neovascularization

Cell proliferation

Myocardial Repair