

FIGURE 4. Pin1 inhibition leads to the aberrant cell differentiation of human iPS cells. *A*, human iPS cells were cultured for 5 days before forming colonies and then treated with either DMSO or juglone (10 μM) for 3 days. The cells were then stained with AP (red). Representative images of phase-contrast microscopy and fluorescent immunocytochemistry are shown. Scale bar, 200 μm . *B*, mouse ES cells were cultured for 2 days before forming colonies and then infected with an adenovirus vector encoding either GFP or GFP-dnPin1 (3000 viral particles/cell). After 48 h, the cells were then stained with AP (red) and DAPI (blue) and analyzed by immunofluorescent microscopy. Scale bar, 50 μm .

ion trap (LTQ) Orbitrap hybrid mass spectrometer. Peptide mass fingerprinting with the Mascot and Aldente search algorithms subsequently identified 23 Pin1 interacting proteins in human iPS cells (Fig. 5*B*). Notably, these Pin1-binding proteins included the pluripotent transcription factor Oct4. Because Oct4 has been shown to be a master regulator of pluripotency (29), we decided to further analyze the Oct4-Pin1 interaction.

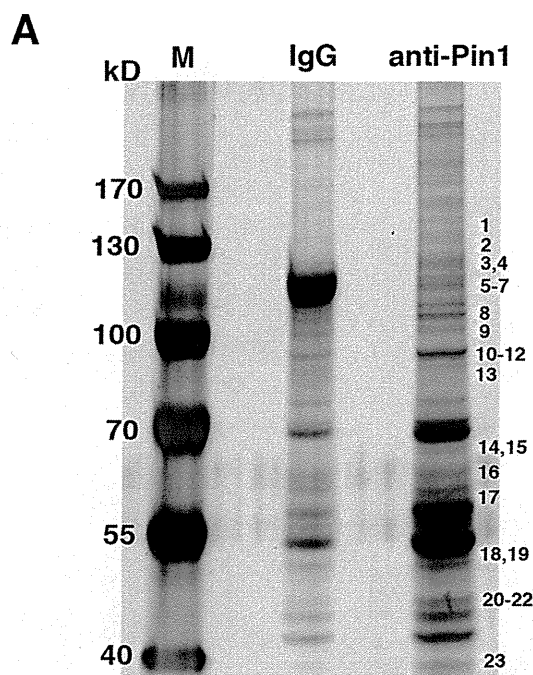
Pin1 Binds and Regulates Protein Stability of Oct4—To further characterize the Oct4-Pin1 interaction, a GST pull-down analysis was initially performed. We found that recombinant GST-Pin1, but not GST alone, binds Oct4. This association was completely abolished by pretreatment of the cell lysates with calf intestine alkaline phosphatase (Fig. 6*A*), indicating that Pin1 binds phosphorylated Oct4. Immunofluorescence analysis further demonstrated that Pin1 co-localizes with Oct4 in the nuclei of iPS cells (Fig. 6*B*). Pin1 has been shown to regulate the stability of its substrate proteins upon binding (17), and we thus addressed whether this was the case for Oct4. Cycloheximide analysis using HeLa cells transfected with Oct4 alone or co-transfected with Oct4 and Pin1 revealed that the protein half-life of Oct4 is significantly enhanced in cells co-expressing Pin1 (Fig. 6*C*). Moreover, immunoprecipitation analysis with cells co-transfected with Oct4 and Myc-tagged ubiquitin, with or without Pin1 co-transfection, further revealed that Pin1 overexpression significantly reduces the polyubiquitination of the Oct4 protein (Fig. 6*D*). Consistently, the Oct4 protein expression level was significantly reduced in human iPS cells treated with juglone as compared with control cells (Fig. 6*E*). These results together confirm that Pin1 enhances the protein stability of Oct4 by suppressing ubiquitin proteasome-mediated proteolysis.

We next investigated the gene expression profile of Oct4 during the inhibition of Pin1. Murine ES cells were transfected

with pGL4-Oct4-2601 promoter (harboring a genomic fragment of the Oct4 gene 5'-upstream region) and treated or not with juglone. Pin1 inhibition by juglone did not affect the transcriptional activity of the Oct4 promoter (Fig. 6*F*). Consistently, the results of parallel quantitative RT-PCR analysis demonstrated that the Oct4 mRNA level was not significantly altered by Pin1 inhibition (Fig. 6*G*), whereas the Oct4 protein level was significantly reduced by juglone treatment, as revealed by immunoblot analysis (Fig. 6*H*). These results together indicate that Pin1 regulates the protein stability of Oct4 but not Oct4 transcription.

We next addressed whether Pin1 enhances the transcriptional activity of the Oct4 protein. A luciferase reporter assay using the Oct-Sox enhancer region derived from the FGF4 gene was performed in HeLa cells co-transfected with Oct4, SOX2 or Pin1. Although the sole expression of Pin1 had no significant effects, the co-expression of Oct4 and Pin1 produced a significant increase in reporter activity in a dose-dependent fashion (Fig. 6*I*). This indicated that Pin1 promotes Oct4-mediated transcriptional activation. We performed a parallel experiment using the W34A and K63A Pin1 mutants. Neither of these mutants up-regulated the transcriptional activity of Oct4 to the levels seen with wild-type Pin1 (Fig. 6*J*), indicating that both the WW and PPIase domains are required for this function.

Pin1 Interacts with Ser¹²-Pro motif of Oct4—To identify the specific Pin1 binding site within the Oct4 protein, we generated several Oct4 deletion mutants and performed GST-pull-down analysis. These experiments revealed that a C-terminal Oct4 deletion mutant (representing amino acids 1–297) could still bind Pin1, but that three extended N-terminal deletion mutants (amino acids 138–360, 113–360, or 34–360) failed to do so (Fig. 7*A*). These data indicate that Pin1 binds to Oct4 in the region between amino acids 1 and 34. Previous reports have indicated



B

No.	Accession No.	Gene description	Predicted size
1	ADCY5_HUMAN	Adenylate cyclase type 5	138818
2	CCD40_HUMAN	Coiled-coil domain-containing protein 40	130033
3	PK3CA_HUMAN	Phosphatidylinositol-4,5-bisphosphate 3-kinase catalytic subunit alpha	124203
4	ZEB1_HUMAN	Zinc finger E-box-binding homeobox 1	123997
5	VINC_HUMAN	Vinculin	123722
6	RADIL_HUMAN	Ras-associating and dilute domain-containing protein	117351
7	UBP2L_HUMAN	Ubiquitin-associated protein 2-like	114465
8	DSG1_HUMAN	Desmoglein-1	113644
9	ENPP3_HUMAN	Ectonucleotide pyrophosphatase/phosphodiesterase family member 3	100059
10	ZN337_HUMAN	Zinc finger protein 337	86819
11	NASP_HUMAN	Nuclear autoantigenic sperm protein	85186
12	ZY11B_HUMAN	Protein zyg-11 homolog B	83921
13	MPEG1_HUMAN	Macrophage-expressed gene 1 protein	78587
14	FA13C_HUMAN	Protein FAM13C	65687
15	VPS45_HUMAN	Vacuolar protein sorting-associated protein 45	65036
16	ANR53_HUMAN	Ankyrin repeat domain-containing protein 53	59493
17	RPA34_HUMAN	DNA-directed RNA polymerase I subunit RPA34	54951
18	VIME_HUMAN	Vimentin	53619
19	KCAB1_HUMAN	Voltage-gated potassium channel subunit beta-1	46534
20	PRS8_HUMAN	26S protease regulatory subunit 8	45597
21	FKBP8_HUMAN	Peptidyl-prolyl cis-trans isomerase FKBP8	44534
22	PO5F1_HUMAN	POU domain, class 5, transcription factor 1 (OCT4)	38571
23	THAP1_HUMAN	THAP domain-containing protein 1	24928

FIGURE 5. Identification of Pin1-binding proteins in human iPS cells. A and B, lysates of human iPS cells were subjected to immunoprecipitation with either non-immunized control mouse IgG (IgG) or mouse anti-Pin1 monoclonal antibodies. Proteins bound to protein A/G-agarose beads were isolated, resolved by SDS-PAGE, and detected by silver staining (A). M indicates protein marker. Excised gel bands were digested with trypsin and analyzed on a linear ion trap (LIT) Orbitrap hybrid mass spectrometer followed by peptide mass fingerprinting with the Mascot and Aldente search algorithms (B).

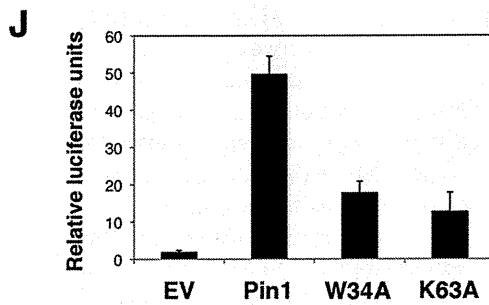
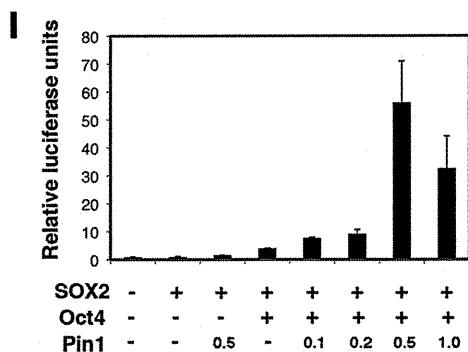
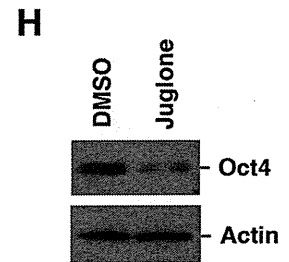
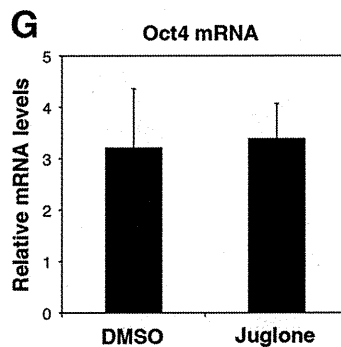
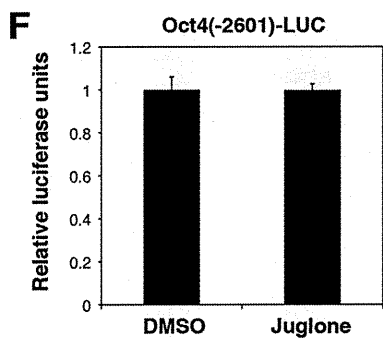
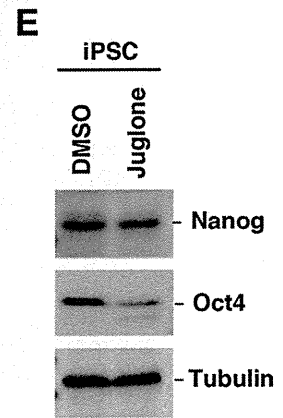
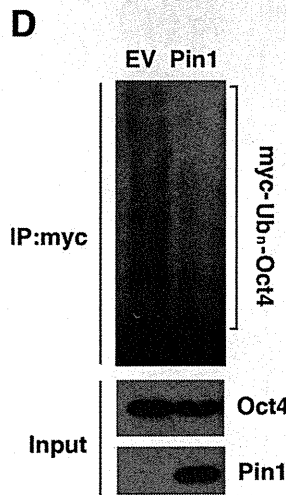
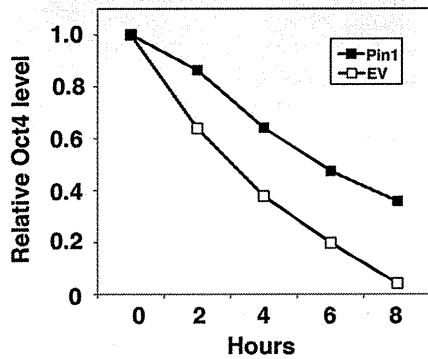
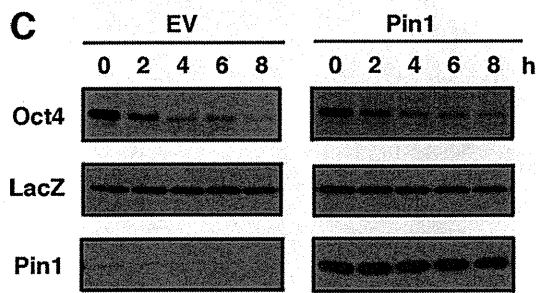
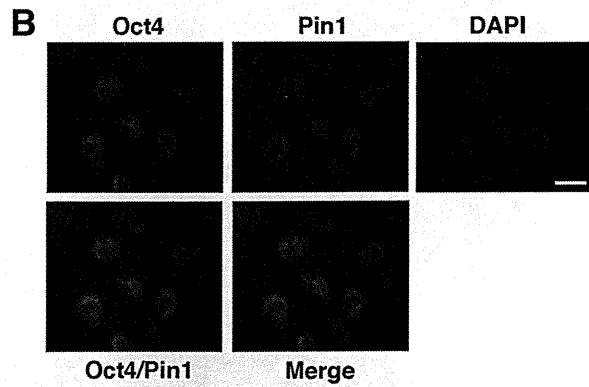
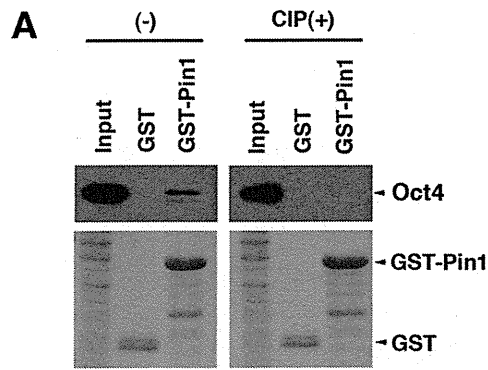
that Pin1 can bind only phosphorylated Ser/Thr-Pro motifs (17, 27) of which only one (Ser¹²-Pro) exists between residues 1 and 34 in the Oct4 protein. Interestingly, this motif is conserved between various species including human, mouse, rat, and rabbit (Fig. 7B). We generated an Oct4 site-directed mutant at this site by substituting serine 12 for alanine (S12A). GST pulldown analysis subsequently revealed that Pin1 binds wild-type Oct4, but not its S12A mutant (Fig. 7C). These results confirm that Pin1 indeed bind the phosphorylated Ser¹²-Pro motif of Oct4.

To further examine the functional interactions between Pin1 and Oct4 on this site, we next investigated the nature of the S12A mutant in terms of its protein expression in the presence

of Pin1. HeLa cells were transfected with either wild-type Oct4 or its S12A mutant and co-transfected with Pin1. This was followed by immunoblotting analysis. We found that Pin1 increased the expression levels of wild-type Oct4, but not the S12A mutant (Fig. 7D).

DISCUSSION

In our present study, we report that Pin1 is an essential regulator of the self-renewal and maintenance of pluripotent stem cells. We further found the following: 1) Pin1 is induced upon the induction of human iPS cells; 2) the co-expression of Pin1 with defined reprogramming factors significantly enhances the



Pin1 Regulates Cellular Stemness

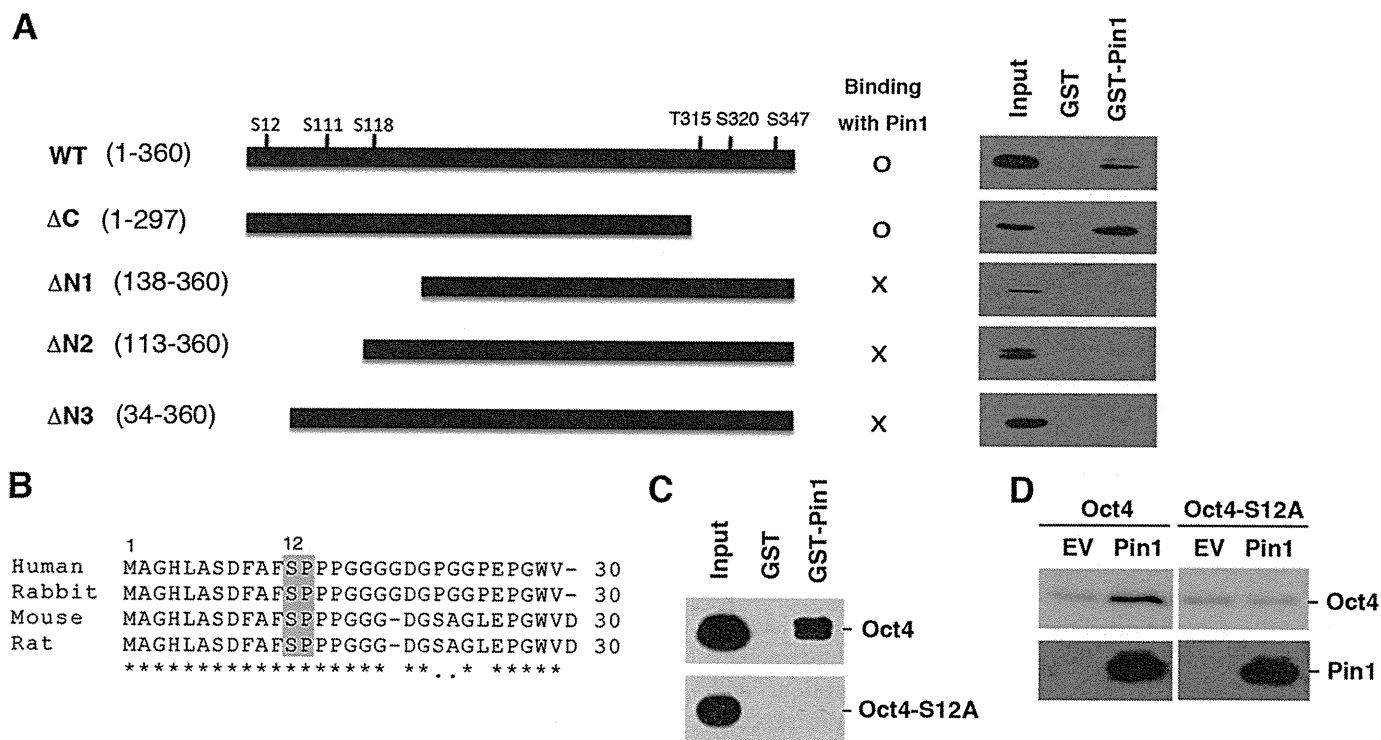


FIGURE 7. Pin1 interacts with the Ser¹²-Pro motif of Oct4. *A*, schematic representation of the Oct4 deletion mutants generated in this study (*left panel*). HeLa cells were transfected with the indicated Oct4 deletion mutants for 24 h. Cell lysates were then prepared and subjected to GST pull-down analysis with either GST or GST-Pin1 followed by immunoblotting analysis with Oct4 antibodies (*right panel*). *B*, amino acid sequence alignment of the human, rabbit, mouse, and rat Oct4 proteins. The conserved Ser¹²-Pro motifs are boxed. *C*, HeLa cells were transfected with the Oct4 site-directed mutant Oct4-S12A and subjected to GST pull-down analysis with wild-type Oct4 or its S12A mutant with or without Pin1. After 24 h, the cells were subjected to immunoblotting analysis with an anti-Oct4 antibody.

frequency of iPS cell induction; 3) the blockade of Pin1 significantly inhibits the colony formation of dissociated human iPS cells and murine ES cells; 4) Pin1 inhibition leads to the aberrant cell differentiation in human iPS cells and murine ES cells after forming colonies; 5) Oct4 is a putative Pin1 substrate in human iPS cells; and 6) Pin1 interacts with Oct4 at its Ser¹²-Pro motif and facilitates its stability and enhanced transcriptional activity. Our findings thus uncover a novel role of Pin1 as a putative regulator of the self-renewal and survival of pluripotent stem cells via Oct4 function.

Our current results add to previous findings indicating that Pin1 is a multifunctional protein that mediates various phosphorylated

proteins involved in divergent cellular processes (17). This implicates Pin1 as a modulator of multiple signaling pathways depending on the cell type and biological context. Indeed, we demonstrate in our present study that Pin1 is a crucial regulator of the phosphorylation-dependent intracellular signaling network that controls cellular stemness and pluripotency. Moreover, iPS cells induced by the expression of four Yamanaka factors (Oct4, SOX2, Klf4, and *c-Myc*) led to a high expression level of Pin1, and these cells were found to be dependent on Pin1 function. This suggests that Pin1 could be one of the crucial factors in the induction of iPS cells from somatic cells that functions by cooperating with reprogramming transcription factors.

FIGURE 6. Pin1 interacts with phosphorylated Oct4 and enhances its transcriptional activity. *A*, human iPS cell lysates treated or untreated with calf intestine alkaline phosphatase were subjected to GST pull-down analysis with either GST or GST-Pin1, followed by immunoblotting analysis with anti-Oct4 antibody (*upper panel*). Coomassie staining for the GST or GST-Pin1 used in the assay is shown in the *lower panel*. *B*, human iPS cells were fixed with 4% paraformaldehyde and then co-immunostained with monoclonal antibodies against Oct4 (*green*) and polyclonal antibodies against Pin1 (*red*). Cells were then analyzed by confocal microscopy. Scale bar, 10 μ m. *C*, HeLa cells transfected with the indicated vectors and HA-LacZ cells were treated with cycloheximide and harvested at the indicated time points. This was followed by immunoblotting analysis with Oct4, Pin1, and HA antibodies (*upper panel*). Quantitative data are shown in the *lower panel*. *D*, HeLa cells were transfected with Myc-tagged ubiquitin, Oct4, and co-transfected with either empty vector (EV) or Pin1. Cells were then treated with MG-132 for 12 h, and lysates were prepared and immunoprecipitated with anti-Myc antibody followed by immunoblotting analysis with anti-Oct4 antibody. Total cell lysates prior to immunoprecipitation (input) were immunoblotted with anti-Pin1 or anti-Oct4 antibody. *E*, human iPS cells were plated on Matrigel-coated feeder-free dishes and treated with either DMSO or juglone (20 μ M) for 24 h. Cell lysates were then processed for immunoblotting analysis with anti-Nanog, anti-Oct4, or anti-tubulin antibodies. *F*, a plasmid containing the luciferase (*LUC*) gene flanked with 2601 bp of the Oct4 5'-upstream region was transfected into murine ES cells. The resulting cells were cultured in Matrigel-coated feeder-free dishes and treated with either DMSO or juglone (10 μ M) for 24 h, and analyzed by gene reporter assay. *G*, murine ES cells were cultured in Matrigel-coated feeder-free dishes and treated with either DMSO or juglone (10 μ M) for 24 h. Total RNAs were then extracted and reverse-transcribed. These preparations were then subjected to quantitative RT-PCR analysis for Oct4. The transcript levels were normalized using GAPDH. *H*, murine ES cells were cultured in Matrigel-coated feeder-free dishes and treated with either DMSO or juglone (10 μ M) for 24 h. Cell lysates were then processed for immunoblotting analysis with either anti-Oct4 or anti- β -actin antibody. *I*, HeLa cells were transiently transfected with plasmids encoding Oct4, SOX2, or Pin1 and co-transfected with Oct-SOX reporter gene and pRL-CMV. At 24 h post-transfection, the cells were collected and subjected to a gene reporter assay. *J*, HeLa cells were transiently transfected with an Oct-SOX reporter gene and co-transfected with plasmids encoding wild-type Pin1 or its W34A or K63A mutants, together with Oct4 and SOX2. At 24 h post-transfection, the cells were collected and subjected to a gene reporter assay.

The molecular mechanisms underlying the regulation of Pin1 in the induction and maintenance of pluripotency are likely to be highly complex given that Pin1 interacts with multiple substrates in pluripotent stem cells, as revealed by our proteomics analysis. However, our current findings also indicate that Pin1 is involved in the growth and maintenance of pluripotency in stem cells through its phosphorylation-dependent prolyl isomerization of substrates such as Oct4. In this regard, a recent report by Moretto-Zita *et al.* (30) has demonstrated that Pin1 can also associate with another pluripotent transcription factor, Nanog, in murine ES cells and sustain the self-renewal and teratoma formation of these cells in immunodeficient mice. These results indicate that Pin1 is a crucial modulator of the transcription factor network governing cellular stemness. It is possible also that Pin1 could regulate this process by modulating the function of other substrates. Further studies of Pin1 function in stem cells at various stages might shed new light on the underlying molecular pathways and factors that control self-renewal and multipotency.

It has been demonstrated that Pin1 knock-out mice develop normally but display some proliferation abnormalities, including a decreased body weight, retinal degeneration, and impaired mammary gland development (31, 32). Pin1 knock-out mice also exhibit testicular atrophy with a significantly impaired proliferation of primordial germ cells and the progressive loss of spermatogenic cells (33). These phenotypes can now be attributed to the impaired maintenance and proliferation of germ-related stem cells due to the loss of Pin1 function.

In many circumstances, Pin1 acts as either a repressor or an enhancer of the degradation of substrate proteins (15–17, 34). Our current data now additionally demonstrate that Pin1 can also prolong the protein half-life of Oct4, thereby enhancing its transcriptional activity. Oct4 has been shown to be regulated by post-translational modifications such as SUMOylation (35). Our current findings reveal that Oct4 is also regulated by phosphorylation and subsequent prolyl isomerization. Identification of the kinase(s) responsible for the association of Pin1 and Oct4 will enhance our understanding of the regulatory pathways that operate during and after the induction of pluripotency.

It is desirable to utilize pluripotent stem cells such as iPS cells for future regenerative medicine applications. However, there are already concerns surrounding the use of iPS cells in a clinical setting because prior studies have suggested that they are likely to develop cancers (4, 36). Our current findings suggest, however, that the Pin1 inhibition could effectively block the proliferation of iPS cells in an undifferentiated state. Pin1 could therefore act as a molecular switch that can reversibly control the proliferation and survival of iPS cells, thereby reducing the risk of cell transformation and tumor formation.

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Coronary vein infusion of multipotent stromal cells from bone marrow preserves cardiac function in swine ischemic cardiomyopathy via enhanced neovascularization

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Few reports have examined the effects of adult bone marrow multipotent stromal cells (MSCs) on large animals, and no useful method has been established for MSC implantation. In this study, we investigate the effects of MSC infusion from the coronary vein in a swine model of chronic myocardial infarction (MI). MI was induced in domestic swine by placing beads in the left coronary artery. Bone marrow cells were aspirated and then cultured to isolate the MSCs. At 4 weeks after MI, MSCs labeled with dye ($n = 8$) or vehicle ($n = 5$) were infused retrogradely from the anterior interventricular vein without any complications. Left ventriculography (LVG) was performed just before and at 4 weeks after cell infusion. The ejection fraction (EF) assessed by LVG significantly decreased from baseline up to a follow-up at 4 weeks in the control group ($P < 0.05$), whereas the cardiac function was preserved in the MSC group. The difference in the EF between baseline and follow-up was significantly greater in the MSC group than in the control group ($P < 0.05$). The MSC administration significantly promoted neovascularization in the border areas compared with the controls ($P < 0.0005$), though it had no effect on cardiac fibrosis. A few MSCs expressed von Willebrand factor in a differentiation assay, but none of them expressed troponin T. In quantitative gene expression analysis, basic fibroblast growth factor and vascular endothelial growth factor (VEGF) levels were significantly higher in the MSC-treated hearts than in the controls ($P < 0.05$, respectively). Immunohistochemical staining revealed VEGF production in the engrafted MSCs. *In vitro* experiment demonstrated that MSCs significantly stimulated endothelial capillary network formation compared with the VEGF protein ($P < 0.0001$). MSC infusion via the coronary vein prevented the progression of cardiac dysfunction in chronic MI. This favorable effect appeared to derive not from cell differentiation, but from enhanced neovascularization by angiogenic factors secreted from the MSCs.

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KEYWORDS: angiogenesis; bone marrow; chronic myocardial infarction; coronary vein; multipotent stromal cells

In recent years, cell-based therapy has emerged as a potential new strategy for organ repair. The optimal source of cells for repairing damaged tissue is a topic of intense research. Bone marrow mononuclear cells (BMNCs) have been the cell source most frequently used for the angiogenic cell therapies

applied for cardiovascular disease.^{1–5} Two randomized controlled trials in Europe, however, failed to prove any functional benefit of intracoronary applied BMNCs in patients with acute myocardial infarction (MI).^{4,5} The results of these trials suggest that the effects of BMNC implantation may be

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insufficient to improve cardiac function. A new cell therapy using other cell types, a therapy more effective than BMNC implantation, is expected to be established soon.

Our group previously demonstrated the important contributions of the stem/progenitor cells among the BMNCs in improving limb ischemia.⁶ There has been considerable interest in the development of a new therapy with a non-hematopoietic subset of adult bone marrow (BM) stem/progenitor cells referred to as the mesenchymal stem cells or multipotent stromal cells (MSCs) in cardiac repair or regeneration.^{7–11} MSCs have been shown to protect against ischemic injury through both direct prevention of cell death and through the stimulation of angiogenesis.^{7,11} In experiments comparing cultured human MSCs with BM hematopoietic stem/progenitor cells, Iso *et al*⁷ found that the former expressed higher mRNA levels for angiogenic factors such as vascular endothelial growth factor (VEGF) and adrenomedullin. Encouragingly, translational studies on small and large models toward clinical implication have shown that MSC administration in the acute phase of MI improves cardiac function.^{8,9,12–14} It remains undetermined, however, whether MSC implantation is effective in a chronic MI or heart failure model.

As the selection of cell types advances, methods and devices for cell delivery will also have to be developed for clinical cell therapies. The methods used for cell implantation have included direct injection into myocardium after thoracotomy,¹⁴ transendocardial injection via an injection catheter,⁸ and intracoronary injection via a balloon catheter.⁹ The first of these methods, via thoracotomy, poses an increased risk of morbidity and mortality, as it requires highly aggressive intervention and general anesthesia. The second method, transendocardial injection with a catheter, also has flaws, including risks such as cardiac tamponade and ventricular arrhythmia. As hopeful alternatives, new methods of cell transplantation have recently been reported.^{15–18} We previously demonstrated the safety and efficacy of retrograde injection of BMNCs via the coronary vein into the myocardium through a single balloon infusion catheter in a swine MI model.¹⁵

Our group conducted this study for two purposes: first, to investigate whether the low invasive delivery of MSCs via the coronary vein restores cardiac function in chronic ischemic cardiomyopathy model; second, to explore the mechanism behind the improvement, if any improvement is found.

MATERIALS AND METHODS

Isolation of Swine MSCs

This study was conducted according to the Guide for the Care and Use of Laboratory Animals published by the United States National Institutes of Health. The experimental protocol was approved by the Animal Care and Use Committee of Showa University School of Medicine.

Swine BM cells were harvested from the femurs of the swine as the MI model was being created (Figure 1a). These

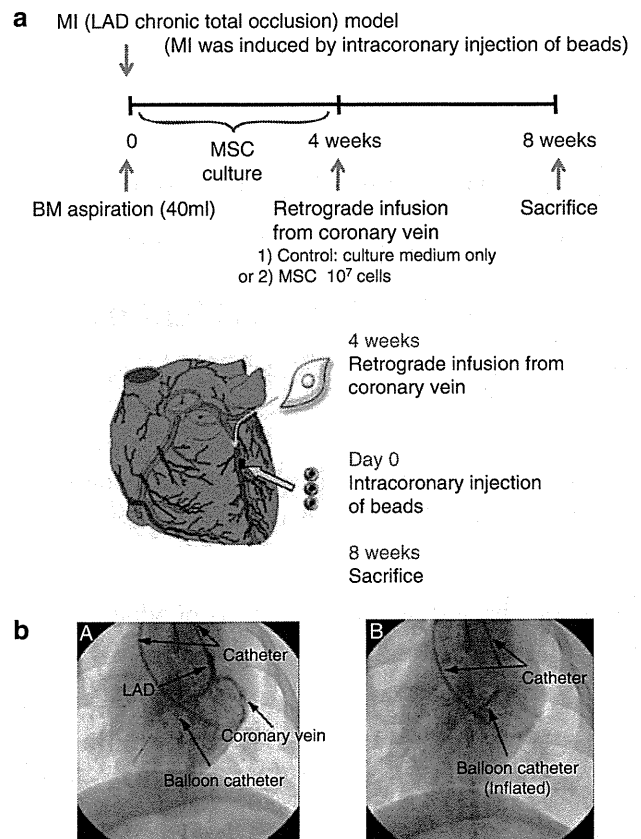


Figure 1 Experimental protocol and procedure. (a) Schedule and scheme for the experimental protocol. (b) Left coronary angiography and coronary venography (left), and X-ray image at balloon inflation with cell infusion (right). MI, myocardial infarction. BM, bone marrow. MSC, multipotent stromal cell.

cells were resuspended in MSC culture medium (10% fetal bovine serum in Dulbecco's modified Eagle's medium containing 4.5 g/l glucose) with antibiotic/antimycotic supplements (Invitrogen, Carlsbad, CA, USA), and the cultures were maintained at 37°C in a humidified atmosphere containing 95% air and 5% CO₂. When the cultures reached subconfluence, the cells were harvested with 0.25% trypsin and 1 mM EDTA, then one-half of the harvested cells were replaced. After one series of passages, the attached MSCs were devoid of hematopoietic cells. After two series of passages, the cells were harvested again with 0.25% trypsin and 1 mM EDTA, washed with phosphate buffer saline, labeled with Celltracker Orange (Invitrogen), and resuspended in 10 ml RPMI Medium 1640 (Invitrogen).

Chronic MI Model and Cell Implantation

In all, 19 male domestic swine, aged 2–4 months and weighing about 25 kg, were used. After receiving an intramuscular injection of ketamine (20 mg/kg) and atropine (60 µg/kg), each animal was intubated, ventilated, and maintained with a mixture of halothane (2%) and oxygen.

Electrocardiograph monitoring was performed continuously, and a coronary angiography was carried out after inserting an introducer from the right cervical artery. Next, the guidewire was inserted into the left anterior descending coronary artery and sterilized beads were placed in the mid left anterior descending coronary artery by microcatheter (Figure 1a and b) according to a method previously described.¹⁵ Peripheral blood was collected before and 1 and 4 days after MI. Serum creatine kinase (CK) levels were determined.

At 4 weeks after the induction of MI by permanent coronary occlusion with the beads, the 13 surviving animals were anesthetized again by the same procedure. After coronary angiography and left ventriculography (LVG), a balloon catheter (2.5 mm in diameter) was advanced through the coronary sinus into the anterior intraventricular vein and positioned just beside the occlusive site of the left anterior descending coronary artery (Figure 1a and c). The swine were randomly assigned to either an MSC infusion group (MSC group: $n=8$) or medium infusion group (control group: $n=5$). In the MSC group, MSCs ($1.02 \pm 0.34 \times 10^7$ cells) labeled with the red fluorescent dye were suspended in 10 ml culture medium and infused retrogradely through the balloon catheter from the anterior intraventricular vein over a 10-min period of balloon inflation. The same volume of medium was infused in the control group by the same procedure.

LVG was performed before and at 4 weeks after the cell implantation to determine cardiac function as previously described.¹⁹ Later, on the completion of follow-up coronary angiography and LVG at 4 weeks after the treatment, the animals were killed and their hearts were harvested. Samples were collected from the infarcted, border, and remote areas in the treated hearts. The tissue was prepared as previously described.¹⁹

Flow Cytometric Analysis of the Cultured Cells

The characteristics of the cultured MSCs were confirmed by flow cytometric analyses using purified or directly conjugated antibodies against SWC3a (BD PharMingen, San Diego, CA, USA), CD44 (BD PharMingen), SLA-class I (BD PharMingen), CD29 (BD PharMingen), CD44H (FITC-labeled, BD PharMingen), SLA-DQ (BD PharMingen), CD31 (BD PharMingen), and CD90 (PE-labeled; BD PharMingen). The cells were detached and stained for 30 min at 4°C with primary antibodies and immunofluorescent secondary antibodies. After washing, the cells were analyzed on a Cytomics FC500 analyzer (Beckman Coulter, Fullerton, CA, USA).

Immunostaining and Morphometric Analysis

Immunohistochemistry and the morphometric analysis were performed as previously described.¹⁹ The primary antibodies were anti-von Willebrand factor (vWF) for endothelial cells at a 1:400 dilution (Santa Cruz Biotechnology, Santa Cruz, CA, USA), anti- α -smooth muscle actin (SMA) for small arteries at a 1:300 dilution (Sigma, Saint Louis, MO, USA)

and phospho-signal transducer and activator of transcription 3 (phospho-STAT3, Cell Signaling Technology, MA, USA) at a 1:50 dilution.

Images of the Mallory-stained sections were recorded, digitized, and analyzed with Win ROOF image analysis software (Mitani, Fukui, Japan) to measure the fibrotic area. The measurements were taken in 10 sections for each animal. To evaluate neovascularization, the numbers of α -SMA- and vWF-positive vessels in the border areas were manually counted in a randomly selected high-power field ($\times 400$). The measurements were taken in 10 areas for each animal. The investigator performing this analysis was blinded to the treatment.

Immunofluorescence

Before staining, frozen sections were fixed for 10 min in chloroform at room temperature. The sections were incubated with the first antibody: either anti-vWF antibody (Santa Cruz Biotechnology) at a 1:300 dilution for 60 min at room temperature, or anti-troponin T antibody (NeoMarkers, Fremont, CA, USA) at a 1:100 dilution for 60 min at room temperature, or anti-VEGF antibody (Santa Cruz Biotechnology) at a 1:800 dilution for 24 h at 4°C. After incubation with the first antibody, the sections were incubated with the second antibody: Alexa 488-labeled IgG (green color; Molecular Probes, Eugene, OR, USA; 1:400 dilution) for 1 h. Nuclear staining was performed by DAPI.

Quantitative mRNA Expression Analysis for Basic Fibroblast Growth Factor (bFGF) and VEGF

An RNA extraction, reverse transcription-polymerase chain reaction (RT-PCR) analysis and quantification of the PCR products were performed as previously described.¹⁹ The PCR primers were as follows: bFGF, forward 5'-TCAAAG GAGTGTGTGCGAAC-3' and reverse 5'-CAGGGCCACATA CCAACTG-3'; VEGF, forward 5'-ATGCGGATCAAACC TCACC-3' and reverse 5'-ATCTGGTTCCCGAAACGCTG-3'; GAPDH, forward 5'-TCACCATCTTCCAGGAGCGA-3' and reverse 5'-CACAATGCCGAAGTGGTCGT-3'. PCR reactions were carried out as follows: for bFGF, 35 cycles of denaturation for 60 s at 94°C, annealing for 45 s at 59°C, and extension for 45 s at 72°C; for VEGF, 27 cycles of denaturation for 30 s at 94°C, annealing for 1 min at 60°C, and extension for 2 min at 68°C, followed by a final extension for 7 min at 68°C; for GAPDH, 30 cycles of denaturation for 1 min at 94°C, annealing for 90 s at 55°C, and extension for 2 min at 72°C.

The sizes and quantities of the PCR products were detected by an Agilent 2100 Bioanalyzer (Agilent Technologies, CA, USA) according to the manufacturer's instructions. In brief, 9 ml of gel-dye mix was fed into each of 16 wells and then forced into a micro channel network. Each of two buffer wells was also filled with 9 ml of the gel-dye mix, and sample and ladder wells were filled with 5 ml of marker mix followed by 1 ml of DNA sizing ladder and sample, respectively. The

prepared microchip was vortexed and placed into the bio-analyzer for analysis. All experiments were performed using Agilent biosizing software (Version A.02.12).

Immunoblotting

The immunoblotting was performed by a method previously described,²⁰ with minor modifications. A total of 20 µg of protein was separated by SDS-PAGE. The protein was transferred from gel to a clear blot P membrane sheet (Atto,

Tokyo, Japan) by horizontal electrophoresis at 108 mA for 90 min at room temperature. Immunostaining was carried out with anti-VEGF (Santa Cruz Biotechnology) and bFGF (Millipore, CA, USA) antibodies.

In Vitro Angiogenesis Assay

Tubule formation experiments were conducted using an Angiogenesis kit (Kurabo, Osaka, Japan), according to the manufacturer's instructions. Briefly, human umbilical vein

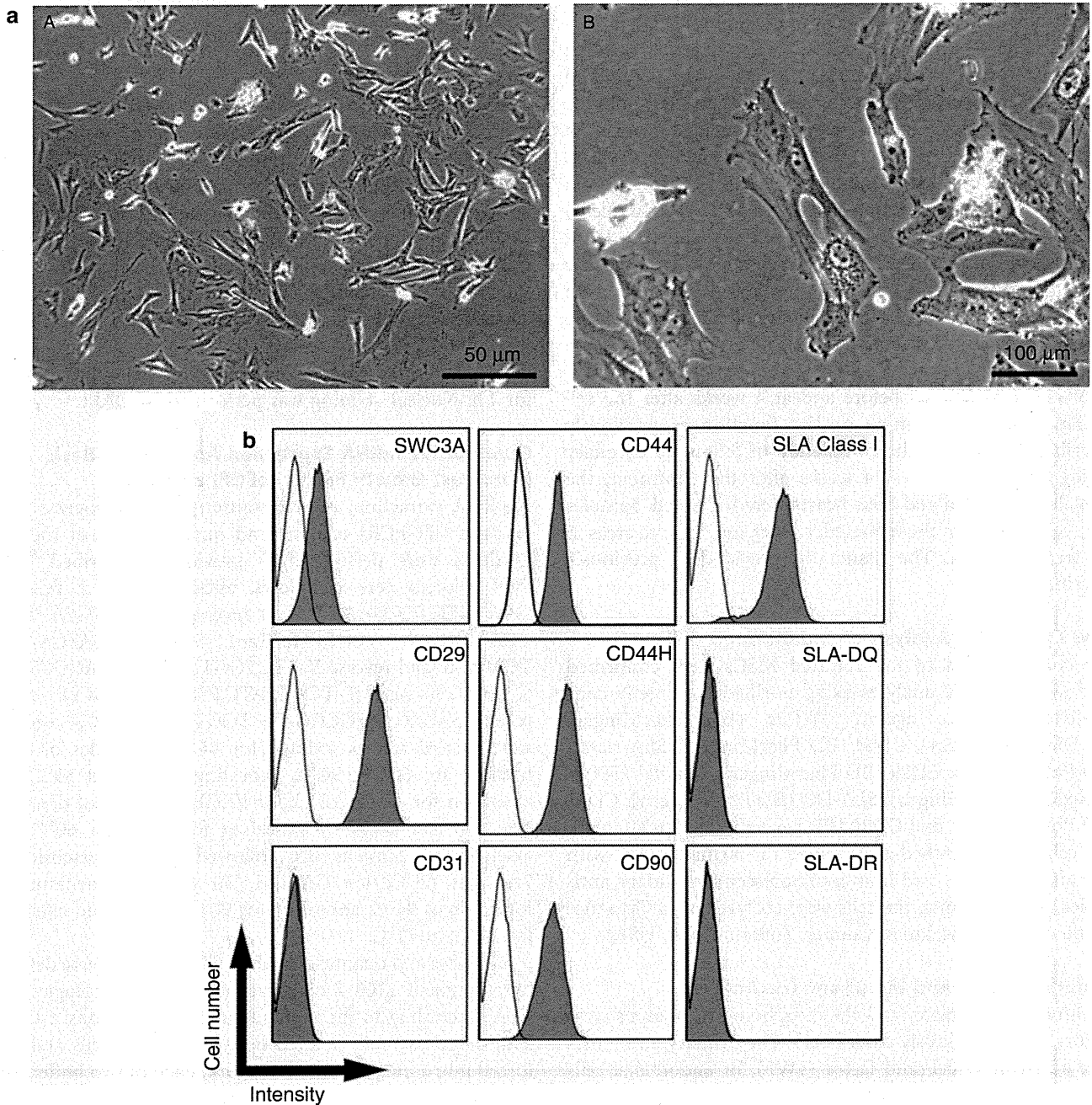


Figure 2 Phenotype of swine MSCs. (a) Phase contrast microscopic images of MSCs (A, low magnification; B, high magnification). (b) Flow cytometric analysis of swine MSCs. MSCs are positive for SWC3a, CD44, SLA class I, CD29, CD44H, and CD90, and negative for SLA-DQ, CD31, and SLA-DR.

endothelial cells (HUVECs) and human fibroblasts were admixed and seeded into 24-well plates. Human MSCs (5×10^4 cells/well) were applied and co-cultured for the experiments. After 10 days of culture, the HUVECs were fixed with 70% ethanol at 4°C and immunostained with an anti-human CD31 antibody using BCIP/NBT as a substrate for the secondary antibody. Five fields per well were selected for digital photography under a microscope (Olympus, Tokyo, Japan), and the areas of the tubule-like structures were measured quantitatively using angiogenesis image analyzer software (Kurabo).

Statistical Analysis

All values are expressed as means \pm s.e.m. The means were compared by the unpaired Student's *t*-test. The paired *t*-test was used for comparison of temporal changes in the ejection fraction (EF). Comparisons among the three groups were conducted by one-way analysis of variance followed by Scheffé's multiple comparison test. A *P*-value of <0.05 was considered statistically significant.

RESULTS

Phenotype of Swine MSCs

Bone marrow was aspirated from the swine femurs as the MI model was being created (Figure 1a). The mononuclear cell

layer from the BM was cultured, and the MSCs adhering to the culture plastic after 24–48 h were isolated. The cultured swine MSCs were phenotypically similar to human MSCs. They had a fibroblastic spindle-like shape typical of mesenchymal cells and were positive for CD44, SLA class I, CD29, and CD90, and negative for CD31 (Figure 2).

Cardiac Function in Chronic MI after the MSC Implantation

We measured the serum CK levels to assess acute myocardial damage after the sterilized beads were implanted in the swine LADs. The levels of CK peaked at 1 day after the infarction, and the peak levels were similar in the two groups (3338.6 ± 538.5 mg/dl in the control group, 3447.7 ± 561.7 mg/dl in the MSC group).

At 4 weeks after the MI, the swine were treated with the MSCs ($1.02 \pm 0.34 \times 10^7$ cells, $n=8$) or vehicle ($n=5$) via coronary vein infusion (Figure 1c). Coronary venography confirmed that the vessels were free of complications such as vein rupture or embolization during the procedure. To determine cardiac function, LVG was performed immediately before and at 4 weeks after the treatment. The EF assessed by the LVG did not significantly differ between the two groups at baseline. The EF significantly decreased in the

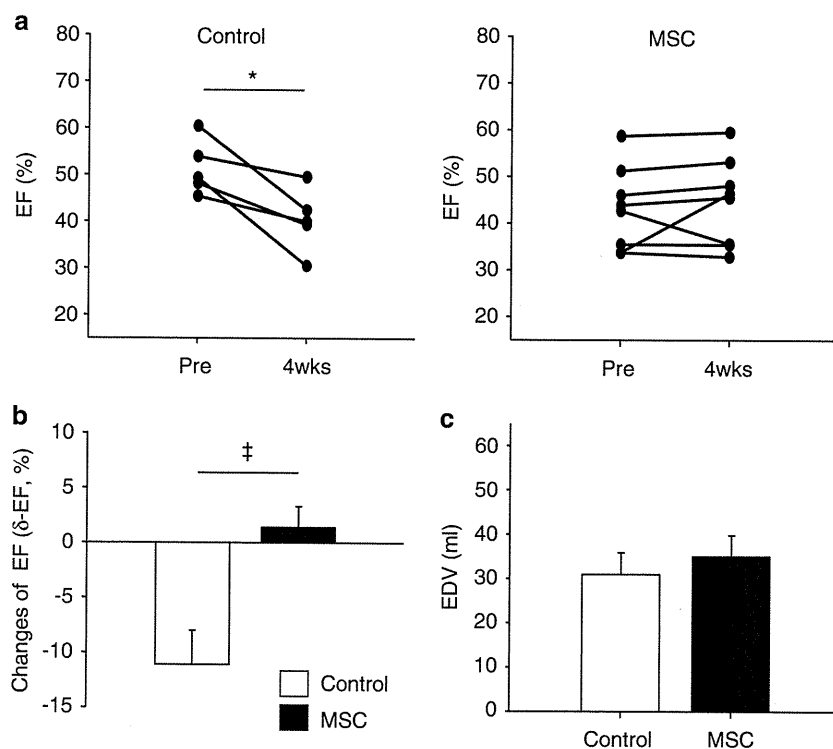


Figure 3 Cardiac function assessed by left ventriculography. (a) Time course changes in ejection fraction (EF) immediately before and at 4 weeks after treatment. Left, control group ($n=5$); right, MSC infusion group ($n=8$). (b) Differences between pre-treatment EF and EF after 4 weeks (δ -EF). (c) Endodiastolic volumes at 4 weeks after treatment. No significant differences between the two groups were observed. Data are means \pm s.e.m.* $P < 0.05$; ‡ $P < 0.01$.

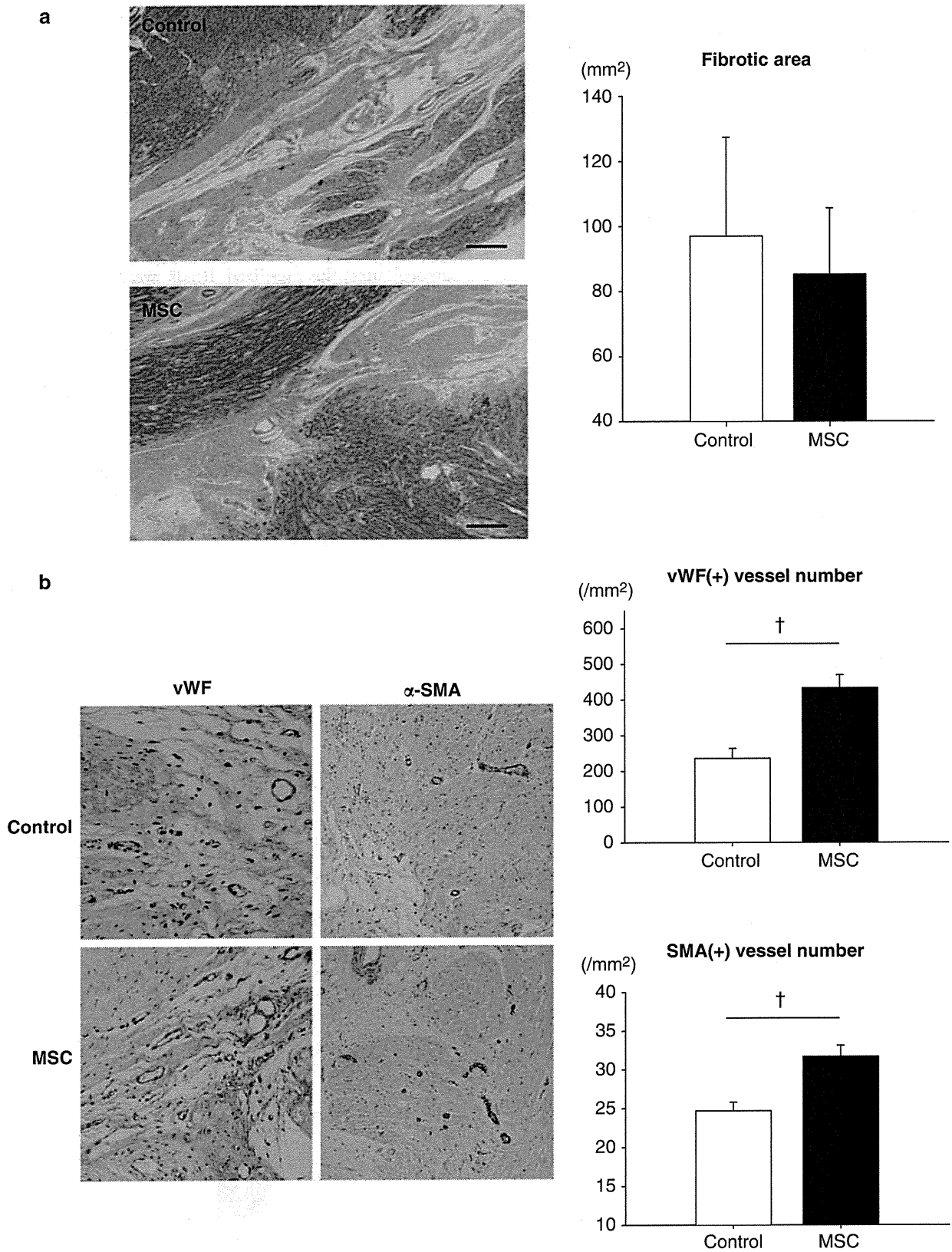


Figure 4 Cardiac fibrosis and neovascularization in hearts after cell infusion. (a) Left, blue staining indicates fibrosis in the Azan-Mallory-stained sections. Right, the morphometric analysis shows no significant differences in the fibrotic area between the two groups. (b) Left, neovessels are evaluated in the von Willebrand factor (vWF)- and the α -smooth muscle actin (α -SMA)-stained sections. Right, the numbers of vWF- and α -SMA-positive vessels in the border areas. Data are means \pm s.e.m. $^\dagger P < 0.0005$.

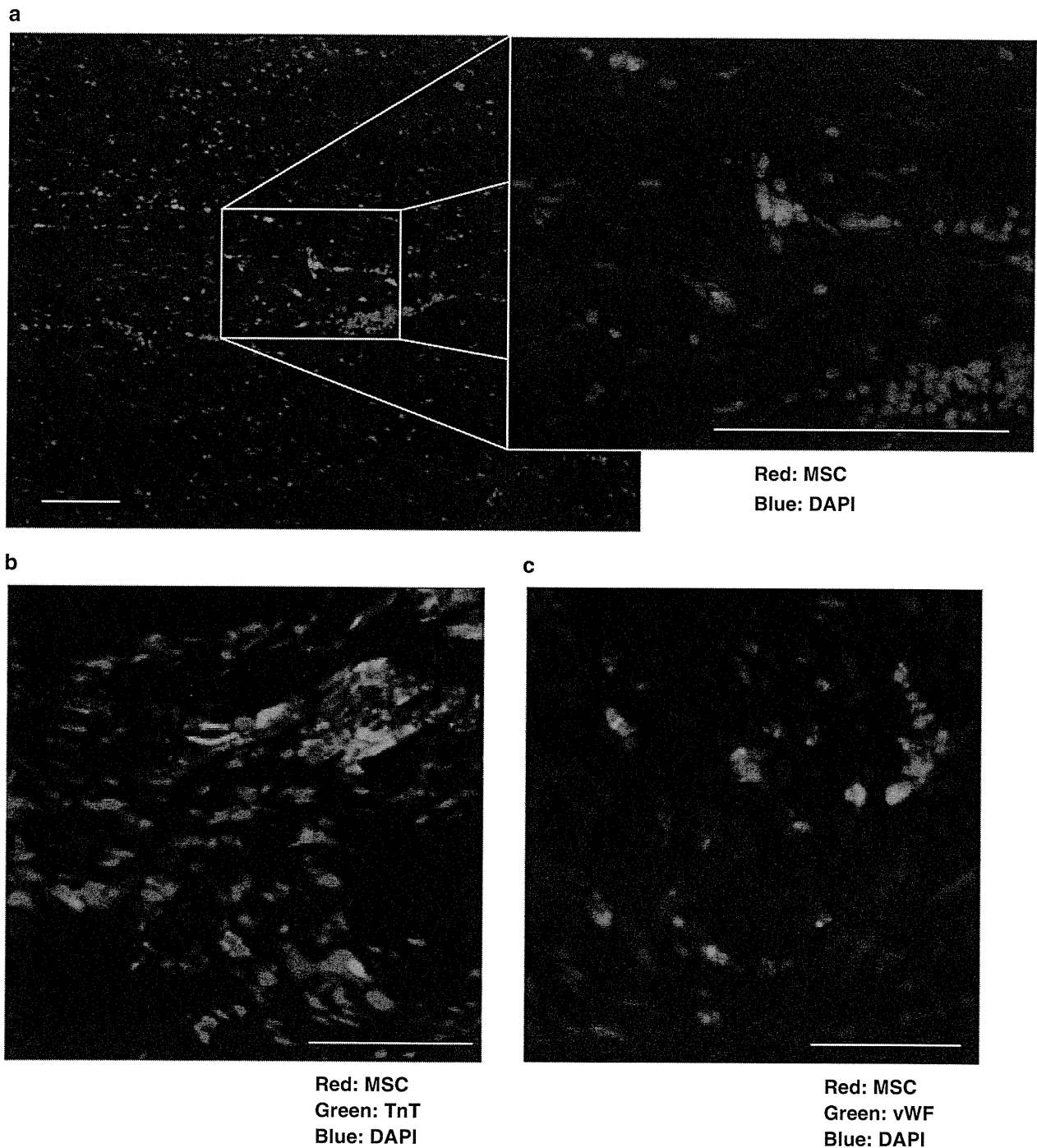


Figure 5 MSC engraftment and differentiation. **(a)** Engraftment of MSCs implanted in the border area. Red fluorescence indicates MSCs. Blue fluorescence indicates DAPI nuclear staining. **(b)** Lack of cardiomyocyte differentiation of the engrafted MSCs. Implanted Red fluorescence indicates MSCs. Green fluorescence indicates myocardium (TnT = troponin T). Blue fluorescence indicates DAPI nuclear staining. **(c)** Endothelial cell differentiation of the engrafted MSCs. Red fluorescence indicates MSCs. Green fluorescence indicates endothelial cells (vWF). Yellow (red and green) fluorescence indicates MSC differentiation. Blue fluorescence indicates DAPI nuclear staining. Bar = 100 μ m.

control group over time ($P < 0.05$), whereas the cardiac function was preserved in the MSC group (Figure 3a). The difference in the EF (δ -EF) between the baseline and follow-up was significantly smaller in the controls than in the MSC animals ($P < 0.01$, Figure 3b). There were no statistically

significant differences between the two groups in the EF or end-diastolic volume at follow-up (Figure 3c).

These findings from the LVG studies indicate that retrograde delivery of the MSCs via the coronary vein suppresses the progression of systolic LV dysfunction in chronic MI.

Effects of the Cell Infusion on Cardiac Fibrosis and Neovascularization

We conducted histopathological studies to address the effects of the MSC administration on cardiac fibrosis and neovascularization. Azan-Mallory-stained sections of hearts from the MSC-treated swine showed somewhat less fibrosis than those from the controls, but the difference was not significant in quantitative analysis (Figure 4a). The numbers of vWF- and α -SMA-positive vessels were assessed in the border areas (Figure 4b). The number of vWF-positive vessels was significantly higher in the MSC-treated swine hearts than in the control group ($P < 0.0005$). Likewise, α -SMA-positive vessel counts were also significantly higher in the MSC group than in the control group ($P < 0.0005$).

The effects of the MSC infusion were limited in terms of modification of cardiac fibrosis in the chronic post-infarcted myocardium, while the cell administration significantly promoted angio/arteriogenesis in the border zones. Thus, the favorable effects of the MSCs on cardiac function appeared to derive, at least in part, from enhanced neovascularization.

In a previous report from another group,²¹ freshly isolated BM cell implantation induced a calcified lesion formation in the infarcted myocardium of rat. Noting this, we carried von Kossa staining to detect abnormal calcium deposition in the frozen sections. This staining, however, revealed no calcified lesions (data not shown).

Engraftment and Differentiation of the Implanted MSCs

We assessed MSC engraftment and differentiation by immunofluorescence. MSCs marked by red fluorescence dye were sparsely observed in an interstitial area of the border area at 4 weeks after the infusion, but we could not detect any cells differentiated to cardiomyocytes (Figure 5a and b). A few MSCs expressed vWF and were incorporated into the vessel structure (Figure 5c).

The effect of the engrafted MSCs in promoting neovascularization seemed to be conferred partly through endothelial differentiation. The extent of the differentiation, however, was far too low to account for the significant increases in the vessel numbers.

Enhanced Expression of Angiogenic Factors after the MSC Implantation

Noting the reduced extent of cell incorporation to neovessels, we examined whether factors related to angiogenesis contributed to the increased neovascularization in the treated myocardium.

We determined the gene expression levels of bFGF and VEGF relative to GAPDH by quantitative RT-PCR analysis.

The bFGF and VEGF levels were significantly higher in the MSC-treated hearts than in the control hearts ($P < 0.05$, respectively; Figure 6a). Immunoblotting analysis confirmed the increased protein expressions of both bFGF and VEGF in the MSC group compared with the controls (Figure 6b). Of note, the MSC infusion enhanced the transcriptional levels not only in the border and infarcted areas, but also in the remote areas with little to no MSC engraftment (Figure 6a). In response to the MSC infusion, resident cardiac cells in the remote areas appeared to produce these angiogenic cytokines.

Immunohistochemical staining revealed that the MSCs engrafted in the border zones produced VEGF (Figure 7a).

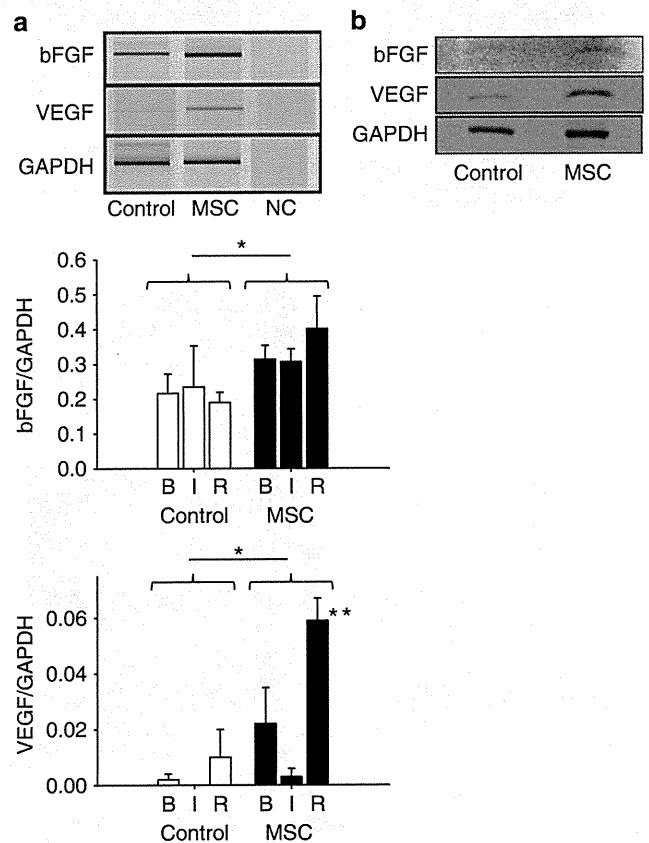
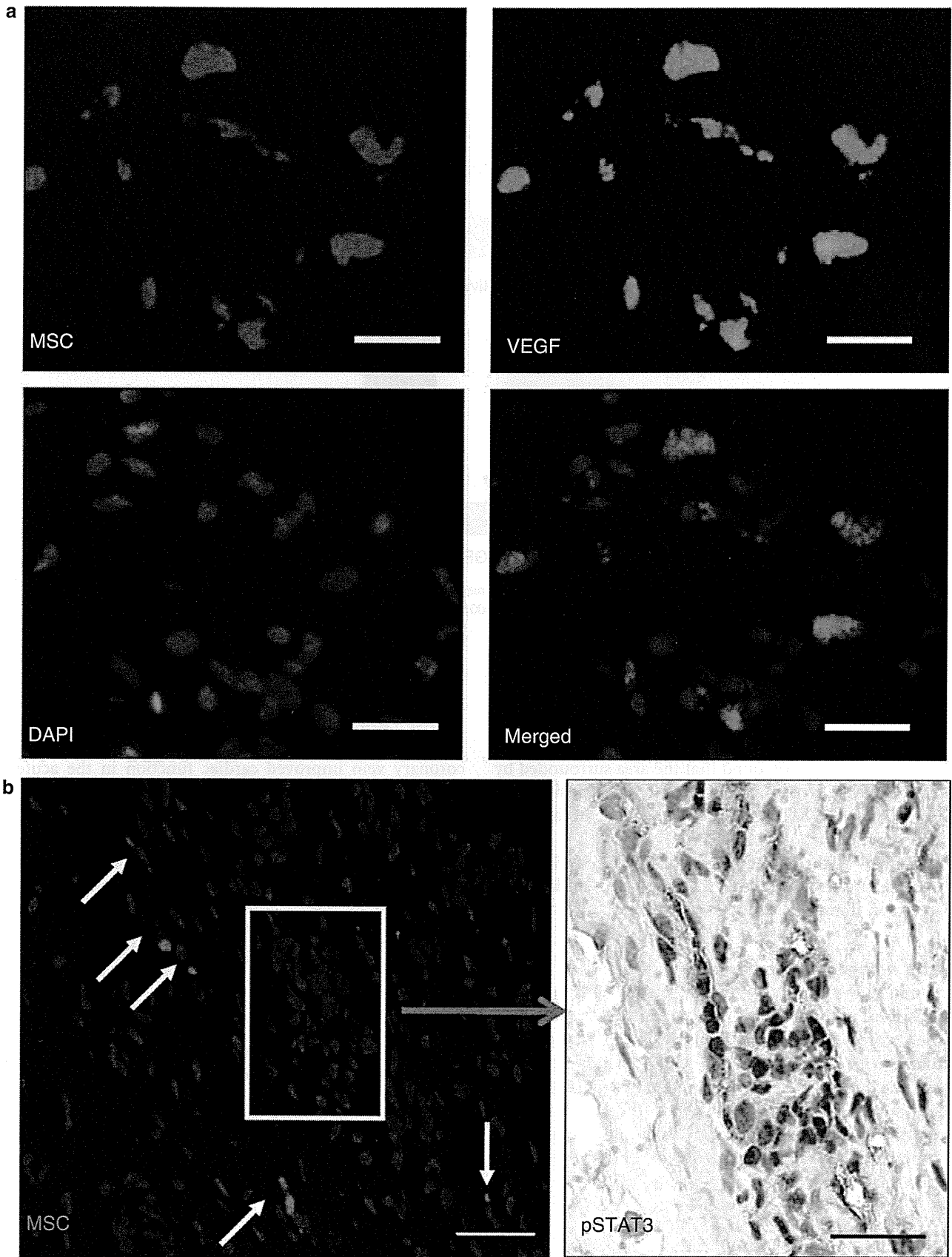


Figure 6 Expressions of bFGF and VEGF in the hearts after cell infusion. (a) Representative images of electrophoretic gels after PCR amplification of bFGF and VEGF mRNA in the border areas (upper). Quantitative analysis of bFGF mRNA expression relative to GAPDH mRNA in each area of the heart (middle). Quantitative analysis of VEGF mRNA expression relative to GAPDH mRNA in each area of the heart (bottom). Data are means \pm s.e.m., $n = 3$ in each area. * $P < 0.05$. ** $P < 0.05$ vs infarcted area in the MSC group. B, border area. I, infarcted area. R, remote area. (b) Immunoblotting analysis for bFGF and VEGF protein expressions in the border area. bFGF, basic fibroblast growth factor. VEGF, vascular endothelial growth factor.

Figure 7 Expressions of VEGF and pro-angiogenic signal in the MSC-treated hearts. (a) VEGF production from the engrafted MSCs. Red fluorescence indicates MSCs. Green fluorescence indicates VEGF. Blue fluorescence indicates DAPI nuclear staining. Merged image (yellow fluorescence) shows VEGF expression in the MSCs. Bar = 20 μ m. (b) STAT3 activation in the resident cardiac cells. Left, MSC engraftment (red fluorescence and white arrow). Blue fluorescence indicates DAPI nuclear staining. Right, clusters of cardiac cells with activated STAT3. Brown indicates positive staining for phospho-STAT3. Bar = 50 μ m. STAT3, signal transducer and activator of transcription 3.



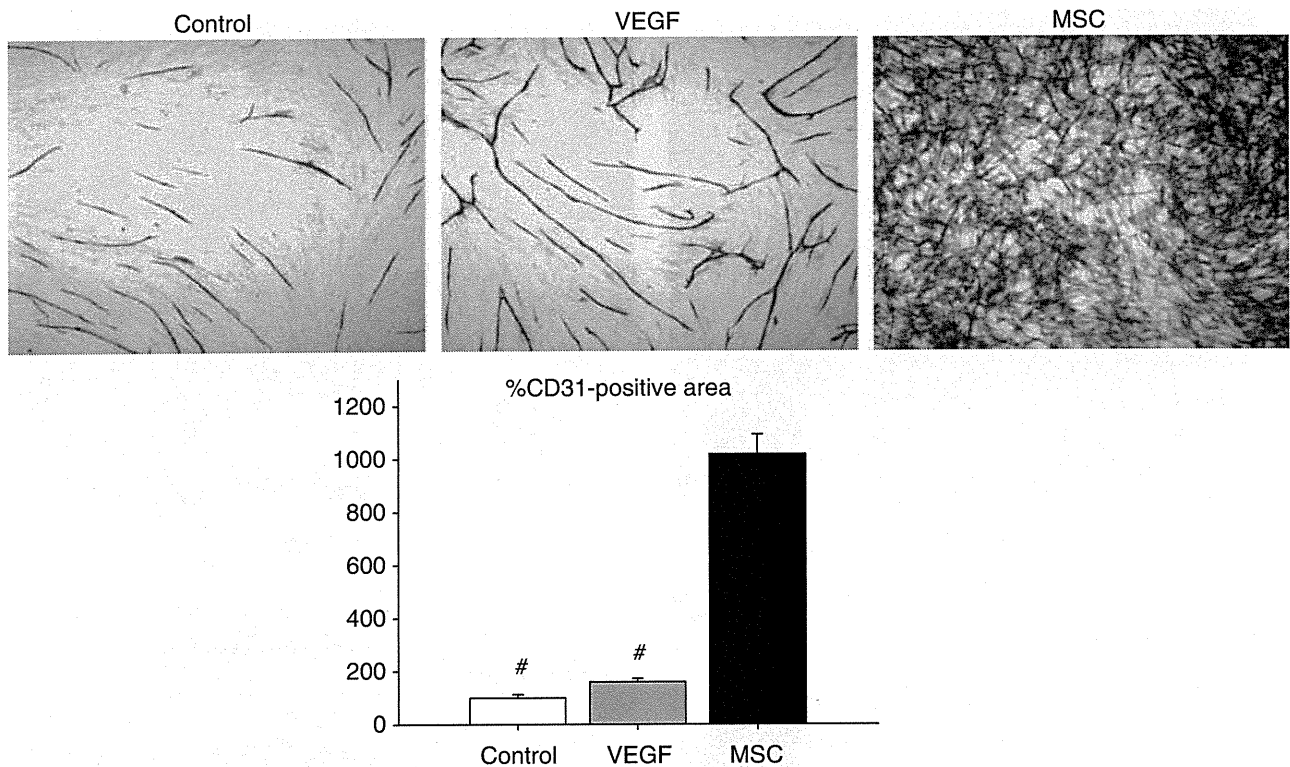


Figure 8 *In vitro* angiogenesis assay. Comparison of %CD31 areas among the growth medium (100%), the growth medium supplemented with VEGF 10 ng/ml, and the MSC co-culture. Data are means \pm s.e.m., $n = 3-4$. * $P < 0.0001$ vs MSC.

MSCs have been shown to secrete IL-6 and leukemia inhibitory factor.^{7,22} The transcriptional factor STAT3 is activated by IL-6 family members, and its activation promotes capillary growth in adult heart via acceleration of VEGF transcription.²³ We found that the area surrounded by the engrafted MSCs contained clusters of cells positive for phosphorylated + -STAT3 in sequential sections of the swine infarcted myocardium (Figure 7b).

Thus, VEGF might be produced not only the MSCs, but also by resident cardiac cells with STAT3 activated by the MSC-secreted factors.

In Vitro Angiogenesis Assay

We examined the culture system by an angiogenesis assay. HUVECs were immunostained with an anti-CD31 antibody, and the colored areas were quantified as capillary growth. Co-culture with human MSCs (5×10^4 cells) prominently induced the capillary network formation (Figure 8). The capillary area was significantly larger in the MSCs than in the control medium or the control medium supplemented with a high concentration (10 ng/ml) of VEGF ($P < 0.0001$, respectively).

DISCUSSION

Various reports have demonstrated a potential relationship between cardiac angiogenesis and cardiac function.²⁴⁻²⁶

Disruption of the balance between myocyte growth and angiogenesis leads to contractile dysfunction and heart failure. VEGF plays a critical role in this process.

In an earlier study by our group, BMNCs implanted via the coronary vein improved cardiac function in the acute and subacute phase of MI.¹⁵ MSCs have been shown to be a robust source of angiogenic factors compared with hematopoietic cells in BM.^{7,27} The low-invasive local delivery of MSCs preserved cardiac function in the swine chronic MI model in our present study. In comparisons against our controls, swine hearts implanted with MSCs exhibited increased expression of VEGF and bFGF and larger numbers of vessels in vWF and α -SMA staining. The enhanced neovascularization induced by the increased VEGF may help to prevent the progression of cardiac dysfunction in the chronic MI model.

Immunohistochemical staining revealed that the elevated VEGF levels were at least partly due to VEGF release from the engrafted MSCs. MSCs significantly promoted capillary network formation in our endothelial tube formation assay *in vitro*. The results of this *in vitro* study may support the hypothesis that factors secreted by the MSCs *in vivo* are responsible for enhanced neovascularization and preserved contractile function. Several previous studies support the paracrine hypothesis for MSC-mediated angiogenesis and cardioprotection.^{7,11,27,28} Moreover, our RT-PCR analysis showed significant increases in the VEGF levels in the remote

areas wholly devoid of MSCs. The MSC-treated heart contained clusters of resident cells positive for phospho-STAT3, the regulator of VEGF production. Human MSCs expressed higher mRNA levels of both VEGF and IL-6 family members than the human BM CD133+ progenitors, and secreted those factors *in vitro*.⁷ On this basis, we speculate that MSC-secreted factors, such as VEGF and IL-6, promote neovascularization both directly through endothelial proliferation and indirectly through activation of STAT3 in the resident cells.

Our previous study demonstrated that the safety and feasibility of the cell delivery via the coronary vein in a pre-clinical trial with the pig models.¹⁵ For the reasons explained below, we also selected coronary vein infusion as the route for MSC delivery into the myocardium in the present study. We did not, however, compare it with the other delivery methods (eg, intracoronary infusion and intramyocardial injection) in our hands. Intracoronary infusion of MSCs may cause distal embolization and consequent no-reflow phenomena. Freyman *et al*²⁹ reported that intracoronary MSC infusion impaired coronary flow distal to the infusion site in half of their treated pigs. Similar to this report, the intracoronary administration showed elevated cardiac enzymes and micro infarctions in dogs with normal coronary arteries.³⁰ Hoshino *et al*¹⁸ compared three methods for the injection of gelatin hydrogel microspheres, namely, antegrade injection, retrograde injection, and direct injection. The greatest number of microspheres was retained in the target area after the antegrade injection, but this method induced microinfarctions and failed to show any angiogenic effect. An increased coronary flow reserve was observed in the animals receiving retrograde injection and direct injection therapy, thus confirming the superior efficacy of these methods compared with antegrade injection. Importantly, retrograde injection was much less invasive than direct intramyocardial injection.

Coronary vein infusion of MSCs is a promising therapeutic strategy for the treatment and prevention of heart failure. Yet, the cell implantation failed to reduce cardiac fibrosis or achieve reverse remodeling in this study. Further studies in large animals to explore the methods to enhance or modify MSC function, such as gene transfer¹¹ and pharmacological preconditioning,³¹ will be necessary.

Taken together, our results show that MSC implantation via the coronary vein is feasible and prevents cardiac contractile dysfunction in ischemic cardiomyopathy of pigs. The mechanism behind this benefit appears to be partly attributable to increased local expression of angiogenic factors such as VEGF and enhanced neovascularization in response to the cell implantation.

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DISCLOSURE/CONFLICT OF INTEREST

The authors declare no conflicts of interest.

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