

においては、発生過程での心室中隔形成や胃腸管の血管形成に重要であることが報告されているが、成体での心血管疾患における役割についてはほとんどわかっていなかった。そこで、本研究では、虚血組織におけるSDF-1/CXCR4システムの役割の解明と治療への応用を目的として研究を行った。

## 【方法と結果】

### 1. 実験プロトコル

C57BL/6 (♂、8-12週齢)、ヌードマウス、(京都大学再生医科学研究所・長澤丘司教授より供与)を用いた。骨髄細胞膜表面上分子の発現はフローサイトメトリー法を用いて評価した。虚血モデルとしてはマウス右大腿動脈結紮による下肢虚血を作製して、レーザードップラー法により血流を評価し、局所におけるサイトカイン/増殖因子の発現は免疫組織染色法やreal-time RT-PCR法、ELISA法にて解析した。

### 2. 培養による骨髄由来CXCR4発現増強

骨髄細胞上のCXCR4発現がその血管新生能を増強する可能性があることから、まず骨髄細胞CXCR4発現に影響を与える因子のスクリーニングを行ったところ、37度で10%ウシ胎児血清(FCS)/DMEMでの培養条件によりCXCR4発現が24-36時間にかけて有意に増強することを明らかとした(CXCR4陽性率:24時間培養80.1 ± 5.8%、新鮮骨髄細胞2.1 ± 0.9%) (図1A)。この発現増強作用は骨髄細胞に発現している他の分子(CCR7、Gr-1、CD3、Mac-1、CD34、Sca-1、Flk-1)では認められず、CXCR4に特異的な作用であると考えられた(図1B)。また、この発現増強はGr-1(顆粒球)、Mac-1(単球・顆粒球)、CD3細胞(リンパ球)系のそれぞれの細胞で認められた(図1C、D)。そこで、新鮮骨髄細胞および24時間培養骨髄細胞をそれぞれCXCR4<sup>Cul</sup>とCXCR4<sup>Fr</sup>として以下の検討を行った。

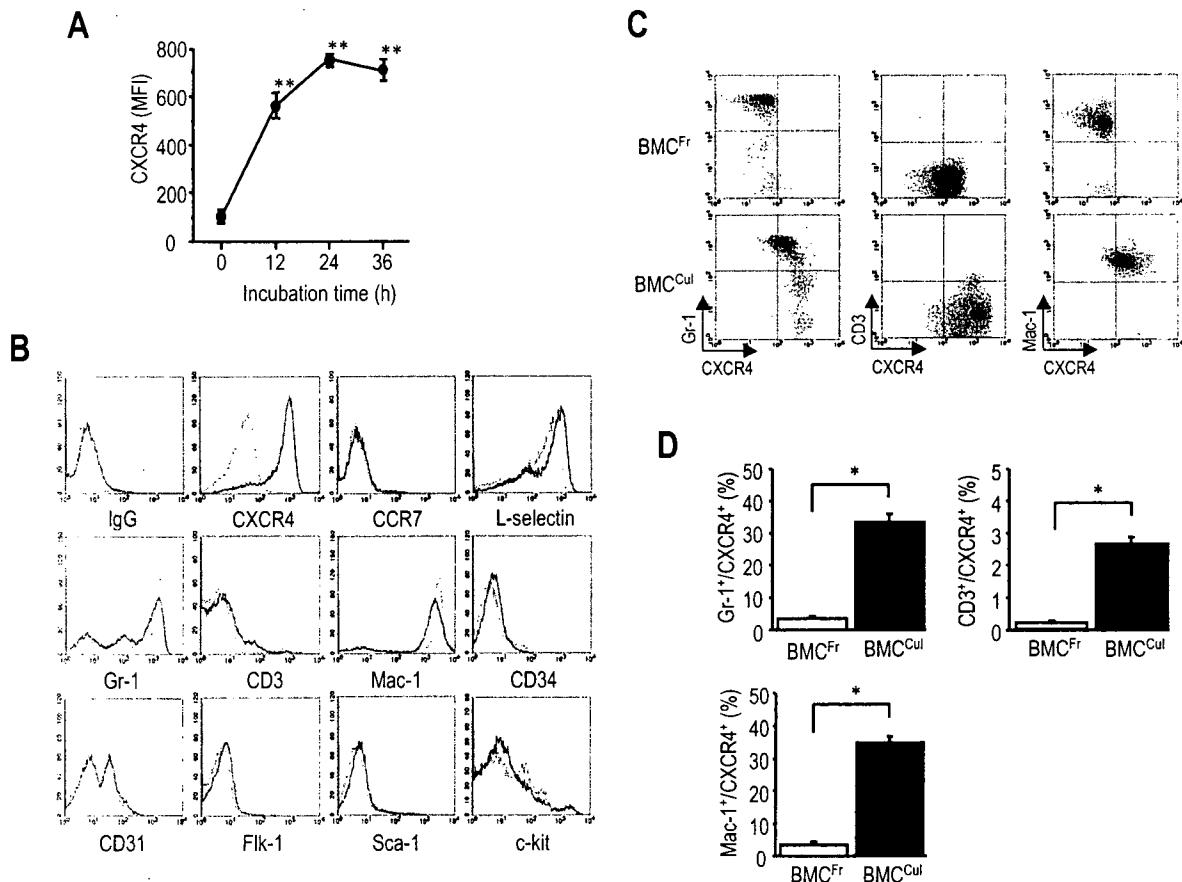


図1 培養による骨髄細胞におけるCXCR4発現への影響

### 3. マウス下肢虚血モデルにおけるCXCR4+骨髄細胞移植の効果

マウス下肢虚血モデルを作製して、以下の3群に分けて血流や血管新生について検討した。(1) Control (生理食塩水) 群、(2) CXCR4<sup>Fr</sup>群 (wild-typeマウス新鮮骨髄細胞) (3) CXCR4<sup>Cul</sup>群 (wild-typeマウス培養骨髄細胞)。CXCR4<sup>Cul</sup>群では、Control およびCXCR4<sup>Fr</sup>群に比較して血流の改善が有意に促進された (図2A,B)。また、CD31およびVE-cadherin抗体を用いた免疫組織染色により、CXCR4<sup>Cul</sup>群では他群に比較して毛細血管形成も有意に促進されていることがわかった (図2C,D)。

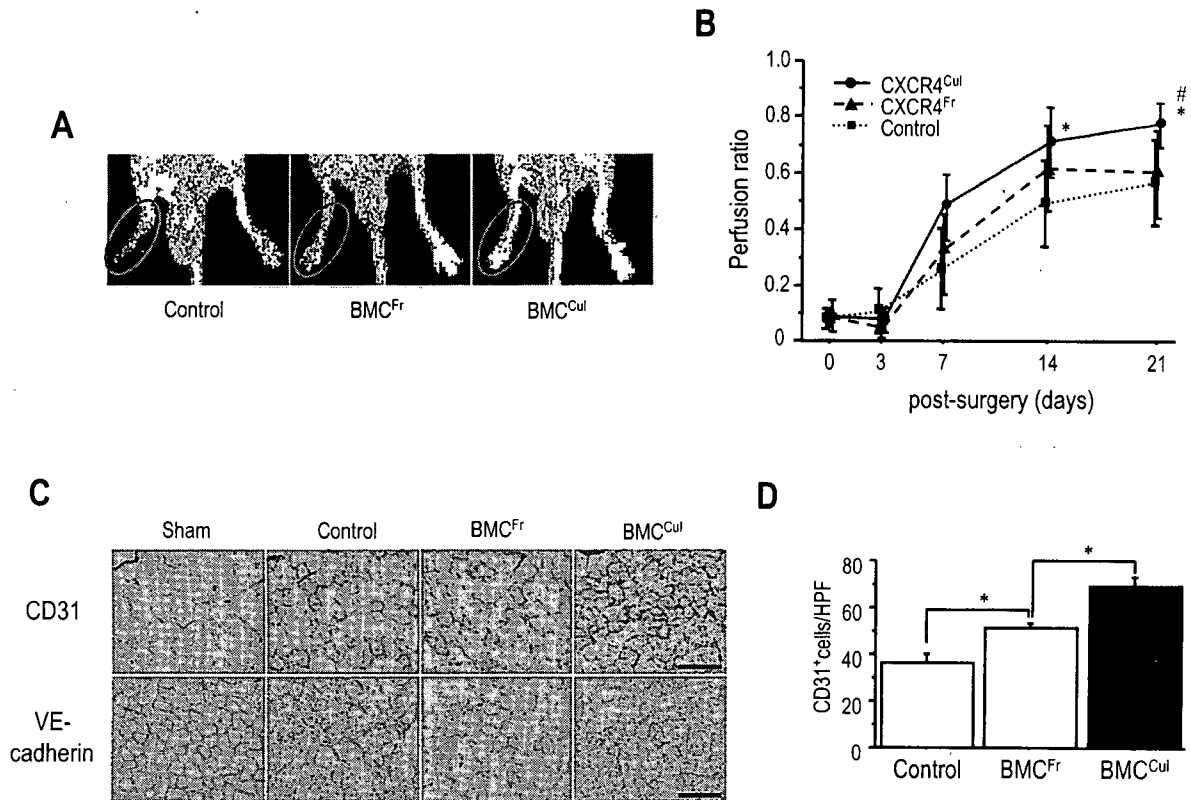


図2 CXCR4<sup>+</sup>骨髄細胞移植による虚血の改善と血管新生作用

この血流改善効果が骨髄細胞CXCR4発現に関わっているかを検討するため、CXCR4<sup>+/-</sup>マウス (CXCR4<sup>-/-</sup>マウスは胎生致死となる) の骨髄細胞を用いて検討したところ、wild-typeマウス由来CXCR4<sup>Cul</sup>骨髄細胞移植群と比較して、CXCR4<sup>+/-</sup>マウス由来CXCR4<sup>Cul</sup>骨髄細胞移植群では、その血流改善効果が有意に低下していた (図3)。

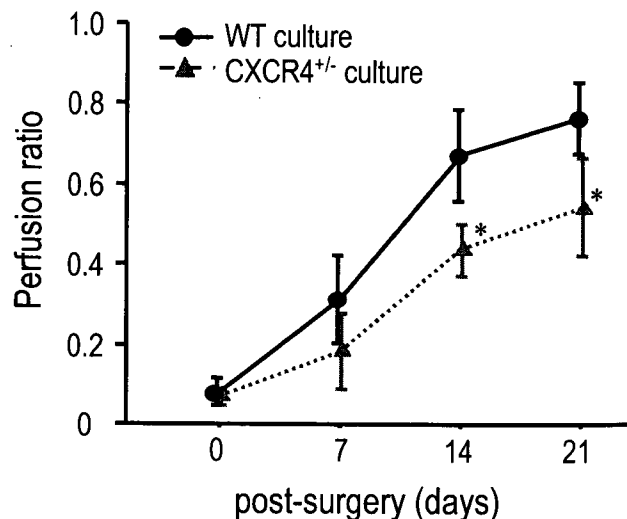


図3 CXCR4<sup>+/-</sup>マウス由来骨髄細胞による虚血改善効果の減弱

#### 4. 移植骨髄細胞の虚血部位への停留

CXCR4<sup>Cul</sup>骨髄細胞による血流改善促進効果の機序としては、以下の2つが考えられる。(1) CXCR4リガンドであるSDF-1は虚血により発現が亢進することから移植骨髄細胞が局所により長く留まり(停留)して血管新生作用を示す可能性と(2) CXCR4<sup>Cul</sup>骨髄細胞がより多くのサイトカイン・増殖因子を産生する可能性である。

まず、マウス下肢虚血モデルにおいてSDF-1が発現しているかどうかを検討した。Real-time RT-PCR法および免疫組織染色法により、マウス虚血部位ではSDF-1の発現亢進が認められた(図4)。

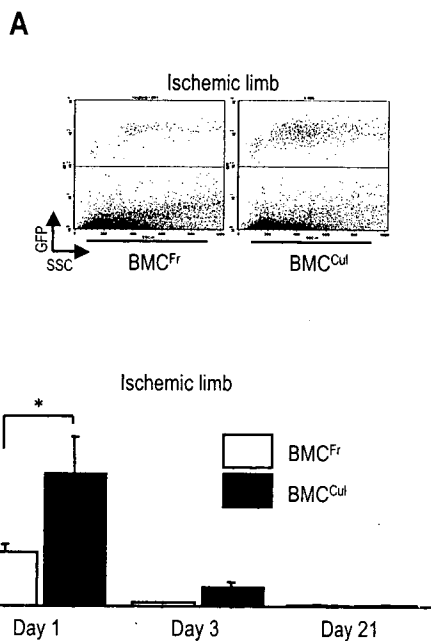


図4 虚血部位におけるSDF-1の発現

さらに、移植骨髄細胞が停留しているかどうかを検討するため、GFPマウス由来CXCR4<sup>Cul</sup>骨髄細胞を虚血部位へと移植し、1、3、21日目に移植部位の骨格筋を採取してコラゲナーゼ処理してGFP<sup>+</sup>細胞をフローサイトメトリー法で解析した。CXCR4<sup>Cul</sup>群では、CXCR4<sup>Fr</sup>群と比較して移植後1日目での移植骨髄細胞の有意な停留の増加を認めた(図5A,B)。しかしながら、この増加は3~21日目において消失しており、特に移植21日目では移植骨髄細胞はほとんど認められなかった。

#### 5. サイトカイン・増殖因子の関与

最後に、移植部位でのIL-1 $\beta$ とVEGF-Aの発現をreal-time RT-PCR法で評価した。Sham群に比較して、Control群でもこれら因子の有意な発現亢進を認めた。CXCR4<sup>Fr</sup>群ではIL-1 $\beta$ の発現が増加傾向にあり、CXCR4<sup>Cul</sup>群ではIL-1 $\beta$ およびVEGF-AともSham群とCXCR4<sup>Fr</sup>群に比較して増加していた(図6A)。さらに、これら因子の産生細胞を明らかとするため、骨髄細胞を採取・培養してその発現を検討したところ、培養によりIL-1 $\beta$ は3時間をピークとして、またVEGF-Aは6-48時間にかけて徐々に発現が増加していた(図6B)。また、これらIL-1 $\beta$ とVEGF-Aの発現はCXCR4<sup>+</sup>マウス由来の骨髄細胞では低下していた。

#### 【考察】

我々は、これまでSDF-1/CXCR4システムが血管傷害後の新生内膜形成に寄与していることや、心筋梗塞後の血管新生や血管リモデリングにも寄与していることを報告してきた。本研究では、その血管新生作用に着目して研究を行い、骨髄細胞において培養によりその表面のCXCR4発現が特異的に亢進され、このCXCR4発現が亢進した骨髄細胞を虚血部位に移植するとより効果的に血管新生が誘導されて血流が改善することを明らかとした。また、その血流改善の機序として、虚血部位で発現するCXCR4リガンドであるSDF-1と移植した骨髄細胞上のCXCR4の相互作用が生じて、移植骨髄細胞がより多く虚血部位へと留まり、さらにIL-1 $\beta$ やVEGF-Aといった血管新生作用のある因子をより多く発現することが考えられた。

近年、心血管疾患への治療法は長足の進歩を遂げているにもかかわらず、現在でも重症の虚血性心疾患や末梢動脈疾患では有効な治療法もなく、救命できなかつたり、四肢の切断に至る症例も少なくない。今後、骨髄細胞によって誘導される血管新生過程におけるSDF-1/CXCR4システムの詳細な解析が行われ、このシステムを利用したより効果的な血管新生療法が確立されることが期待される。

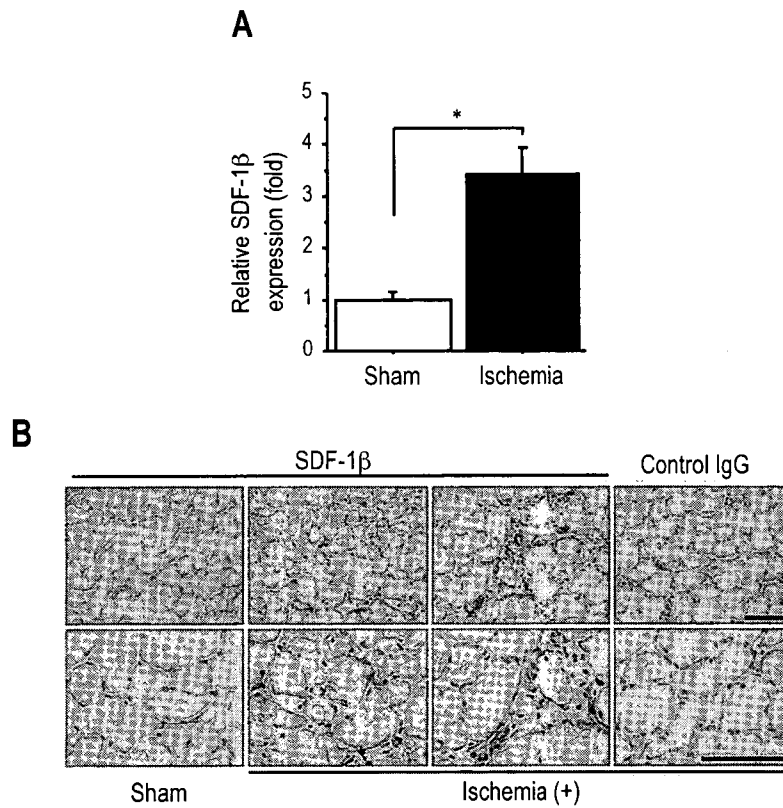


図5 移植骨髄細胞の虚血部位への停留

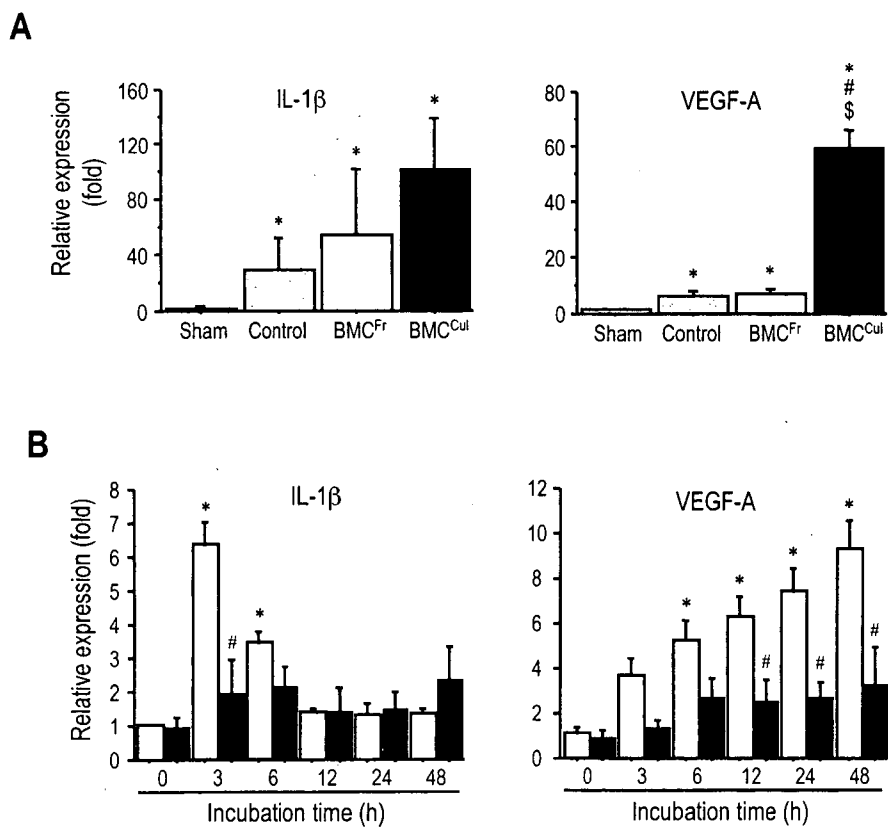


図6 骨髄細胞移植部位 (vivo) および培養骨髄細胞 (vitro) における IL-1 $\beta$  と VEGF-A の発現

## 【文 献】

1. Shiba Y, Takahashi M, Yoshioka T, Yajima N, Morimoto H, Izawa A, Ise H, Hatake K, Motoyoshi K, Ikeda U. M-CSF accelerates neointimal formation in the early phase after vascular injury in mice: The critical role of the SDF-1-CXCR4 system. *Arterioscler Thromb Vasc Biol* 27: 283-289, 2007
2. Morimoto H, Takahashi M, Shiba Y, Izawa A, Ise H, Hongo M, Hatake K, Motoyoshi K, Ikeda U. Bone marrow-derived CXCR4+ cells mobilized by M-CSF participate in the prevention of cardiac dysfunction and remodeling after myocardial infarction in mice. *Am J Pathol* 171: 755-766, 2007
3. Shiba Y, Takahashi M, Morimoto H, Izawa A, Ise H, Ikeda U: Bone marrow-derived CXCR4-positive cells as a new source for therapeutic angiogenesis. American Heart Association Scientific Sessions (abstract 1304), Orlando, Florida, 200

## 研究成果の刊行に関する一覧表

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雑 誌

発表者氏名	論文タイトル	発表誌名	巻号	ページ	出版年
Morimoto H, Takahashi M, Shiba Y, Izawa A, Ise H, Hongo M, Hatake K, Motoyoshi K, Ikeda U	Bone marrow-derived CXCR4+ cells mobilized by M-CSF participate in the prevention of cardiac dysfunction and remodeling after myocardial infarction in mice.	<i>Am J Pathol</i>	171	755-766	2007
Aso S, Ise H, Takahashi M, Kobayashi S, Morimoto H, Izawa A, Goto M, Ikeda U.	Effective uptake of N-acetylglucosamine-conjugated liposome by cardiomyocytes in vitro.	<i>J Control Release</i>	122	189-198	2007
Ito T, Okada T, Miyashita H, Nemoto T, Nonaka-Sarukawa M, Uchibori R, Maeda Y, Urabe M, Mizukami H, Kume A, Takahashi M, Ikeda U, Shimada K, Ozawa K.	Interleukin-10 expression mediated by an adeno-associated virus vector prevents monocrotaline-induced pulmonary arterial hypertension in rats.	<i>Circ Res</i>	101	734-741	2007
Ito T, Okada T, Mimuro J, Miyashita H, Uchibori R, Urabe M, Mizukami H, Kume A, Takahashi M, Ikeda U, Sakata Y, Shimada K, Ozawa K.	Adenoassociated virus-mediated prostacyclin synthase expression prevents pulmonary arterial hypertension in rats.	<i>Hypertension</i>	50	531-536	2007
Nonaka-Sarukawa M, Yamamoto K, Aoki H, Nishimura Y, Tomizawa H, Ichida M, Eizawa T, Muroi K, Ikeda U, Shimada K.	Circulating endothelial progenitor cells in congestive heart failure.	<i>Int J Cardiol</i>	119	344-348	2007
Shiba Y, Takahashi M, Yoshioka T, Yajima N, Morimoto H, Izawa A, Ise H, Hatake K, Motoyoshi K, Ikeda U.	M-CSF accelerates neointimal formation in the early phase after vascular injury in mice: The critical role of the SDF-1-CXCR4 system.	<i>Arterioscler Thromb Vasc Biol</i>	27	283-289	2007
川人浩之、中村英夫、的場聖明、辰巳哲也、松原弘明	骨髓細胞移植による血管再生療法	<i>Heart View</i>	11(13)	84-89	2007
Tateishi K, Ashihara E, Honsho S, Takehara N, Nomura T, Takahashi T, Ueyama T, Yamagishi M, Yaku H, Matsubara H, Oh H	Human cardiac stem cells exhibit mesenchymal features and are maintained through Akt/GSK-3 $\beta$ signaling.	<i>Biochem Biophys Res Comm</i>	352	635-641	2007
Tatsumi T, Ashihara E, Yasui T, Matsunaga S, Kido A, Sasada Y, Nishikawa S, Hadase M, Koide M, Nakamura R, Irie H, Ito K, Matsui A, Matsi H, Katamura M, Kusuoka S, Matoba S, Okayama S, Horii M, Uemura S, Shimazaki C,	Intracoronary transplantation of Non-Expanded peripheral blood-derived mononuclear cells promotes improvement of cardiac function in patients with acute myocardial infarction.	<i>Circ J</i>	71	1199-1207	2007

Ysuji H, Saito Y, Matsubara H					
Nomura T, Ashihara E, Tateishi K, Asada S, Ueyama T, Takahashi T, Matsubara H, Oh H	Skeletal myosphere-derived progenitor cell transplantation promotes neovascularization in $\delta$ -sarcoglycan knockdown cardiomyopathy.	<i>Biochem Biophys Res Comm</i>	352	668-674	2007
Tateishi K, Ashihara E, Takehara N, Nomura T, Honsho S, Nakagami T, Morikawa S, Takahashi T, Ueyama T, Matsubara H, Oh H	Clonally amplified cardiac stem cells are regulated by Sca-1 signaling for efficient cardiovascular regeneration.	<i>J Cell Science</i>	120	1791-1800	2007
松永晋作、五十殿弘二、浅田 聡、辰巳哲也、松原弘明	急性冠症候群への再生医療—細胞治療と造血性サイトカイン治療—	治療学	41 (8)	78-84	2007
木下英吾、浦岡真希、中村英夫、西澤信也、松原弘明	心筋梗塞への再生医療の現状と展望	心臓	39 (11)	1023-1027	2007
辰巳哲也、的場聖明、木戸淳道、安威俊秀、松原弘明	閉塞性動脈硬化症	総合臨床	56	933-943	2007
五十殿弘二、浅田 聡、高橋知三郎、辰巳哲也、松原弘明	心筋梗塞への再生医療 Update—骨髄単核球を利用した細胞治療の現状	内科	100 (3)	529-535	2007
Kamata Y, Takahashi Y, Iwamoto M, Matsui K, Murakami Y, Muroi K, Ikeda U, Shimada K, Yoshio Y, Okazaki H, Minota S	Local implantation of autologous mononuclear cells from bone marrow and peripheral blood for treatment of ischaemic digits in patients with connective tissue diseases.	<i>Rheumatology</i>	46	882-884	2007
Mitsuma W, Kodama M, Hitono S, Ito M, Ramadan M M, Tanaka K, Hoyano M, Saigawa T, Kashimura T, Fuse K, Okura Y, Aizawa Y	Angiotensin-1, Angiotensin-2 and Tie-2 in the coronary circulation of patients with and without coronary collateral vessels.	<i>Circ J</i>	71	343-347	2007
Mitsuma W, Kodama M, Hanawa H, Ito M, Ramadan M M, Hirono S, Obata H, Okada S, Sanada F, Yanagawa T, Kashimura T, Fuse K, Tanabe N, Aizawa Y	Serum endostatin in the coronary circulation of patients with coronary heart disease and its relation to coronary collateral formation.	<i>Am J Cardiol</i>	In press	494-498	2007
Ozawa T, Kato K, Yoba K, Oda M, Isobe M, Asami F, Ikarashi N, Yanagawa T, Motiyama M, Higashimura M, Kitajima T, Otaki K, Takayama T, Hirono S, Okura Y, Hanawa H, Kodama M, Aizawa Y	Serum erythropoietin level as a marker of limb ischemia.	<i>Int J Cardiol</i>	In press		2007
米川基樹、堀江 卓、津田一郎、河村明夫	透析患者の幹細胞治療	臨床透析	23	85-91	2007



## 研究成果の刊行物・別冊

Cardiovascular, Pulmonary and Renal Pathology

# Bone Marrow-Derived CXCR4<sup>+</sup> Cells Mobilized by Macrophage Colony-Stimulating Factor Participate in the Reduction of Infarct Area and Improvement of Cardiac Remodeling after Myocardial Infarction in Mice

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**The monocyte/macrophage lineage might affect the healing process after myocardial infarction (MI). Because macrophage colony-stimulating factor (M-CSF) stimulates differentiation and proliferation of this lineage, we examined the effect of M-CSF treatment on infarct size and left ventricular (LV) remodeling after MI. MI was induced in C57BL/6J mice by ligation of the left coronary artery. Either recombinant human M-CSF or saline was administered for 5 consecutive days after MI induction. M-CSF treatment significantly reduced the infarct size ( $P < 0.05$ ) and scar formation ( $P < 0.05$ ) and improved the LV dysfunction (percent fractional shortening,  $P < 0.001$ ) after the MI. Immunohistochemistry revealed that M-CSF increased macrophage infiltration (F4/80) and neovascularization (CD31) of the infarct myocardium but did not increase myofibroblast accumulation ( $\alpha$ -smooth muscle actin). M-CSF mobilized CXCR4<sup>+</sup> cells into peripheral circulation, and the mobilized CXCR4<sup>+</sup> cells were then recruited into the infarct area in which SDF-1 showed marked expression. The CXCR4 antagonist AMD3100 deteriorated the infarction and LV function after the MI in the M-CSF-treated mice. In conclusion,**

**M-CSF reduced infarct area and improved LV remodeling after MI through the recruitment of CXCR4<sup>+</sup> cells into the infarct myocardium by the SDF-1-CXCR4 axis activation; this suggests that the SDF-1-CXCR4 axis is as a potential target for the treatment of MI. (Am J Pathol 2007, 171:755–766; DOI: 10.2353/ajpath.2007.061276)**

Myocardial infarction (MI) is accompanied by inflammatory responses that lead to the recruitment of leukocytes and subsequent myocardial damage, healing, and scar formation. The recruitment and activation of monocytes/macrophages in the infarct myocardium have been shown to play an important role in the processes that occur after MI. The activated macrophages lead to the release of cytokines and proteinases that can induce further inflammation and left ventricular (LV) remodeling. Furthermore, recent evidence indicates that some endothelial progenitor cells (EPCs) are derived from cells of the monocytic lineage and participate in the neovascularization of ischemic tissues.<sup>1–3</sup> These cells have also been reported to secrete a large amount of angiogenic factors such as the vascular endothelial growth factor and the hepatocyte growth factor<sup>3</sup>; this suggests that monocytes/macrophages could influence LV dysfunction and remodeling after MI.

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The macrophage colony-stimulating factor (M-CSF) is a multifunctional proinflammatory cytokine that regulates the differentiation, proliferation, and survival of monocytic progenitor cells<sup>4</sup> and plays a role in various processes involved in inflammatory diseases. Furthermore, it has been recently demonstrated that M-CSF is expressed by nonhematopoietic cells such as endothelial cells, smooth muscle cells, and cardiomyocytes and that its function extends beyond its role in monocytic progenitor cells. A recent investigation demonstrated that M-CSF is expressed in the infarct heart and plays an important role in myocardial healing postinfarction.<sup>5</sup> However, the effect of M-CSF on cardiac dysfunction and remodeling after MI is not entirely understood. In the present study, we demonstrate that exogenous M-CSF treatment induces macrophage infiltration and capillary formation in the infarct myocardium, thereby improving LV dysfunction and remodeling. This process is mediated through a system that comprises a key stem cell homing factor, the stromal cell-derived factor (SDF-1/CXCL12), and the SDF-1 receptor CXCR4. The findings of this study may provide new insights into the role of M-CSF and the SDF-1-CXCR4 axis in the pathophysiology of MI.

## Materials and Methods

### Animals

All mice (C57BL/6J, male) were purchased from Japan SLC Inc. (Hamamatsu, Japan). Their ages ranged from 8 to 12 weeks. Mice were fed a standard diet and water and maintained on a 12-hour light/dark cycle. All of the experiments in this study were performed in accordance with the Shinshu University Guide for Laboratory Animals, which conforms to the National Institutes of Health Guidelines.

### Experimental Protocols

In the preliminary experiments, we examined the effect of M-CSF (5, 50, and 500  $\mu\text{g}/\text{kg}$ , i.p.) on the number of peripheral monocytes in C57BL/6J mice. We found that the administration of 500  $\mu\text{g}/\text{kg}$  of recombinant human M-CSF (kindly provided by Morinaga Milk Industry Co. Ltd., Kanagawa, Japan) significantly increased the number of peripheral monocytes; this finding was consistent with a previous report.<sup>6</sup> Therefore, in the present study, we used this M-CSF at a dose of 500  $\mu\text{g}/\text{kg}$  per day. We also used recombinant human granulocyte colony-stimulating factor (G-CSF; kindly provided by Chugai Pharmaceutical Co. Ltd., Tokyo, Japan) at a dose of 100  $\mu\text{g}/\text{kg}$  per day.

The mice were anesthetized with an intraperitoneal injection of 50 mg/kg pentobarbital sodium and splenectomized, not only to eliminate spleen-derived stem cells (eg, EPCs and CXCR4<sup>+</sup> cells) but also to prevent the homing of bone marrow-derived stem cells to the spleen.<sup>7</sup> The animals were allowed to recover for 14 days, after which MI was induced. Either recombinant human M-CSF (i.p.,  $n = 60$ ), G-CSF (s.c.,  $n = 12$ ), or saline (i.p., vehicle,  $n = 53$ ) was administered to the splenectomized mice for 5 consecutive days after MI induction. The M-CSF treatment was well tolerated, and

no abnormal behavior was observed. AMD3100 (Sigma, St. Louis, MO) was administered subcutaneously at a concentration of 300  $\mu\text{g}/\text{kg}$  per hour for 7 days after the MI by using a micro-osmotic pump (Alzet model 1007D; Durect Corporation, Cupertino, CA).

### MI Induction Protocol

A murine model of MI was constructed as described previously.<sup>8</sup> Intubation was performed after anesthetizing the mice with isoflurane. The mice were ventilated with a rodent ventilator (MiniVent Type 845; Harvard Apparatus, Holliston, MA). A left thoracotomy was performed through the fourth or fifth intercostal space. An 8-0 nylon suture was placed directly beneath the left atrium in the inter-ventricular groove. Successful coronary occlusion was verified by observing the development of a pale color in the distal myocardium after the ligation of the left coronary artery. The lungs were re-expanded using positive pressure at end expiration, and the thoracotomy and skin incision were closed with a 3-0 silk suture. Extubation was performed when spontaneous respiration resumed.

### Histology and Immunohistochemistry

Histological and immunohistochemical analyses were performed as described previously.<sup>9</sup> In brief, the mice were euthanized after irrigation with saline (Otsuka Pharmaceutical Co. Ltd., Tokushima, Japan), and their blood was completely washed out. The hearts were embedded in optimal cutting temperature compound (Tissue-Tek, Sakura Finetechnical Co. Ltd., Tokyo, Japan) and frozen on dry ice. They were then sectioned transversely from the apex to the site of ligation beneath the left atrium. Tissue sections (10- $\mu\text{m}$  thick) were cut on a cryostat (CM-1900; Leica Microsystems GmbH, Wetzlar, Germany) and histologically examined. Four sections were selected from each heart to perform morphometrical assessments of the LV myocardium and the infarct size. The sections were stained with hematoxylin-eosin (H&E) and Masson's trichrome. Measurements were performed using Scion Image software (Beta 4.03; Scion Corporation, Frederick, MD). The infarct size was calculated as a percentage of the total LV area. The extent of fibrosis in the sections was measured, and the value was expressed as the ratio of the Masson's trichrome-stained area to the total LV free wall area.

For immunohistochemical analysis, the heart sections were incubated with primary antibodies against M-CSF (kindly provided by Morinaga Milk Industry Co. Ltd.), mouse CD31 (clone MEC13.3, BD Bioscience, San Jose, CA), F4/80 (clone A3-1; RDI, Flanders, NJ),  $\alpha$ -smooth muscle actin ( $\alpha$ -SMA; clone 1A4; Sigma), and CD184 (CXCR4, clone 2B11; BD Biosciences). This was followed by incubation with biotin-conjugated secondary antibodies. The sections were washed and treated with avidin peroxidase (ABC kit; Vector Laboratories; Burlingame, CA), and the stain was developed using the 3,3'-diaminobenzidine (DAB) substrate kit (Vector Laboratories). The sections were then counterstained with hematoxylin.

Mouse on mouse (M.O.M.) basic kits (Vector Laboratories) were used to specifically localize mouse primary monoclonal antibodies in the tissues. No signal was detected when an irrelevant IgG was used as the negative control instead of the primary antibody. All of the measurements were performed in a double-blind manner by two independent researchers.

### Cell Cultures and Hypoxia-Reoxygenation

Primary cultures of murine neonatal cardiomyocytes and cardiac fibroblasts were prepared and cultured as described previously.<sup>8</sup> For hypoxia-reoxygenation experiments, cells were exposed to hypoxia induced with AnaeroPack (Mitsubishi Gas Chemical, Tokyo, Japan) for 6 hours, followed by reoxygenation for 18 hours.

### Flow Cytometric Analysis

Blood samples were collected from the mice at baseline, 7 days, and 14 days after the MI. Circulating cells were identified using a nucleated cell fraction. The nucleated cells were double-labeled with the following antibodies: peridinin chlorophyll protein-conjugated anti-CD11b (Mac-1, clone M1/70; BD Biosciences), fluorescein isothiocyanate-conjugated anti-Ly-6G (Gr-1, clone 1A8), fluorescein isothiocyanate-conjugated anti-CD34 (clone RAM34; BD Biosciences), phycoerythrin-conjugated anti-Fli-1 (VEGFR2/KDR, clone Avas 12a1; BD Biosciences), and phycoerythrin-conjugated anti-CXCR4 (clone 2B11; BD Biosciences). For staining with antibody against  $\alpha$ -SMA, cells were permeabilized with Cytotfix/Cytoperm (BD Biosciences) according to the manufacturer's instructions. The cells were examined by flow cytometry (FACSCalibur; Becton Dickinson) and analyzed using CellQUEST software version 3.3 (Becton Dickinson).

### Serum Levels of Inflammatory Cytokines

The serum levels of monocyte chemoattractant protein-1 (MCP-1), interleukin (IL)-6, IL-10, IL-12p70, interferon- $\gamma$  (IFN- $\gamma$ ), and tumor necrosis factor- $\alpha$  (TNF- $\alpha$ ) were assessed using the CBA Mouse Inflammation Kit (BD Biosciences) according to the manufacturer's instructions.

### Echocardiography

Transthoracic echocardiography was performed at baseline, 7 days, and 14 days after the MI by using a Vivid Five system (GE Yokogawa Medical Systems, Tokyo, Japan) as described previously.<sup>10</sup> Ketamine (50 mg/kg) and xylazine (10 mg/kg) were administered intraperitoneally for mild sedation. Two-dimensional targeted M-mode echocardiograms were obtained along the short axis of the left ventricle at the level of the papillary muscles, and at least three consecutive beats were evaluated. The phases in which the smallest and largest area of the left ventricle were obtained were defined as the left ventricular end-systolic diameter (LVESD) and left ventricular end-dia-

stolic diameter (LVEDD), respectively. The percentage fractional shortening (%FS) was calculated using the standard formula:  $\%FS = [(LVEDD - LVESD)/LVEDD] \times 100$ . All measurements were performed in a double-blind manner by two independent researchers.

### Real-Time Reverse Transcription-Polymerase Chain Reaction Analysis

Total cellular RNA was extracted using ISOGEN (Nippon Gene Co. Ltd., Toyama, Japan) or RNA-Bee (Tel-Test, Inc., Friendswood, TX), according to the manufacturer's instructions. Real-time reverse transcription-polymerase chain reaction (RT-PCR) analysis was performed by using the ABI Prism 7000 system (Applied Biosystems, Inc.) to detect the mRNA expression of c-fms, G-CSF receptor (G-CSFR), collagen type I and type III, transforming growth factor- $\beta$ 1 (TGF- $\beta$ 1), and  $\beta$ -actin.<sup>11</sup> The following primers (oligonucleotide sequences are provided in parentheses in the order of antisense and sense primers) were used: c-fms (5'-CATGGCCTTCCTGCTTCTAA-3' and 5'-ACATGTCCGCTGGTCAACAG-3'), G-CSFR (5'-GTCCAGCGAGTCCCCAAAG-3' and 5'-AGCATGGGAGGCTCCAATT-3'), collagen type I (5'-CGGAGAAGAA-GGAAAACGAGGAG-3' and 5'-CACCATCAGCACCAG-GGAAAC-3'), collagen type III (5'-CCCAA CCCAGAGATCCCATT-3' and 5'-GAAGCACAGGAGCA GGTGTAGA-3'), TGF- $\beta$ 1 (5'-GCAACATGTGGAAGTCTACCAGA-3' and 5'-GACGTCAAAAGACAGCCACTCA-3'), and  $\beta$ -actin (5'-CCTGAGCGCAAGTACTCTGTGT-3' and 5'-GCTGATCCACATCTGCTGGAA-3'). The expression levels of each target gene were normalized by subtracting the corresponding  $\beta$ -actin threshold cycle ( $C_T$ ) values; this was done by using the  $\Delta\Delta C_T$  comparative method.

### Apoptosis Assessment

Apoptotic cells were identified by the terminal deoxynucleotidyl transferase dUTP nick-end labeling staining kit (Roche Diagnostics, Mannheim, Germany) performed according to the manufacturer's instructions.

### Statistical Analysis

Data are represented as mean  $\pm$  SEM. Multiple group comparison was performed by one-way analysis of variance (analysis of variance), followed by Scheffé's F-test for comparison of the means. The comparison between two groups was analyzed by an F-test, followed by a two-tailed *t*-test. Values of  $P < 0.05$  were considered statistically significant.

## Results

### Expression of M-CSF in the Myocardium after MI Induction

First, we examined whether the expression of M-CSF was up-regulated in the infarct area after the permanent MI.

Although no M-CSF expression was visualized in the infarct myocardium and vasculatures at baseline, immunohistochemical analysis revealed that the M-CSF expression was clearly up-regulated in these regions 6 to 24 hours after the MI, and it declined 3 to 7 days after the MI (Figure 1A). To identify the cell types that express M-CSF, double-immunohistochemical staining using antibodies against M-CSF and the cardiac-specific marker cTnl or the endothelial marker CD31 was performed. We detected the coexpression of M-CSF and cTnl, but not that of CD31 (Figure 1B). By using RT-PCR, we further examined whether cardiomyocytes express M-CSF mRNA and observed the expression of M-CSF mRNA in the murine heart and cultured cardiomyocytes (Figure 1C). The J774 cell line was used as a positive control for M-CSF expression. These findings suggest that M-CSF plays a role in the processes involved in MI.

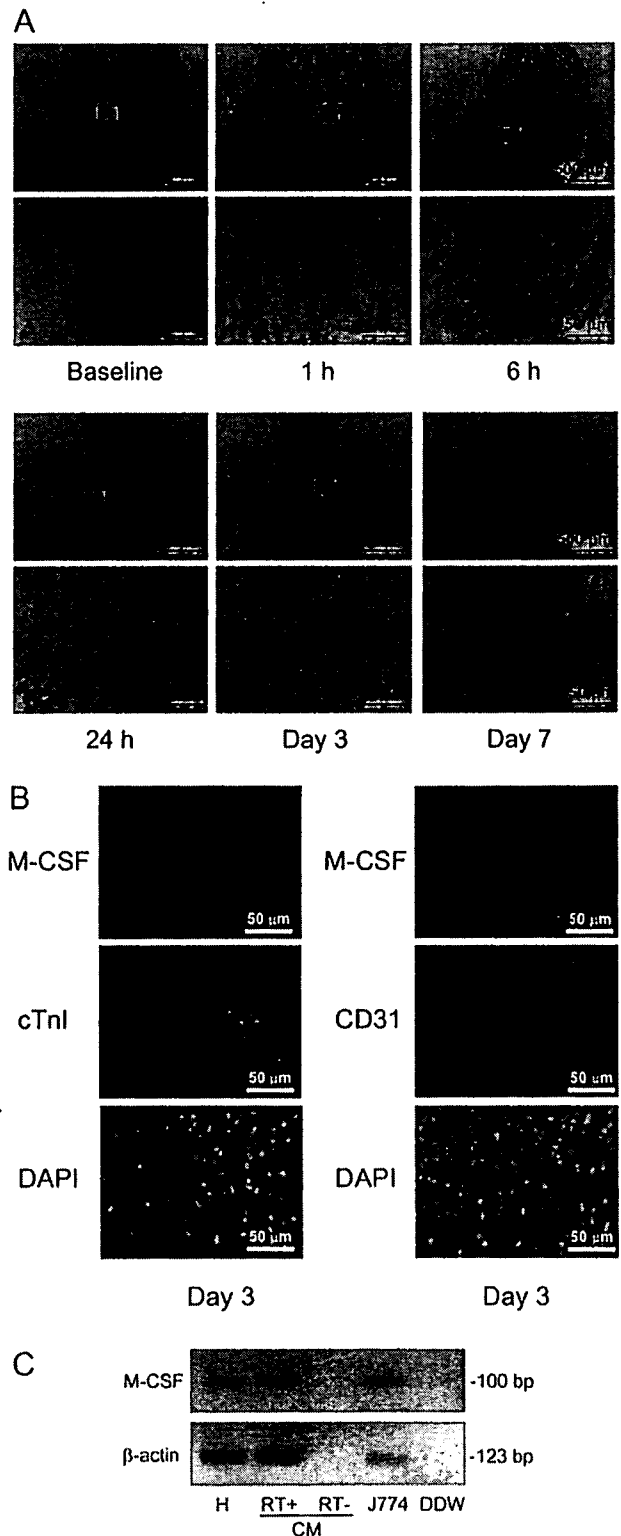
### Effect of M-CSF or G-CSF on the Infarct Area and Scar Formation

Next, we examined the effect of exogenous M-CSF treatment on the infarct area and scar formation after the MI. As shown in Figure 2, A and B, the infarct area at 14 days after MI in the M-CSF-treated mice decreased significantly compared with that in the vehicle-treated mice ( $P < 0.01$ ). Further, Masson's trichrome staining demonstrated that scar formation was significantly reduced in the former compared with that in the latter ( $P < 0.05$ ) (Figure 2, C and D). In addition, because the G-CSF has been shown to improve LV dysfunction and remodeling after MI,<sup>12,13</sup> we tested the effect of G-CSF and confirmed that G-CSF treatment significantly reduced the infarct area and scar formation after the MI (Figure 2). We also assessed the infarct area at the early stage after MI and showed that M-CSF treatment appeared to decrease the infarct area on days 3 and 7 after the MI (Supplemental Figure 1 at <http://ajp.amipathol.org>).

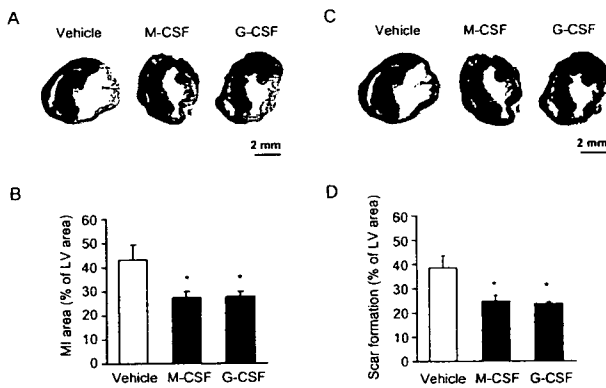
Further, we assessed the LV function after the MI by using echocardiography. No significant difference was observed in the %FS between the vehicle- and M-CSF-treated mice at baseline. In the vehicle-treated mice, a marked decrease in the %FS was observed 7 days after MI, and this decrease was sustained for 28 days. In contrast, the %FS was maintained in the M-CSF-treated mice after the MI (Table 1; 14 days, vehicle:  $25.8 \pm 1.0\%$  versus M-CSF:  $33.5 \pm 0.8\%$ ,  $P < 0.001$ ). As expected, the %FS was also maintained in the G-CSF-treated mice after the MI (14 days, G-CSF:  $34.4 \pm 0.5\%$ ,  $P < 0.001$  versus vehicle).

### Expression of c-fms and G-CSFR in the Myocardium

A previous investigation reported that G-CSF directly activates the Jak-Stat pathway via the G-CSFR in cardiomyocytes and exerts cardioprotective effects.<sup>12</sup> Therefore, we examined the expression of the M-CSF receptor c-fms and G-CSFR in the myocardium after the MI. Real-time RT-PCR analysis revealed that c-fms



**Figure 1.** Expression of M-CSF in the myocardium after MI induction. **A** and **B:** Heart sections were obtained from the mice at baseline, 1 hour, 6 hours, 24 hours, 3 days, and 7 days after MI; these sections were immunohistochemically analyzed by staining with anti-M-CSF antibodies (**A**). Double-immunohistochemical staining for M-CSF (green) and cTnl or CD31 (red) was performed (**B**). Further, nucleic acid was stained with 4,6-diamidino-2-phenylindole (DAPI). The representative photographs are shown ( $n = 3$ ). **C:** Total RNA was extracted from the murine heart (H), cultured cardiomyocytes (CM), and J774 cell line and analyzed for M-CSF and  $\beta$ -actin by RT-PCR analysis. DDW, deionized distilled water.



**Figure 2.** Effect of M-CSF or G-CSF on MI area and scar formation. **A and C:** Heart sections were obtained from the vehicle-, M-CSF-, and G-CSF-treated mice at 14 days after the MI, and these were stained with H&E (**A**) and Masson's trichrome (**C**). **B and D:** Bar graphs indicate the MI area (**B**) and scar formation (**D**). Results are expressed as mean  $\pm$  SEM (each  $n = 10$ ). \* $P < 0.05$  versus vehicle.

expression was decreased by 43% at 6 hours after the MI, and this decrease was sustained for at least 7 days (Figure 3A). In contrast, G-CSFR expression was in-

creased at 6 hours after the MI and reached a peak at 24 hours (approximately 22-fold expression; Figure 3B). Immunohistochemical staining showed the decreased expression of c-fms protein in the heart at 6 hours after the MI (Figure 3C). These results suggest that M-CSF might improve LV dysfunction and remodeling after MI via its effect on monocytes/macrophages rather than that on cardiomyocytes.

### Macrophage Infiltration and Endothelial Cell Density

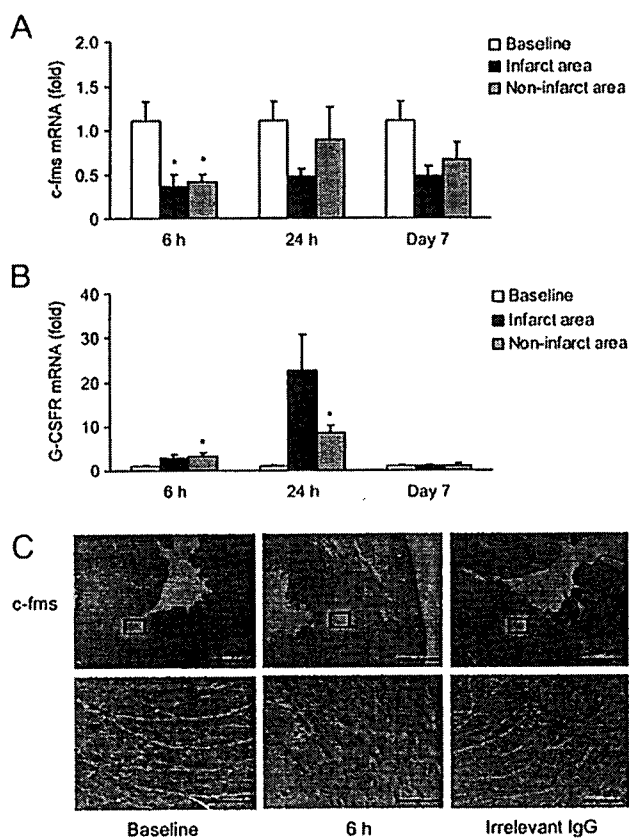
To investigate the mechanism underlying the beneficial effect of M-CSF, we performed an immunohistochemical analysis to detect macrophages (F4/80) and endothelial cells (CD31). The number of infiltrated macrophages in the infarct area of the heart was greater in the M-CSF-treated mice than in the vehicle-treated mice after the MI ( $P < 0.001$ , Figure 4, A and B). The density of endothelial cells that was determined by CD31 expression increased significantly in the M-CSF-treated mice compared with

**Table 1.** Echocardiographic Findings in Vehicle and M-CSF-Treated Mice after MI Induction

	Baseline	Day 7	Day 14	Day 21	Day 28
<b>Vehicle</b>					
<i>n</i>	24	24	23	6	6
B.W. (g)	25.1 $\pm$ 0.3	24.8 $\pm$ 0.5	26.0 $\pm$ 0.4	28.3 $\pm$ 0.2**	28.5 $\pm$ 0.3**
B.W. (%)	100	98.8 $\pm$ 0.9	102.5 $\pm$ 0.9	107.6 $\pm$ 2.0**	108.3 $\pm$ 2.0**
IVSd (mm)	0.74 $\pm$ 0.02	0.67 $\pm$ 0.03	0.65 $\pm$ 0.03	0.60 $\pm$ 0.02	0.55 $\pm$ 0.05*
LVDd (mm)	4.04 $\pm$ 0.11	4.45 $\pm$ 0.10	4.92 $\pm$ 0.12**	5.13 $\pm$ 0.17**	5.22 $\pm$ 0.21**
LVPWd (mm)	0.82 $\pm$ 0.03	0.90 $\pm$ 0.05	0.85 $\pm$ 0.04	0.71 $\pm$ 0.02	0.73 $\pm$ 0.04
IVSs (mm)	1.36 $\pm$ 0.03	1.12 $\pm$ 0.04**	1.04 $\pm$ 0.04**	1.02 $\pm$ 0.03**	0.99 $\pm$ 0.04**
LVDs (mm)	2.24 $\pm$ 0.07	3.20 $\pm$ 0.08**	3.66 $\pm$ 0.12**	3.71 $\pm$ 0.14**	3.83 $\pm$ 0.18**
LVPWs (mm)	1.44 $\pm$ 0.02	1.27 $\pm$ 0.05**	1.23 $\pm$ 0.03**	1.07 $\pm$ 0.03**	1.08 $\pm$ 0.02**
FS (%)	44.9 $\pm$ 0.7	28.2 $\pm$ 0.9**	25.8 $\pm$ 1.0**	27.6 $\pm$ 0.7**	26.7 $\pm$ 1.0**
FS (% of baseline)	100	63.2 $\pm$ 2.1**	58.8 $\pm$ 2.3**	64.2 $\pm$ 1.7**	61.9 $\pm$ 1.9**
<b>M-CSF</b>					
<i>n</i>	25	25	23	5	5
B.W. (g)	24.8 $\pm$ 0.3	24.2 $\pm$ 0.6	25.2 $\pm$ 0.4	27.8 $\pm$ 0.6	28.3 $\pm$ 0.7*
B.W. (%)	100	97.4 $\pm$ 1.9	101.3 $\pm$ 0.9	105.2 $\pm$ 1.2	107.0 $\pm$ 1.2
IVSd (mm)	0.71 $\pm$ 0.02	0.68 $\pm$ 0.02	0.67 $\pm$ 0.02	0.64 $\pm$ 0.02	0.63 $\pm$ 0.04
LVDd (mm)	4.10 $\pm$ 0.06	4.52 $\pm$ 0.06**	4.78 $\pm$ 0.11**	4.97 $\pm$ 0.26**	5.14 $\pm$ 0.19**
LVPWd (mm)	0.80 $\pm$ 0.02	0.79 $\pm$ 0.03	0.80 $\pm$ 0.02	0.72 $\pm$ 0.05	0.70 $\pm$ 0.04
IVSs (mm)	1.29 $\pm$ 0.02	1.18 $\pm$ 0.03	1.19 $\pm$ 0.03 <sup>††</sup>	1.12 $\pm$ 0.05	1.13 $\pm$ 0.09
LVDs (mm)	2.32 $\pm$ 0.05	3.03 $\pm$ 0.06**	3.18 $\pm$ 0.09** <sup>††</sup>	3.22 $\pm$ 0.18**	3.44 $\pm$ 0.14**
LVPWs (mm)	1.42 $\pm$ 0.02	1.28 $\pm$ 0.04*	1.36 $\pm$ 0.03 <sup>††</sup>	1.24 $\pm$ 0.04 <sup>††</sup>	1.17 $\pm$ 0.05*
FS (%)	43.6 $\pm$ 0.6	32.9 $\pm$ 0.8** <sup>†††</sup>	33.5 $\pm$ 0.8** <sup>†††</sup>	35.2 $\pm$ 0.8** <sup>†††</sup>	32.9 $\pm$ 1.4** <sup>††</sup>
FS (% of baseline)	100	75.9 $\pm$ 2.1** <sup>†††</sup>	76.7 $\pm$ 2.3** <sup>†††</sup>	83.1 $\pm$ 1.8** <sup>†††</sup>	77.7 $\pm$ 3.4** <sup>††</sup>
<b>G-CSF</b>					
<i>n</i>	12	10	10		
B.W. (g)	24.9 $\pm$ 0.3	24.0 $\pm$ 0.3	24.8 $\pm$ 0.3 <sup>†</sup>		
B.W. (%)	100	96.3 $\pm$ 1.4**	100.3 $\pm$ 0.5		
IVSd (mm)	0.71 $\pm$ 0.04	0.77 $\pm$ 0.02 <sup>†</sup>	0.72 $\pm$ 0.04		
LVDd (mm)	4.06 $\pm$ 0.09	4.68 $\pm$ 0.23*	5.04 $\pm$ 0.15**		
LVPWd (mm)	0.81 $\pm$ 0.06	1.08 $\pm$ 0.12	0.98 $\pm$ 0.08		
IVSs (mm)	1.27 $\pm$ 0.04	1.31 $\pm$ 0.04 <sup>††</sup>	1.37 $\pm$ 0.04 <sup>†††</sup>		
LVDs (mm)	2.32 $\pm$ 0.06	3.02 $\pm$ 0.19**	3.31 $\pm$ 0.10** <sup>†</sup>		
LVPWs (mm)	1.40 $\pm$ 0.04	1.59 $\pm$ 0.08 <sup>††</sup>	1.57 $\pm$ 0.08 <sup>††</sup>		
FS (%)	42.9 $\pm$ 0.5	35.9 $\pm$ 1.5** <sup>†††</sup>	34.4 $\pm$ 0.5** <sup>†††</sup>		
FS (% of baseline)	100	83.4 $\pm$ 3.0** <sup>†††</sup>	80.5 $\pm$ 1.8** <sup>†††</sup>		

Data represent the mean  $\pm$  S.E.M. *n*, number; B.W., body weight; IVSd, interventricular septal diastolic thickness; LVDd, left ventricular diastolic dimension; LVPWd, left ventricular posterior wall diastolic thickness; IVSs, interventricular septal systolic thickness; LVDs, left ventricular systolic dimension; LVPWs, left ventricular posterior wall systolic thickness; FS, fractional shortening.

\* $P < 0.05$  and \*\* $P < 0.01$  versus baseline.  
<sup>†</sup> $P < 0.05$ , <sup>††</sup> $P < 0.01$ , and <sup>†††</sup> $P < 0.001$  versus vehicle.



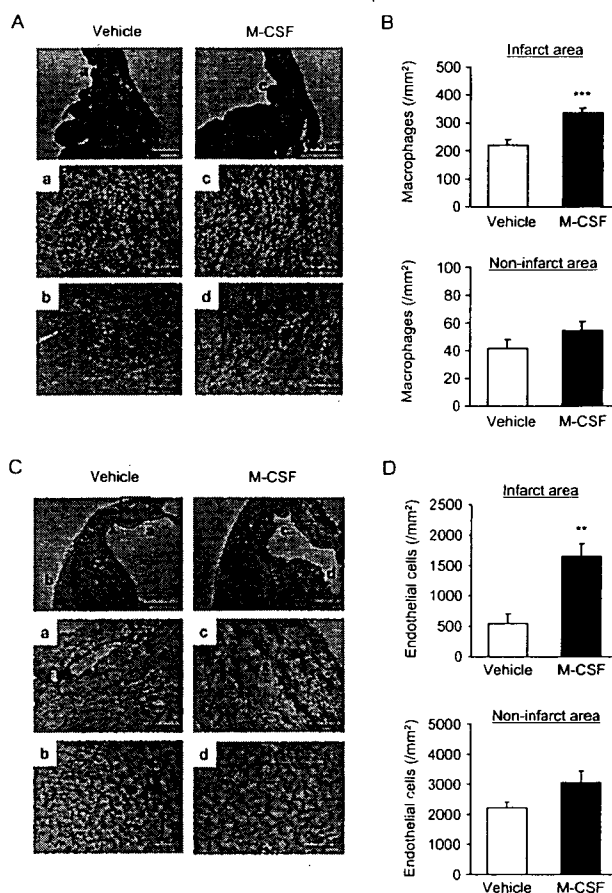
**Figure 3.** Expression of c-fms and G-CSFR in the myocardium. **A** and **B**: Total RNA was extracted from the infarct and noninfarct areas of the heart at baseline (sham operation), 6 hours, 24 hours, and 7 days after the MI and analyzed for c-fms (**A**) or G-CSFR (**B**) by real-time RT-PCR analysis. Results are expressed as mean  $\pm$  SEM (each  $n = 5$ ). \* $P < 0.05$  versus baseline. **C**: Heart sections were obtained from the mice at baseline and 6 hours after MI; these sections were immunohistochemically analyzed by staining with anti-c-fms antibody. Irrelevant IgG was used as a negative control.

that in the vehicle-treated mice ( $P < 0.01$ , Figure 4, C and D). These findings suggest that M-CSF treatment promotes macrophage infiltration and neovascularization in the infarct myocardium.

### Effect of M-CSF on Differentiation into Myofibroblasts and Cardiomyocyte Apoptosis

Cardiac fibroblasts have been shown to differentiate into myofibroblasts during the process of myocardial repair and remodeling after MI.<sup>14</sup> Because myofibroblasts are characterized by the presence of  $\alpha$ -SMA, immunohistochemical analysis of  $\alpha$ -SMA was performed. The number of  $\alpha$ -SMA-positive myofibroblasts was notably increased at the infarct area compared with that at the noninfarct area (Figure 5, A and B). However, no change was observed in the number of myofibroblasts in the M-CSF-treated mice compared with that in the vehicle-treated mice ( $P = 0.245$ ).

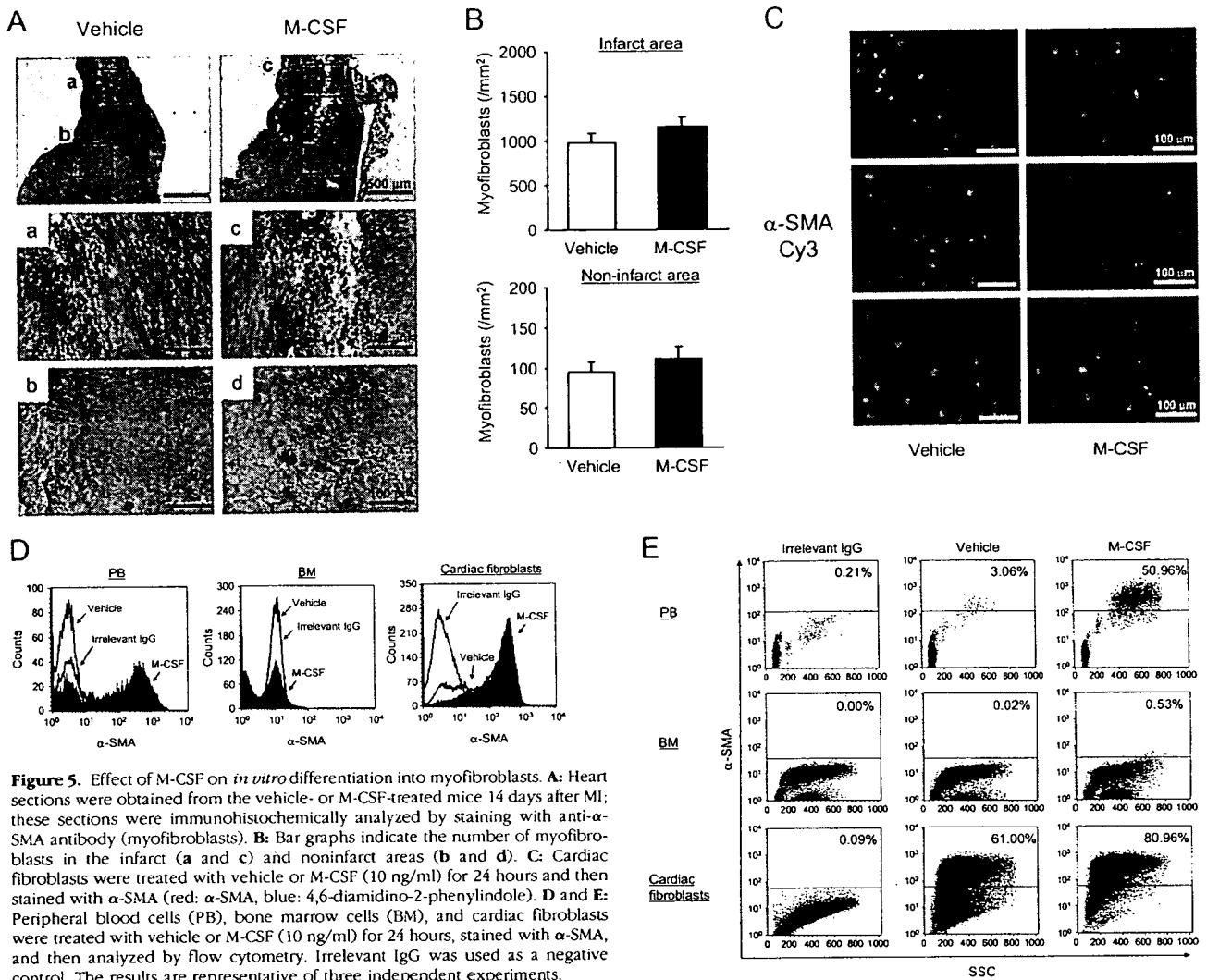
To examine the effect of M-CSF on the differentiation into myofibroblasts *in vitro*, we used murine neonatal cardiac fibroblasts. M-CSF accelerated the *in vitro* differentiation of cardiac fibroblasts into myofibroblasts (Figure 5C). We performed flow cytometry analysis to quantify



**Figure 4.** Macrophage infiltration and endothelial cell density. **A** and **C**: Heart sections were obtained from the vehicle- or M-CSF-treated mice 14 days after MI; these sections were immunohistochemically analyzed by staining with F4/80 (macrophages; **A**) and anti-CD31 antibody (endothelial cells; **C**). **B** and **D**: Bar graphs indicate the number of macrophages (**B**) and endothelial cells (**D**) in the infarct (**a** and **c**) and noninfarct areas (**b** and **d**). Results are expressed as mean  $\pm$  SEM (each  $n = 5$ ). \*\* $P < 0.01$ , and \*\*\* $P < 0.001$  versus vehicle.

this differentiation. Interestingly, M-CSF clearly stimulated the *in vitro* differentiation of not only cardiac fibroblasts but also peripheral blood cells and bone marrow-derived cells (Figure 5, D and E).

To assess the involvement of apoptosis after MI in the vehicle- and M-CSF-treated mice, terminal deoxynucleotidyl transferase dUTP nick-end labeling staining was performed; however, no differences were found in the number of apoptotic cells between the vehicle- and M-CSF-treated mice (Supplemental Figure 2A at <http://ajp.amipathol.org>). To investigate further whether M-CSF confers an anti-apoptotic effect on cardiomyocytes, *in vitro* experiments using murine cultured cardiomyocytes were performed. We exposed cardiomyocytes to hypoxia-reoxygenation in the absence or presence of M-CSF and examined cardiomyocyte apoptosis. Treatment with M-CSF reduced the number of apoptotic cells induced by hypoxia-reoxygenation compared with cells that were not treated with M-CSF (Supplemental Figure 2B at <http://ajp.amipathol.org>).



**Figure 5.** Effect of M-CSF on *in vitro* differentiation into myofibroblasts. **A:** Heart sections were obtained from the vehicle- or M-CSF-treated mice 14 days after MI; these sections were immunohistochemically analyzed by staining with anti- $\alpha$ -SMA antibody (myofibroblasts). **B:** Bar graphs indicate the number of myofibroblasts in the infarct (a and c) and noninfarct areas (b and d). **C:** Cardiac fibroblasts were treated with vehicle or M-CSF (10 ng/ml) for 24 hours and then stained with  $\alpha$ -SMA (red;  $\alpha$ -SMA, blue; 4,6-diamidino-2-phenylindole). **D and E:** Peripheral blood cells (PB), bone marrow cells (BM), and cardiac fibroblasts were treated with vehicle or M-CSF (10 ng/ml) for 24 hours, stained with  $\alpha$ -SMA, and then analyzed by flow cytometry. Irrelevant IgG was used as a negative control. The results are representative of three independent experiments.

*Involvement of Inflammatory Cytokines, Collagen, and TGF- $\beta$ 1*

To investigate whether cytokines and collagen synthesis are involved in reducing the infarct area and scar formation after MI, we determined the serum level of MCP-1, IL-6, IL-10, IL-12p70, IFN- $\gamma$ , and TNF- $\alpha$ . Serum IL-6 and MCP-1 levels tended to increase 24 hours after the MI (Figure 6, A and B), whereas no increase was observed in the IL-12p70 level (Figure 6C). The other inflammatory cytokines were under detectable limits (<20 pg/ml, data not shown). Real-time RT-PCR analysis revealed that the mRNA expression of collagen type I and type III was clearly up-regulated in the infarct area of the vehicle- and M-CSF-treated mice 14 days after the MI, and no difference was observed in these expression levels between the two groups (Figure 6, D and E). In addition, no significant increase was observed in the TGF- $\beta$ 1 mRNA expression in the infarct area (Figure 6F).

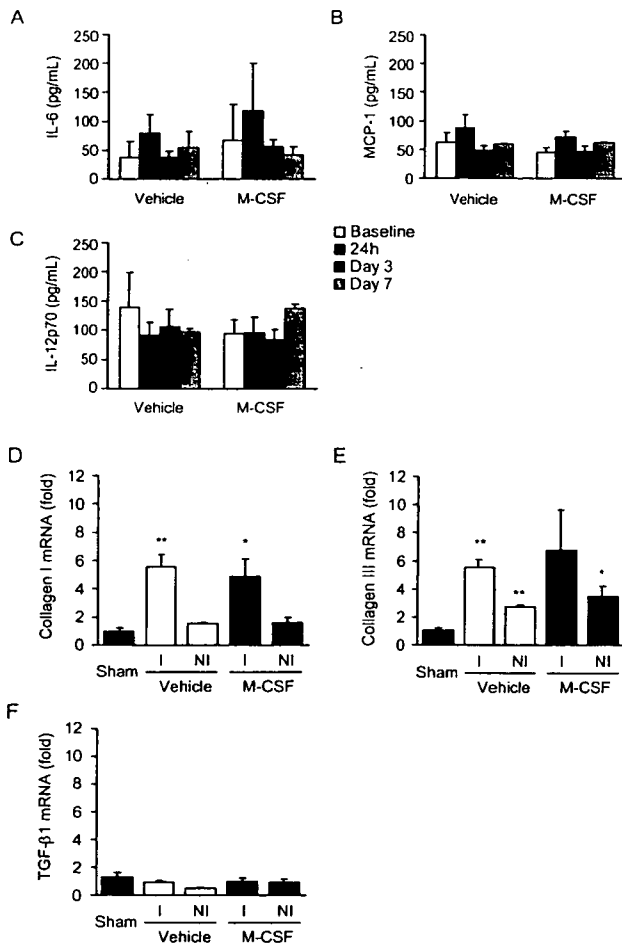
*Mobilization of Bone Marrow-Derived Cells*

To determine the types of bone marrow-derived cells that were mobilized by M-CSF treatment, we assessed the percentage of Mac-1<sup>+</sup>/Gr-1<sup>-</sup> cells (monocytic marker), CD34<sup>+</sup>/Flk-1<sup>+</sup> cells, and CXCR4<sup>+</sup> cells. Flow cytometric analysis revealed that M-CSF treatment resulted in a significant increase in the Mac-1<sup>+</sup>/Gr-1<sup>-</sup> ( $P < 0.05$ ) and CXCR4<sup>+</sup> cells ( $P < 0.01$ ) but not in the CD34<sup>+</sup>/Flk-1<sup>+</sup> cells in peripheral circulation 72 hours after the MI (Figure 7, A–C). Furthermore, M-CSF treatment preferentially increased the Mac-1<sup>+</sup>/CXCR4<sup>+</sup> cells (Figure 7D).

*Involvement of the SDF-1-CXCR4 Axis*

Because the peripheral CXCR4<sup>+</sup> cells were observed to increase substantially in the M-CSF-treated mice after the MI, we performed an immunohistochemical analysis to detect CXCR4 and its ligand SDF-1. The expression of SDF-1 was predominantly observed in the infarct and border areas but not in the noninfarct area (Figure 8A).

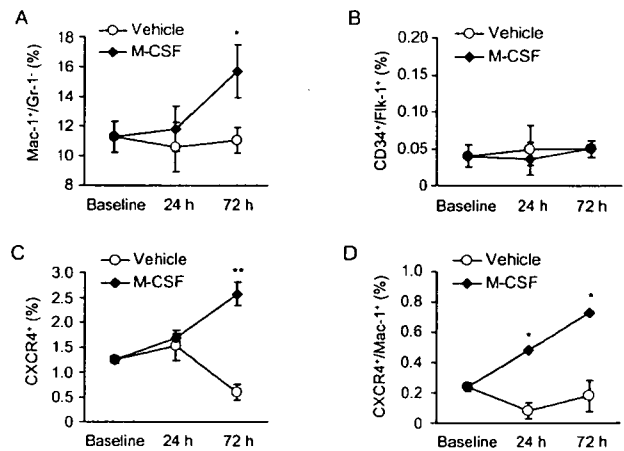




**Figure 6.** Involvement of inflammatory cytokines, collagen, and TGF- $\beta$ 1. **A–C:** Blood samples were obtained from the vehicle- or M-CSF-treated mice at baseline, 24 hours, 3 days, and 7 days after the MI. Serum levels of IL-6 (**A**), MCP-1 (**B**), and IL-12p70 (**C**) were assessed. Data are represented as mean  $\pm$  SEM ( $n = 5$ ). **D–F:** Total RNA was extracted from the infarct (I) or noninfarct (NI) area in the sham-operated, vehicle-, and M-CSF-treated mice 14 days after the MI and analyzed for collagen type I, collagen type III, and TGF- $\beta$ 1 mRNA expression by real-time RT-PCR analysis. Results are expressed as mean  $\pm$  SEM ( $n = 3$ ). \* $P < 0.05$ , and \*\* $P < 0.01$  versus sham.

No difference was observed in the levels of SDF-1 expression between the vehicle- and M-CSF-treated mice. Similarly, the number of infiltrated CXCR4<sup>+</sup> cells increased in the infarct and border areas but not in the noninfarct area (Figure 8B). Quantitative analysis revealed that M-CSF treatment significantly increased the number of CXCR4<sup>+</sup> cells increased in the border area of MI mice (Figure 8C,  $P < 0.01$ ).

To explore the role of the SDF-1-CXCR4 axis, we used a novel CXCR4 antagonist, namely, AMD3100. AMD3100 was administered subcutaneously for 7 consecutive days after the MI by using a micro-osmotic pump. The administration of AMD3100 significantly increased the number of circulating white blood cells, particularly neutrophils (data not shown); this finding was consistent with that in previous reports.<sup>15</sup> As shown in Figure 8, D–F, M-CSF treatment improved LV function at 7 and 14 days after the MI, and additional treatment with AMD3100 deteriorated the infarction ( $P = 0.054$ ) and LV function ( $P < 0.05$ ) in the M-CSF-treated mice.



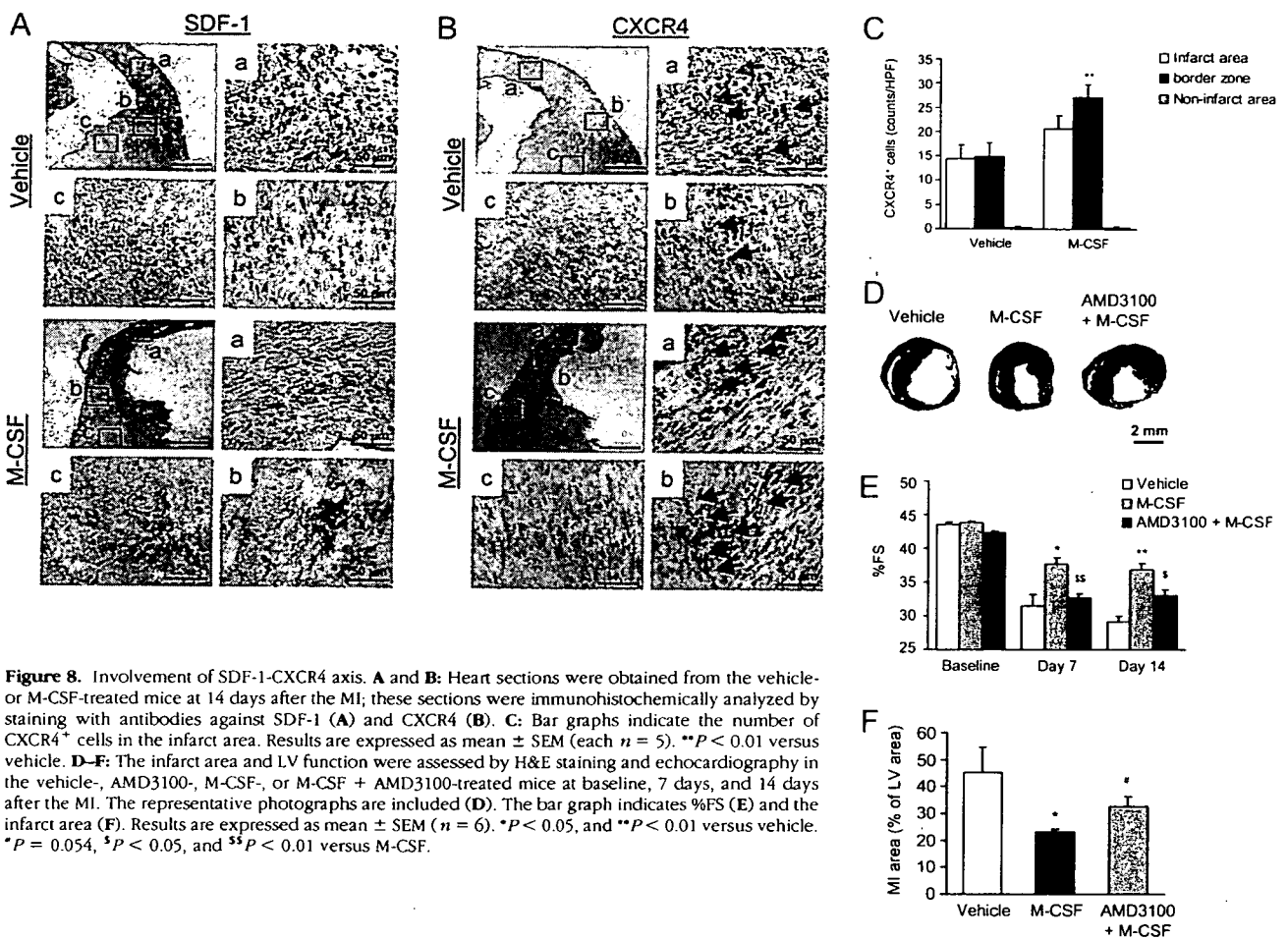
**Figure 7.** Mobilization of bone marrow-derived cells. **A–D:** Blood samples were collected from the vehicle- or M-CSF-treated mice at baseline, 24 hours, and 72 hours after the MI. The percentage of Mac-1<sup>+</sup>/Gr-1<sup>+</sup>, CD34<sup>+</sup>/Flk-1<sup>+</sup>, and CXCR4<sup>+</sup> cells was assessed by flow cytometric analysis. Results are expressed as mean  $\pm$  SEM ( $n = 3$ ). \* $P < 0.05$ , and \*\* $P < 0.01$  versus vehicle.

## Discussion

The major findings of this study are as follows: i) M-CSF expression was up-regulated in the infarct myocardium; ii) M-CSF treatment reduced the infarct size and scar formation and improved LV dysfunction and remodeling after the MI; iii) c-fms expression decreased in the infarct myocardium; iv) M-CSF treatment also induced macrophage infiltration and capillary formation but not myofibroblast accumulation in the infarct area; v) M-CSF treatment mobilized the number of CXCR4<sup>+</sup> cells in peripheral circulation; vi) SDF-1 expression was marked in the infarct myocardium, and CXCR4<sup>+</sup> cells were recruited to the infarct area in the M-CSF-treated mice; and vii) the CXCR4 antagonist AMD3100 deteriorated the infarction and LV function after the MI in the M-CSF-treated mice. These findings suggest that M-CSF reduces infarct size and scar formation and improves LV dysfunction and remodeling after the MI via the activation of SDF-1-CXCR4 axis.

### Effect of M-CSF Treatment on the Infarct Area and LV Dysfunction after the MI

Increasing evidence indicates the importance of bone marrow-derived stem/progenitor cells in the pathophysiology of cardiovascular diseases. In particular, bone marrow stem cells could promote neovascularization and cardiac regeneration after MI and thereby improve LV dysfunction and remodeling<sup>16,17</sup>; this suggests the therapeutic potential of bone marrow stem cell transplantation for the treatment of cardiovascular diseases. Because colony-stimulating factors could mobilize bone marrow stem cells into peripheral circulation, the use of G-CSF and the granulocyte-macrophage colony-stimulating factor has recently been reported as a clinical application of stem cell therapy<sup>18,19</sup>; however, the effect of M-CSF treatment on cardiac dysfunction and remodeling after permanent MI has not been entirely understood.



**Figure 8.** Involvement of SDF-1-CXCR4 axis. **A** and **B**: Heart sections were obtained from the vehicle- or M-CSF-treated mice at 14 days after the MI; these sections were immunohistochemically analyzed by staining with antibodies against SDF-1 (**A**) and CXCR4 (**B**). **C**: Bar graphs indicate the number of CXCR4<sup>+</sup> cells in the infarct area. Results are expressed as mean  $\pm$  SEM (each  $n = 5$ ). \*\* $P < 0.01$  versus vehicle. **D-F**: The infarct area and LV function were assessed by H&E staining and echocardiography in the vehicle-, AMD3100-, M-CSF-, or M-CSF + AMD3100-treated mice at baseline, 7 days, and 14 days after the MI. The representative photographs are included (**D**). The bar graph indicates %FS (**E**) and the infarct area (**F**). Results are expressed as mean  $\pm$  SEM ( $n = 6$ ). \* $P < 0.05$ , and \*\* $P < 0.01$  versus vehicle. <sup>†</sup> $P = 0.054$ , <sup>§</sup> $P < 0.05$ , and <sup>§§</sup> $P < 0.01$  versus M-CSF.

We clearly demonstrated that M-CSF treatment improved LV dysfunction, reduced the infarct size, and improved LV remodeling after permanent MI. Recently, Yano et al<sup>20</sup> reported that M-CSF treatment attenuated LV dysfunction after myocardial ischemia-reperfusion in rats; however, M-CSF had no effects on the infarct size, infarct transmural, and the number of factor VIII-positive vessels in the infarct and noninfarct myocardium. In their study,<sup>20</sup> because M-CSF treatment increased TGF- $\beta$ 1 expression and the collagen content in the myocardium, they speculated that the beneficial effect of M-CSF was mediated through the acceleration of the myocardial repair processes. Although the reason for the discrepancy in the effect of M-CSF is unclear, there are a number of differences between the pathophysiology of permanent MI and ischemia-reperfusion injury.<sup>21-23</sup> Reperfusion releases a large excess of oxygen-derived free radicals and produces "reperfusion injury." Inflammatory responses such as the infiltration of neutrophils and macrophages are much stronger in the reperfused heart than in the infarct heart. Because the inflammatory response after MI is determinative for tissue healing,<sup>23</sup> the processes involved in myocardial healing, such as collagen deposition, are accelerated in the reperfused heart. Furthermore, reperfusion enhances neovascularization to a greater extent in the reperfused heart than in the infarct heart. Thus, we postulate that although M-CSF has benefi-

cial effects on the repair processes of both permanent MI and ischemia-reperfusion, its mechanism of action might differ between these two conditions.

### Effect of M-CSF Treatment on Macrophage Infiltration and Neovascularization

We demonstrated that M-CSF treatment increased macrophage infiltration and endothelial cell density after the MI. Monocytes/macrophages have been shown to promote the healing and repair processes of MI by several mechanisms. Macrophages produce cytokines and growth factors, which influence the process of myocardial healing.<sup>24</sup> In addition, macrophages may regulate extracellular matrix metabolism through the synthesis of matrix metalloproteinases and their inhibitors.<sup>25</sup> Thus, M-CSF-induced macrophage infiltration might play a role in the post-MI healing process. Previous reports showed that myofibroblasts are the predominant source of collagen and that TGF- $\beta$ 1 acts as a key mediator for myofibroblast differentiation during wound healing by regulating  $\alpha$ -SMA expression.<sup>23,26</sup> Using a rat model of myocardial ischemia-reperfusion, Yano et al<sup>20</sup> recently reported that M-CSF treatment increased TGF- $\beta$ 1 expression and collagen content. However, in our study, M-CSF treatment did not increase myofibroblast accumulation and the expression of colla-

gen and TGF- $\beta$ 1 in the infarct area. Interestingly, M-CSF accelerated the *in vitro* differentiation of cardiac fibroblasts, peripheral blood cells, and bone marrow-derived cells. Furthermore, M-CSF decreased hypoxia-reoxygenation-induced apoptosis in cultured cardiomyocytes, although there was no difference in the number of apoptotic cells in the heart after MI between the vehicle- and M-CSF-treated mice. Although the reason for the discrepancy between *in vitro* and *in vivo* experiments is currently unclear, further investigation is required to elucidate the precise mechanisms underlying the beneficial effect of M-CSF. Taken together, our findings suggest that in a permanent MI model, the beneficial effect of M-CSF might at least in part depend on macrophage infiltration and neovascularization.

Recent evidence indicates that monocytes/macrophages promote neovascularization in ischemic tissues through several possible mechanisms. First, recent reports demonstrated that monocytes/macrophages produce matrix metalloproteinase-dependent tunnels and directly promote neovascularization.<sup>1,27,28</sup> Second, bone marrow-derived cells of monocytic lineage are suggested to function as EPCs and participate in the neovascularization of ischemic tissues.<sup>2,3</sup> In our study, M-CSF treatment increased the Mac-1<sup>+</sup>/CXCR4<sup>+</sup> cells but not the CD34<sup>+</sup>/Flk-1<sup>+</sup> cells (ordinary EPC marker) in peripheral circulation after the MI. In this regard, Harraz et al<sup>2</sup> reported that CD34<sup>-</sup> angioblasts are a subset of CD14<sup>+</sup> monocytic cells and that these monocytes have the potential to transdifferentiate into endothelial cells; this suggests that EPCs derived from monocytic cells might have other surface markers.

There are several reports describing the role of SDF-1-CXCR4 axis in neointimal formation after vascular injury. Weber and his colleagues<sup>29,30</sup> reported that the SDF-1-CXCR4 axis contributed to the recruitment of bone marrow-derived smooth muscle progenitor cells and neointimal formation after vascular injury in apoE<sup>-/-</sup> mice. Zhang et al<sup>31</sup> also reported that the SDF-1-CXCR4 axis contributed to neointimal formation after the ligation of carotid artery in endothelial nitric-oxide synthase-deficient mice. Furthermore, we recently reported that M-CSF treatment accelerated neointimal formation after vascular injury via the SDF-1-CXCR4 axis.<sup>32</sup> These investigations suggest that the CXCR4<sup>+</sup> cells act as smooth muscle progenitor cells. In contrast, Walter et al<sup>33</sup> reported that impaired CXCR4 signaling contributes to the reduced neovascularization capacity of EPCs, suggesting that the CXCR4<sup>+</sup> cells function as EPCs in ischemic tissue. Furthermore, several studies demonstrated that bone marrow-derived CXCR4<sup>+</sup> cells promote neovascularization in ischemic tissue by the release of angiogenic factors.<sup>34,35</sup> Thus, we speculate that M-CSF-mobilized monocytic CXCR4<sup>+</sup> cells are retained by SDF-1 and accelerate neovascularization in the infarct area. Taken together, we postulate that the CXCR4<sup>+</sup> cells have the potential to function as both EPCs and smooth muscle progenitor cells according to the circumstances, and the SDF-1-CXCR4 system may contribute to the pathogenesis of cardiovascular diseases.

### Role of the SDF-1-CXCR4 Axis in the Pathophysiology of MI

We demonstrated that M-CSF increased the CXCR4<sup>+</sup> cells in peripheral circulation, whereas MI induced SDF-1 expression in the infarct area. The mobilized CXCR4<sup>+</sup> cells were recruited to the infarct myocardium, and they interacted with SDF-1. The inhibitory effect of AMD3100 indicates the contribution of the SDF-1-CXCR4 axis to the beneficial effect of M-CSF. Interestingly, recent investigations demonstrated that the AMD3100 treatment rapidly mobilizes CD34<sup>+</sup> hematopoietic stem cells from the bone marrow into peripheral circulation<sup>36</sup> and that G-CSF combined with single injection of AMD3100 promoted angiogenesis in a murine model of hindlimb ischemia.<sup>37</sup> In our previous study,<sup>32</sup> however, continuous AMD3100 infusion had no effect on the re-endothelialization after vascular injury; this suggests that a continuous CXCR4 inhibition abrogates the chemotactic activity of SDF-1 and homing of CXCR4<sup>+</sup> cells to the SDF-1-expressing site. Supporting this, a recent study demonstrated that CXCR4<sup>+</sup> cells could not accumulate at the SDF-1-expressing site in animals harboring an osmotic pump releasing AMD3100.<sup>35</sup>

Several previous studies demonstrated that the SDF-1-CXCR4 axis participated in the neovascularization of ischemic tissues.<sup>33,38</sup> Consistent with our finding, by using a rabbit model of myocardial ischemia-reperfusion, Misao et al<sup>39</sup> very recently reported that the SDF-1-CXCR4 axis might be responsible for the G-CSF-induced improvement of LV dysfunction. Taken together, these findings suggest that the SDF-1-CXCR4 axis plays a crucial role in the beneficial effect of M-CSF in MI. Further studies are required to elucidate its precise role of the SDF-1-CXCR4 axis in the pathophysiology of MI.

### Conclusion

We demonstrated that M-CSF treatment reduced infarct area and scar formation and improved LV dysfunction and remodeling after MI through the recruitment of CXCR4<sup>+</sup> cells into the infarct myocardium by the activation of SDF-1-CXCR4 axis. These findings suggest that M-CSF has a therapeutic potential for MI and that the SDF-1-CXCR4 axis could be new target for the treatment of ischemic heart diseases.

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## References

- Schmeisser A, Garlich CD, Zhang H, Eskafi S, Graffy C, Ludwig J, Strasser RH, Daniel WG: Monocytes coexpress endothelial and macrophagocytic lineage markers and form cord-like structures in Matrigel under angiogenic conditions. *Cardiovasc Res* 2001, 49:671-680
- Harrasz M, Jiao C, Hanlon HD, Hartley RS, Schattman GC: CD34-blood-derived human endothelial cell progenitors. *Stem Cells* 2001, 19:304-312
- Rehman J, Li J, Orschell CM, March KL: Peripheral blood "endothelial progenitor cells" are derived from monocyte/macrophages and secrete angiogenic growth factors. *Circulation* 2003, 107:1164-1169
- Motoyoshi K: Biological activities and clinical application of M-CSF. *Int J Hematol* 1998, 67:109-122
- Frangogiannis NG, Mendoza LH, Ren G, Akivakis S, Jackson PL, Michael LH, Smith CW, Entman ML: MCSF expression is induced in healing myocardial infarcts and may regulate monocyte and endothelial cell phenotype. *Am J Physiol* 2003, 285:H483-H492
- Misawa E, Sakurai T, Yamada M, Tamura Y, Motoyoshi K: Administration of macrophage colony-stimulating factor mobilized both CD11b<sup>+</sup>CD11c<sup>+</sup> cells and NK1.1<sup>+</sup> cells into peripheral blood. *Int Immunopharmacol* 2004, 4:791-803
- Werner N, Junk S, Laufs U, Link A, Walenta K, Bohm M, Nickenig G: Intravenous transfusion of endothelial progenitor cells reduces neointima formation after vascular injury. *Circ Res* 2003, 93:e17-e24
- Morimoto H, Takahashi M, Izawa A, Ise H, Hongo M, Kolattukudy PE, Ikeda U: Cardiac overexpression of monocyte chemoattractant protein-1 in transgenic mice prevents cardiac dysfunction and remodeling after myocardial infarction. *Circ Res* 2006, 99:891-899
- Yoshioka T, Takahashi M, Shiba Y, Suzuki C, Morimoto H, Izawa A, Ise H, Ikeda U: Granulocyte colony-stimulating factor (G-CSF) accelerates reendothelialization and reduces neointimal formation after vascular injury in mice. *Cardiovasc Res* 2006, 70:61-69
- Kamiyoshi Y, Takahashi M, Yokoseki O, Yazaki Y, Hirose S, Morimoto H, Watanabe N, Kinoshita O, Hongo M, Ikeda U: Mycophenolate mofetil prevents the development of experimental autoimmune myocarditis. *J Mol Cell Cardiol* 2005, 39:467-477
- Jia L, Takahashi M, Morimoto H, Takahashi S, Izawa A, Ise H, Iwasaki T, Hattori H, Wu KJ, Ikeda U: Changes in cardiac lipid metabolism during sepsis: the essential role of very low-density lipoprotein receptors. *Cardiovasc Res* 2006, 69:545-555
- Harada M, Qin Y, Takano H, Minamino T, Zou Y, Toko H, Ohtsuka M, Matsuura K, Sano M, Nishi J, Iwanaga K, Akazawa H, Kunieda T, Zhu W, Hasegawa H, Kunisada K, Nagai T, Nakaya H, Yamauchi-Takahara K, Komuro I: G-CSF prevents cardiac remodeling after myocardial infarction by activating the Jak-Stat pathway in cardiomyocytes. *Nat Med* 2005, 11:305-311
- Sugano Y, Anzai T, Yoshikawa T, Maekawa Y, Kohno T, Mahara K, Naito K, Ogawa S: Granulocyte colony-stimulating factor attenuates early ventricular expansion after experimental myocardial infarction. *Cardiovasc Res* 2005, 65:446-456
- Virag JI, Murry CE: Myofibroblast and endothelial cell proliferation during murine myocardial infarct repair. *Am J Pathol* 2003, 163:2433-2440
- Martin C, Burdon PC, Bridger G, Gutierrez-Ramos JC, Williams TJ, Rankin SM: Chemokines acting via CXCR2 and CXCR4 control the release of neutrophils from the bone marrow and their return following senescence. *Immunity* 2003, 19:583-593
- Orlic D, Kajstura J, Chimenti S, Jakoniuk I, Anderson SM, Li B, Pickel J, McKay R, Nadal-Ginard B, Bodine DM, Leri A, Anversa P: Bone marrow cells regenerate infarcted myocardium. *Nature* 2001, 410:701-705
- Asahara T, Masuda H, Takahashi T, Kalka C, Pastore C, Silver M, Kearne M, Magner M, Isner JM: Bone marrow origin of endothelial progenitor cells responsible for postnatal vasculogenesis in physiological and pathological neovascularization. *Circ Res* 1999, 85:221-228
- Zohlnhofer D, Ott I, Mehilli J, Schomig K, Michalk F, Ibrahim T, Meisetschlag G, von Wedel J, Bollwein H, Seyfarth M, Dirschinger J, Schmitt C, Schwaiger M, Kastrati A, Schomig A: Stem cell mobilization by granulocyte colony-stimulating factor in patients with acute myocardial infarction: a randomized controlled trial. *JAMA* 2006, 295:1003-1010
- Deng Z, Yang C, Deng H, Yang A, Geng T, Chen X, Ma A, Liu Z: Effects of GM-CSF on the stem cells mobilization and plasma C-reactive protein levels in patients with acute myocardial infarction. *Int J Cardiol* 2006, 113:92-96
- Yano T, Miura T, Whittaker P, Miki T, Sakamoto J, Nakamura Y, Ichikawa Y, Ikeda Y, Kobayashi H, Ohori K, Shimamoto K: Macrophage colony-stimulating factor treatment after myocardial infarction attenuates left ventricular dysfunction by accelerating infarct repair. *J Am Coll Cardiol* 2006, 47:626-634
- Kloner RA, Jennings RB: Consequences of brief ischemia: stunning, preconditioning, and their clinical implications: part 1. *Circulation* 2001, 104:2981-2989
- Vandervelde S, van Amerongen MJ, Tio RA, Petersen AH, van Luyn MJ, Harmsen MC: Increased inflammatory response and neovascularization in reperfused vs. non-reperfused murine myocardial infarction. *Cardiovasc Pathol* 2006, 15:83-90
- Frangogiannis NG, Smith CW, Entman ML: The inflammatory response in myocardial infarction. *Cardiovasc Res* 2002, 53:31-47
- Weihrauch D, Arras M, Zimmermann R, Schaper J: Importance of monocytes/macrophages and fibroblasts for healing of microcircles in porcine myocardium. *Mol Cell Biochem* 1995, 147:13-19
- Lindsey ML: MMP induction and inhibition in myocardial infarction. *Heart Fail Rev* 2004, 9:7-19
- Desmoulière A, Geinoz A, Gabbiani F, Gabbiani G: Transforming growth factor- $\beta$ 1 induces  $\alpha$ -smooth muscle actin expression in granulation tissue myofibroblasts and in quiescent and growing cultured fibroblasts. *J Cell Biol* 1993, 122:103-111
- Anghelina M, Krishnan P, Moldovan L, Moldovan NI: Monocytes/macrophages cooperate with progenitor cells during neovascularization and tissue repair: conversion of cell columns into fibrovascular bundles. *Am J Pathol* 2006, 168:529-541
- Moldovan NI, Goldschmidt-Clermont PJ, Parker-Thornburg J, Shapiro SD, Kolattukudy PE: Contribution of monocytes/macrophages to compensatory neovascularization: the drilling of metalloelastase-positive tunnels in ischemic myocardium. *Circ Res* 2000, 87:378-384
- Schober A, Knarren S, Lietz M, Lin EA, Weber C: Crucial role of stromal cell-derived factor-1 $\alpha$  in neointima formation after vascular injury in apolipoprotein E-deficient mice. *Circulation* 2003, 108:2491-2497
- Zernecke A, Schober A, Bot I, von Hundelshausen P, Liehn EA, Mopps B, Mericskay M, Gierschik P, Biessen EA, Weber C: SDF-1 $\alpha$ /CXCR4 axis is instrumental in neointimal hyperplasia and recruitment of smooth muscle progenitor cells. *Circ Res* 2005, 96:784-791
- Zhang LN, Wilson DW, da Cunha V, Sullivan ME, Vergona R, Rutledge JC, Wang YX: Endothelial NO synthase deficiency promotes smooth muscle progenitor cells in association with upregulation of stromal cell-derived factor-1 $\alpha$  in a mouse model of carotid artery ligation. *Arterioscler Thromb Vasc Biol* 2006, 26:765-772
- Shiba Y, Takahashi M, Yoshioka T, Yajima N, Morimoto H, Izawa A, Ise H, Hatake K, Motoyoshi K, Ikeda U: M-CSF accelerates neointimal formation in the early phase after vascular injury in mice: the critical role of the SDF-1-CXCR4 system. *Arterioscler Thromb Vasc Biol* 2007, 27:283-289
- Walter DH, Haendeler J, Reinhold J, Rochwalsky U, Seeger F, Honold J, Hoffmann J, Urbich C, Lehmann R, Arenzana-Seisdesdos F, Aicher A, Heeschen C, Fichtlscherer S, Zeiher AM, Dimmeler S: Impaired CXCR4 signaling contributes to the reduced neovascularization capacity of endothelial progenitor cells from patients with coronary artery disease. *Circ Res* 2005, 97:1142-1151
- Jin DK, Shido K, Kopp HG, Petit I, Shmelkov SV, Young LM, Hooper AT, Amano H, AVECILLA ST, Heissig B, Hattori K, Zhang F, Hicklin DJ, Wu Y, Zhu Z, Dunn A, Salari H, Werb Z, Hackett NR, Crystal RG, Lyden D, Rafii S: Cytokine-mediated deployment of SDF-1 induces neovascularization through recruitment of CXCR4<sup>+</sup> hemangiocytes. *Nat Med* 2006, 12:557-567
- Grunewald M, Avraham I, Dor Y, Bachar-Lustig E, Itin A, Jung S, Chimenti S, Landsman L, Abramovitch R, Keshet E: VEGF-induced adult neovascularization: recruitment, retention, and role of accessory cells. *Cell* 2006, 124:175-189
- Broxmeyer HE, Orschell CM, Clapp DW, Hangoc G, Cooper S, Plett PA, Liles WC, Li X, Graham-Evans B, Campbell TB, Calandra G, Bridger G, Dale DC, Srour EF: Rapid mobilization of murine and