Cardiovasc Thorac Ann, 2009, Itzhaki-Alfia et al. Circulation, 2009)、若齢、高血圧、女性、右房という4つの因子が心臓組織から得られる C-Kit 陽性細胞数の上昇に関与するという事が示唆されている。しかしながら、例えば、性差に関して2つの報告の結果が一致していない等、未だ共通の見解は見出されていないと考えられる。ちなみに、若齢、女性という因子に関しては、我々の今年度の結果と一致していないが、右房という因子に関しては一致している(上述)。何れにせよ、臨床応用を見据えると、この病態と心臓幹細胞の分離効率等に関する情報は重要であると考えられる事から、今後更なる検討が必要と考えられる。

最後に、心臓幹細胞シート作製条件の検討を行った。前年度までに最低播種細胞密度は大まかに検討できていたが、より高い播種細胞密度において細胞シートが作製可能かどうか検討を行った。その結果、3 ～ 9 x10^6 cells/3.5cm dish という条件で作製可能であると考えられた。その実験を行った際には、1.5 x10^6 cells/3.5cm dishという条件で失敗したが、別の機会には成功する事も確認された(データは示していない)。よって、今年度までの検討結果と、一般的により高い播種細胞密度において細胞シートは作製可能と考えられる事を考慮すると、心臓幹細胞を用いた細胞シートの作製条件は 1.5 x10^6 cells/3.5cm dish(1.7 x10^5 cells/cm^2)という条件以上において任意に定める事が可能と考えられた。但し、9.0 x10^6 cells/3.5cm dish 以外の条件では 17 時間の培養で浮遊していた事、また、接着細胞が浮遊状態になると生存率が悪化すると考えられる為、9.0 x10^6 cells/3.5cm dish 以外の条件では、播種後の培養時間を 17 時間よりも短くするか、FBS を用いての予備培養を30分よりも長く行う必要性が考えられた。臨床応用の際に重要な事は治療効果であるから、今後、上記条件で作製された細胞シートの治療効果に関する検討を行う必要があると考えられた。さらに、細胞シートの複層化や、用いる細胞の分化程度も併せて検討する必要があると考えられた。

上述の通り、今年度は、心臓幹細胞の分離・培養条件を詳細に検討し、ほぼ確立できた。現実的に臨床応用可能かどうかに関しては、今後詳細に検討する必要があるものの、現時点において最も効率的なヒト心臓幹細胞の分離・培養方法としては、『ヒト右房から筋肉組織を採取し、12 時間以内のコラゲナーゼ反応で細胞を分離した後、それらの細胞を 4 ～ 5 日間培養(APP0, APP0 pieces, APP0 o/n, APP0 o/n pieces)し、回収する。再び 10^4 cells/10cm dish, 4 days/passage という条件で複数回継代した後(P1～)、心臓幹(C-Kit 陽性)細胞を単離し、2 x10^4 cells/10cm dish, 5 days/passage という条件で培養する』という方法が考えられた。

E. 健康危険情報　特記なし

F. 研究発表

1) 論文発表　特記なし

2) 学会発表

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G. 知的財産権の出願・登録状況

1. 特許取得　特記なし。

2. 実用新案登録　特記なし。

3.その他　特記なし。

厚生労働科学研究費補助金（再生医療実用化研究事業）

分担研究報告書

心筋幹/前駆細胞シート移植による梗塞心修復機序の検討

分担研究者　清水　達也　東京女子医科大学　先端生命科学研究所　准教授

研究要旨；成体マウス心臓由来心筋前駆細胞シート移植による心筋梗塞マウス心機能改善効果が確認されている。この効果の一つの機序として、移植細胞の分泌する蛋白によるパラクリン効果について着目し検討を行った。心筋前駆細胞は可溶性 VCAM-1 を発現し、可溶性 VCAM-1 はその受容体である VLA-4 を介して、in vitro においては血管内皮細胞の管腔形成を促進し、また心筋細胞死を抑制した。また in vivo においては移植細胞の生着に関与し、細胞シート移植による心機能改善効果に寄与することが明らかとなった。

A. 研究目的

心筋梗塞モデルに対する、細胞シートを用いた心筋幹/前駆細胞移植の有用性及びその機序を明らかすることを主たる目的とする。昨年度までの検討により、心筋前駆細胞シートが移植は、心筋梗塞マウスの心機能改善効果を有すること、また移植した心筋前駆細胞の一部は心筋細胞に分化し、マウス心臓内の心筋細胞の約 5%を新たに創生することは明らかになっている。今年度は、細胞移植によるパラクリン効果を検討した。

B. 研究方法

同数の心筋前駆細胞、脂肪間葉系細胞を無血清培地で培養後、培養上清を回収し、サイトカイン抗体アレイにより分泌蛋白の差異を明らかにし、心筋前駆細胞により多く発現する蛋白を同定する。

心筋前駆細胞培養上清による血管新生促進効果を、血管内皮細胞の遊走、管腔構造促進能の評価により検討する。

心筋前駆細胞培養上清による心筋細胞保護効果を、過酸化水素添加後の新生仔ラット心筋細胞を用いて MTT assay により評価する。また培養上清による心筋細胞内シグナル伝達機構を明らかにする。

心筋前駆細胞培養上清による心筋前駆細胞自身の遊走促進効果について評価する。

心筋前駆細胞シート移植後に、心筋前駆細胞特異的分泌蛋白に対する抗体を投与し、細胞移植による心臓への効果と分泌蛋白との関連を明らかにする。

C. 研究結果

近年、細胞移植による梗塞心の心機能改善効果の一機序として、移植細胞の分泌する増殖因子の関与が報告されている。昨年までの検討により、心筋前駆細胞(CPC)も、対照として用いた脂肪間葉系細胞(ATMC)に比して、移植後の生着率が高いことより、CPC が分泌する因子を介して、血管新生、心筋保護はもちろんのこと、CPC 自身の生着率を向上させることで、その因子の発現は維持され、結果生着した CPC からの心筋細胞や血管への創生に寄与していると考えられる。そこで、CPC 及び ATMC の培養上清を用いて、サイトカイン抗体アレイを行い、CPC において発現の多い因子の同定を行った。その結果、CPC においては可溶性 VCAM-1 の発現が ATMC に比して多いことが確認された。

CPCの培養上清を添加することにより、血管内皮細胞の遊走及び管腔形成能が促進された。可溶性VCAM-1は、すでに血管内皮細胞の遊走を促進することが報告されている。そこでVCAM-1特異的miRNAベクターを作成後、CPCに遺伝子導入し、再度培養上清を回収し、血管内皮細胞に添加したところ、CPC培養上清による血管内皮細胞の遊走、管腔形成能促進効果が抑制されたことから、CPCは自身の分泌する可溶性VCAM-1により血管新生促進効果を示すことが明らかとなった。一方、CPC培養上清は、過酸化水素添加に伴う心筋細胞死を抑制し、またCPC自身の遊走も促進することが明らかとなった。

可溶性VCAM-1は、VLA-4(α4β1インテグリン)のリガンドであり、VLA-4の下流には、細胞生存、増殖、遊走に関連する蛋白であるAkt、ERK、p38MAPKが存在する。新生仔ラット心筋細胞に可溶性VCAM-1を添加するとAkt、ERK、p38MAPKのリン酸化が亢進した。またCPCの培養上清を添加すると、可溶性VCAM-1添加時と同様にAkt、ERK、p38MAPKのリン酸化亢進が確認され、この効果はVLA-4の中和抗体添加によって抑制された。CPC培養上清添加に伴う心筋細胞死抑制効果は、Akt阻害剤、ERK阻害剤、p38MAPK阻害剤いずれの添加においても消失したことから、CPC移植に伴うパラクリン効果として、CPCの分泌する可溶性VCAM-1/VLA-4シグナルを介する心筋細胞死抑制効果の関与が示唆された。CPCのp38MAPKは、通常の培養状態においても、リン酸化状態であることが確認されたが、このリン酸化は可溶性VCAM-1の添加により更に亢進し、一方VLA-4の中和抗体のみの添加により顕著にp38MAPKのリン酸化は抑制される。さらにCPCの遊走は、可溶性VCAM-1添加により促進されるが、p38MAPK阻害剤の添加によって有意に抑制されることから、CPCは、自身の分泌する可溶性VCAM-1によりオートクリン的にVLA-4を介してp38MAPKを活性化し自身の遊走能を促進している可能性が示唆された。

CPCシート移植後に、VLA-4の中和抗体の投与を行うと、移植細胞の生着が著しく減少し、血管新生の抑制と相まって、細胞シート移植に伴う心機能改善効果が消失することが明らかとなっている。以上よりCPCをシート移植することにより、心筋細胞、血管への分化も認められる一方、可溶性VCAM-1/VLA-4を介した血管新生、心筋細胞死抑制効果、CPC自身の遊走、生存促進効果により梗塞後心筋の修復が得られるものと考えられる。

D. 考察

今回の検討は、マウス心臓由来の**Sca-1**陽性心筋前駆細胞における検討であり、ヒト心筋幹細胞における機能は依然不明である。単層の細胞シート移植のみで左室全体の心筋細胞を補填することが物理的に難しく、ヒト心筋幹細胞移植においても、その効果におけるパラクリン効果の検討が必要と考えられる。

E. 結論

F. 健康危険情報　特記なし

G. 研究発表

1. 論文発表

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第 74 回日本循環器学会学術集会　2010 年 3 月 5 日
Circ. J. Vol74, Supple1, 72

H. 知的財産権の出願・登録状況

（予定を含む。）

1. 特許取得　特記なし。
2. 実用新案登録　特記なし。
3.その他　特記なし。

厚生労働科学研究費補助金（再生医療実用化研究事業）

分担研究報告書

心・血管系細胞ハイブリッドシート作成を目的とした心筋幹細胞由来血管内皮前駆細胞の分化増幅法の確立

分担研究者　増田　治史　東海大学　医学部基盤診療学系　再生医療科学　准教授

研究要旨；ヒト心筋細胞・血管内皮細胞ハイブリッド細胞シートによる心・血管再生療法の確立を目的として、心筋幹細胞由来血管内皮前駆細胞分化培養技術を確立する。本年は、心筋幹細胞由来血管内皮前駆細胞の分化増幅法の確立と至適化を行った。採取ヒト心筋幹細胞(心筋組織 c-kit+細胞)を単離・継代培養を行い、特定培養条件における血管内皮前駆細胞への分化促進が可能であった。次年度は、本培養系を用いて分化誘導された血管内皮前駆細胞と代表研究者により確立された心筋幹細胞由来心筋細胞シートとのハイブリッド心筋細胞シート移植による虚血心再生評価を in vivo にて行う。

A. 研究目的

ヒト心筋幹細胞(cardiac stem cell)由来血管内皮前駆細胞の分化培養法の確立を目的とした。

B. 研究方法

1)CSC の増幅培養

①採取ヒト心組織を初代培養し、c-kit+細胞を FACS sorting にて CSC として採取。

②昨年、研究代表者とともに確立した CSC 培養法における基本培地を用いて CSC を bFGF,erythropoietin 添加 Ham's F12 培地を用いて、HYCLONE 社、VITROMEX 社製 FBS にて増幅培養を行った。

2)増幅 CSC の血管内皮系細胞への分化培養法の検討

①FBS 濃度、coating の条件検討

6 well Primaria plate に 100% FBS コーティングまたは 5ug/cm2 ヒト組換え型 collagen type I (hrCI) コーティングを行い、各コーティング培養皿において、研究代表者が確立した CSC の心筋細胞への分化培養法の基本培地に基づき 1%, 2.5%, 5% FBS/L-glu/ α-MEM の培地を用いて hCSC からの内皮系細胞の分化能、増殖能を検討した。

②培養期間の検討

①の培養条件において培養期間(day0, 3, 7, 10)における培養細胞の FACS による内皮細胞抗原陽性率と Matrigel assay を用いて血管網形成数を評価した。

(倫理面への配慮)

東海大学における「医の倫理委員会」の承認を得た。

C. 研究結果

1)FBS 濃度、coating の条件の培養細胞数に与える影響(図1)；

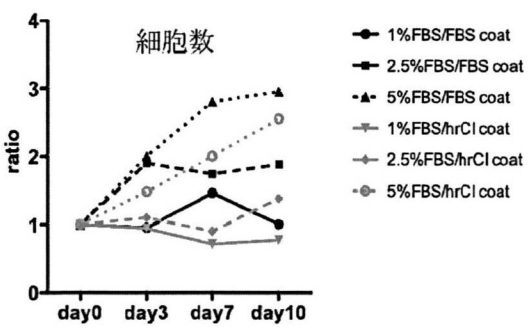

図1.FBS濃度及びコートの検討

①FBS、hrCI コーティングのいずれにおいても各培養期間において FBS 濃度依存性に細胞数の増加が認められた。

②d day0 に比較した場合、培養後細胞数が増加したの

はいずれのコーティングも5%FBSであった。

③day0に比較した場合、培養後1%FBSでは両コーティングにおいて細胞数は減少した。

④ day3ではhrCIコーティングの2.5%FBSが、FBSコーティングよりも細胞数が増加した。

⑤ day7, day10では2.5%及び5%FBSのいずれのFBS濃度でもFBSコーティングの方がhrCIコーティングに比較して細胞数が増加した。

2)FBS濃度、coatingの条件による血管内皮細胞分化能としての血管新生機能に与える影響(図2);

①血管網形成能ではday3において1%FBS以外、hrCIコーティングの方がFBSコーティングよりも優れていた。

②day7, day10ではday0またはhrCIに比較してFBSコーティングの方が、いずれのFBS濃度でも血管網形成能の増強が認められた。

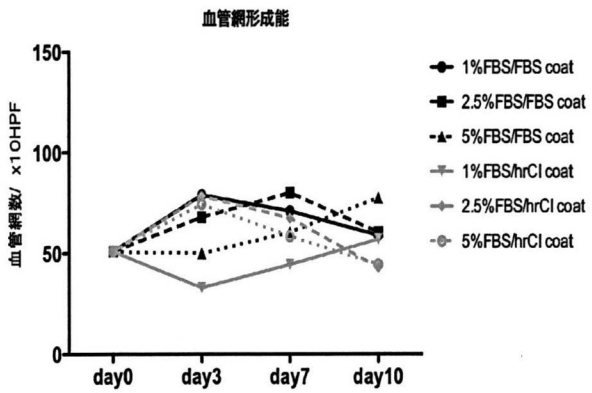

図2. 血管新生能の検討

3)FBS濃度、coatingの条件による血管内皮細胞抗原発現率(図2);

Day7での内皮系抗原(ckit, Tie2, CD31,CD34)のFACSではいずれのFBS濃度でもFBSコーティングの方がhrCIコーティングよりも陽性率が高値であった。

④FBSコーティングにおいてday7では2.5%FBS濃度が、day10では5%FBS濃度が血管網形成能が最高であった。

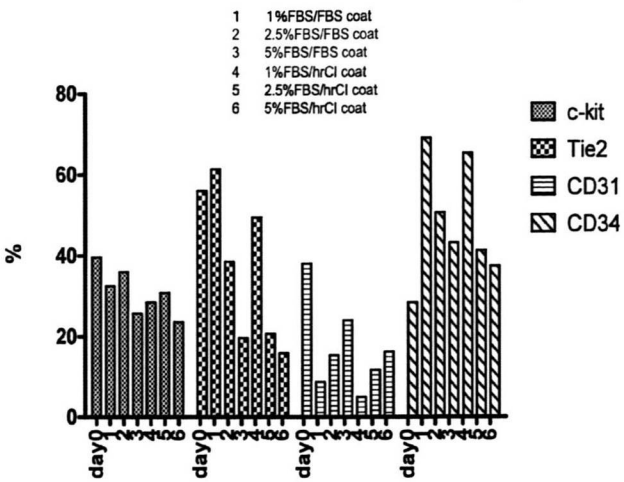

図3. FBS濃度及びコートによる内皮系抗原発現率

D. 考察

CSCから血管内皮系細胞の培養による分化誘導が可能であり、FBSコーティングにおいて2.5%FBS/7日間、5%FBS/10日間の培養条件が内皮増殖、分化能においてより良い培養環境を提供すると考えられた。

F. 健康危険情報　特記なし。

G. 研究発表

1. 論文発表

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H. 知的財産権の出願・登録状況

(予定を含む。)

1. 特許取得　特記なし。

2. 実用新案登録　特記なし。

3.その他　特記なし。

Ⅲ 研究成果の刊行に関する一覧表

原著論文

発表者氏名	論文タイトル	発表誌名	巻号	ページ	出版年
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Ⅳ　研究成果の刊行物・別刷

Research article

Transplantation of cardiac progenitor cells ameliorates cardiac dysfunction after myocardial infarction in mice

Katsuhisa Matsuura,[1,2] Atsushi Honda,[1] Toshio Nagai,[3] Noritoshi Fukushima,[1] Koji Iwanaga,[3] Masakuni Tokunaga,[3] Tatsuya Shimizu,[2] Teruo Okano,[2] Hiroshi Kasanuki,[1] Nobuhisa Hagiwara,[1] and Issei Komuro[3]

[1]Department of Cardiology and [2]Institute of Advanced Biomedical Engineering and Science, Tokyo Women's Medical University, Tokyo, Japan. [3]Department of Cardiovascular Science and Medicine, Chiba University Graduate School of Medicine, Chiba, Japan.

Cardiac progenitor cells are a potential source of cell therapy for heart failure. Although recent studies have shown that transplantation of cardiac stem/progenitor cells improves function of infarcted hearts, the precise mechanisms of the improvement in function remain poorly understood. The present study demonstrates that transplantation of sheets of clonally expanded stem cell antigen 1–positive (Sca-1–positive) cells (CPCs) ameliorates cardiac dysfunction after myocardial infarction in mice. CPC efficiently differentiated into cardiomyocytes and secreted various cytokines, including soluble VCAM-1 (sVCAM-1). Secreted sVCAM-1 induced migration of endothelial cells and CPCs and prevented cardiomyocyte death from oxidative stress through activation of Akt, ERK, and p38 MAPK. Treatment with antibodies specific for very late antigen-4 (VLA-4), a receptor of sVCAM-1, abolished the effects of CPC-derived conditioned medium on cardiomyocytes and CPCs in vitro and inhibited angiogenesis, CPC migration, and survival in vivo, which led to attenuation of improved cardiac function following transplantation of CPC sheets. These results suggest that CPC transplantation improves cardiac function after myocardial infarction through cardiomyocyte differentiation and paracrine mechanisms mediated via the sVCAM-1/VLA-4 signaling pathway.

Introduction

Accumulating evidence has suggested that myocardial regeneration is a promising therapy for various heart diseases (1). Recently, several groups, including our own, have reported that adult hearts contain cardiac stem/progenitor cells that can differentiate into functional cardiomyocytes in vitro and in vivo (2–6). Transplantation of cardiac stem/progenitor cells has been shown to improve cardiac function via newly formed cardiomyocytes and blood vessels (2, 7). On the other hand, it has been reported that when noncardiac stem cells are transplanted, paracrine factors play a major role in the improvement of cardiac function (8, 9). Several preclinical reports and clinical trials have demonstrated that intracoronary or intramyocardial injection of bone marrow–derived cells attenuates cardiac dysfunction following acute and chronic myocardial infarction (MI) (10–12). However, it is not known whether cardiac stem/progenitor cells are superior to other noncardiac stem/progenitor cells. Furthermore, it remains unclear to what extent paracrine effects or transdifferentiation of cardiac stem/progenitor cells contributes to beneficial effects on cardiac function.

Transplanted cells are the source of paracrine factors or newly formed cardiomyocytes, and the survival of grafted cells is a critical issue. The majority of the grafted cells have been reported to disappear within 1 week after transplantation when directly injected into ischemic hearts (9, 13), suggesting that alternative strategies to facilitate survival of grafted cells are required. We developed temperature-responsive culture dishes that were covalently grafted with the temperature-responsive polymer poly(*N*-isopropylacrylamide) (PIPAAm) (14). Lowering the temperature induces a rapid surface transition from hydrophobic (cell adhesive) to hydrophilic (non-cell adhesive), which results in the release of contiguous viable cell sheets with full preservation of cell-to-cell connections and adhesion proteins without using any enzymatic digestion (15). To date, cell sheet transplantations of skeletal myoblasts (16), mesenchymal stem cells derived from adipose tissue (17), and menstrual blood (18) have been reported to improve cardiac function in animal MI models. However, the precise mechanisms of the improvement, which include mutual interactions between host tissue and transplanted cells, remain poorly understood.

The present study demonstrates that transplanted cell sheets of clonally expanded stem cell antigen 1–positive (Sca-1–positive) cells (CPCs) differentiated into cardiomyocytes and vascular cells and prevented cardiac remodeling after MI. CPCs secreted soluble VCAM-1 (sVCAM-1), which facilitated engraftment and migration of CPCs from cell sheets into host myocardium and improved cardiac function after MI via angiogenic and cardioprotective effects mediated by paracrine mechanisms.

Results

Establishment and character of CPCs. Since primary isolated Sca-1–positive cells, which were derived from adult murine hearts, con-

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Nonstandard abbreviations used: ATMC, adipose tissue–derived mesenchymal cell; CM, conditioned medium; CPC, clonally expanded Sca-1–positive cell; FAK, focal adhesion kinase; FS, fractional shortening; IMDM, Iscove's Modified Dulbecco's Medium; LVDd, LV diastolic dimension; LVDs, LV systolic dimension; LVEDP, LV end-diastolic pressure; MI, myocardial infarction; miRNA, microRNA; RFP, red fluorescent protein; Sca-1, stem cell antigen 1; sVCAM-1, soluble VCAM-1; VLA-4, very late antigen-4.

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sisted of several cell populations, including cardiac stem/progenitor cells, hematopoietic cells, and endothelial cells (ref. 4 and our unpublished observations), clonal cells were initially established from cardiac Sca-1–positive cells. A total of 10^4 primary isolated Sca-1–positive cells derived from adult murine hearts were plated onto 10-cm culture dishes. After repeated limited dilutions, clonal cell lines were established. The efficiency of cloning was approximately 0.1%. The clonal cells were expanded for more than 500 population doublings (Figure 1A). Flow cytometric analysis revealed that almost 100% of cells expressed Sca-1, CD29, and CD44, approximately 20% of cells expressed CD34, and no cells expressed CD31, CD45, and c-kit throughout the culture passages (Table 1 and Supplemental Figure 1; supplemental material available online with this article; doi:10.1172/JCI37456DS1), suggesting that the clonal cells were not homogenous. Under culture conditions of 80% confluency, the clonal cells expressed cardiac transcription factors, such as Nkx2.5 and GATA4 (Figure 1B), but not cardiac contractile proteins. These cell phenotypes remained unchanged throughout the culture passages. Gene profiles and cell-surface marker analyses revealed that CPCs were similar to primary isolated Sca-1–positive cells in analyses previously reported (4). Therefore, CPCs possessed features almost identical to those of intrinsic cardiac stem/progenitor cells. When CPCs were cultured under confluent conditions for 4 weeks, expression levels of Nkx2.5 and GATA4 were upregulated and expressions of myocyte enhancer factor 2C (MEF2C), atrial natriuretic peptide (ANP), β-myosin heavy chain (β-MHC), and sarcomeric α-actinin were detected at mRNA and protein levels (Figure 1, B and C). These results suggested that CPCs differentiated into immature cardiomyocytes in vitro. However, the cells did not exhibit spontaneous beating. To examine the cardiac differentiation potency of CPCs in vivo, 2.0×10^6 CPCs labeled with red fluorescent protein (RFP) were injected directly into the infarcted myocardium within 5 minutes after left coronary artery ligation. At 4 weeks after transplantation, several GATA4-expressing RFP^+ cells were recognized in the border areas and some RFP^+ cells expressed sarcomeric α-actinin in a fine striated pattern, which suggested that CPCs differentiated into mature cardiomyocytes in vivo (Figure 1, D and E). However, transplanted CPC-derived RFP expression in the infarcted heart was very weak 1 week after transplantation compared with immediately after transplantation (Supplemental Figure 2). These results suggest that direct intramyocardial injection is not an ideal method for efficient engraftment.

Cell sheet transplantation. To elucidate the functional benefits of CPC transplantation in the infarcted heart compared with effects of noncardiac stem/progenitor cells, cell sheet transplantation methods were utilized. RFP^+ CPCs or adipose tissue–derived mesenchymal cells (ATMCs) isolated from GFP mice were cultured on temperature-responsive culture dishes at 37°C. The monolayered cell sheet was collected by decreasing the temperature at which it was cultured to 20°C. Final cell counts and areas of monolayered cell sheets prior to transplantation were $2.0 \pm 0.2 \times 10^6$ cells and 80.1 ± 3.3 mm² in CPCs and $1.9 \pm 0.1 \times 10^6$ cells and 79.3 ± 2.0 mm² in ATMCs, respectively ($n = 5$). The mice were randomly assigned into 3 groups: mice transplanted with monolayered CPCs (CPC group), mice transplanted with monolayered ATMCs (ATMC group), and mice that were not transplanted (MI group). Within 5 minutes after left coronary artery ligation, monolayered CPC or ATMC sheets were transplanted over the infarcted area and cardiac function was examined by echocardiography every week. Echocardiographic analysis revealed that LV diastolic dimension (LVDd) and LV systolic dimension (LVDs) were significantly decreased at 4 weeks and fractional shortening (FS) was markedly improved 3 weeks after transplantation in the CPC group compared with the MI and ATMC groups. These results suggested that transplantation of CPC sheets inhibited cardiac remodeling and improved cardiac function following MI (Figure 2A). Furthermore, LV end-diastolic pressure (LVEDP) and +dp/dt, as determined by catheter, were markedly improved in the CPC group compared with the remaining 2 groups at 4 weeks (Figure 2B). In contrast, in the ATMC group, LVDs was significantly smaller and FS was better 1 week after transplantation compared with the MI and CPC groups. However, these favorable effects were not observed 2 weeks after transplantation, and cardiac remodeling and dysfunction progressed in a manner similar to that in the MI group (Figure 2A). The fibrotic area, which was evaluated by Masson trichrome staining 4 weeks after transplantation, was significantly smaller in the CPC group compared with the other 2 groups (Figure 3A). At 1 week after transplantation, more vWF-positive blood vessels were observed in the border area of the ATMC group than in the MI and CPC groups (Figure 3B). At 4 weeks, a greater number of vessels were detected in the CPC group than in the MI and ATMC groups (Figure 3C). Furthermore, when lectin perfusion assay was performed at 4 weeks, more lectin-positive blood vessels were detected in the border area of the CPC group compared with the remaining 2 groups (Supplemental Figure 4). In contrast, there were few inflammatory cells in the border area of each group at 4 weeks (Supplemental Figure 5).

Cell survival and differentiation. Immunohistochemical analysis showed that many transplanted CPCs were present in the middle of the LV wall, including the normal and injured areas (normal area, 28.3 ± 9.7 cells/mm²; injured area, 235.9 ± 75.1 cells/mm²), after 4 weeks (Figure 4, A–C). This suggested that CPCs migrated from the epicardial cell sheet into the ventricular myocardium following transplantation. Conversely, GFP^+ ATMCs were not observed in the myocardium (data not shown). Western blot analysis, using Abs against fluorescent proteins, revealed that approximately 20% of transplanted CPCs remained 4 weeks after transplantation, whereas only approximately 0.8% of transplanted ATMCs remained (Supplemental Figure 3). Furthermore, approximately 30% of RFP^+ cells expressed sarcomeric α-actinin in a fine striated pattern, and some RFP^+ cells also formed blood vessel structures (Figure 4, D–F). Because cardiomyocytes have the ability to fuse with surrounding noncardiomyocytes (19), the possibility that CPCs acquired cardiomyogenic features following fusion with existing cardiomyocytes was examined. When RFP^+ CPC sheets were transplanted into hearts of GFP mice immediately following MI, approximately half of the α-actinin–expressing cells expressed GFP in injured areas 4 weeks after transplantation (Supplemental Figure 6, A, B, and E). In the normal areas, all of the α-actinin–expressing RFP^+ cells expressed GFP (Supplemental Figure 6, C–E). To further ensure the occurrence of cell fusion, sheets of nonlabeled CPCs derived from male mice were transplanted into the infarcted hearts of female mice. At 4 weeks after transplantation, cells exhibiting a fine striated pattern possessed 3 X chromosomes and a Y chromosome in the nucleus (Supplemental Figure 7). These findings suggested that CPCs differentiated into cardiomyocytes via cell fusion–dependent and –independent mechanisms. Because approximately 20% of transplanted CPCs remained at 4 weeks (Supplemental Figure 3), approximately

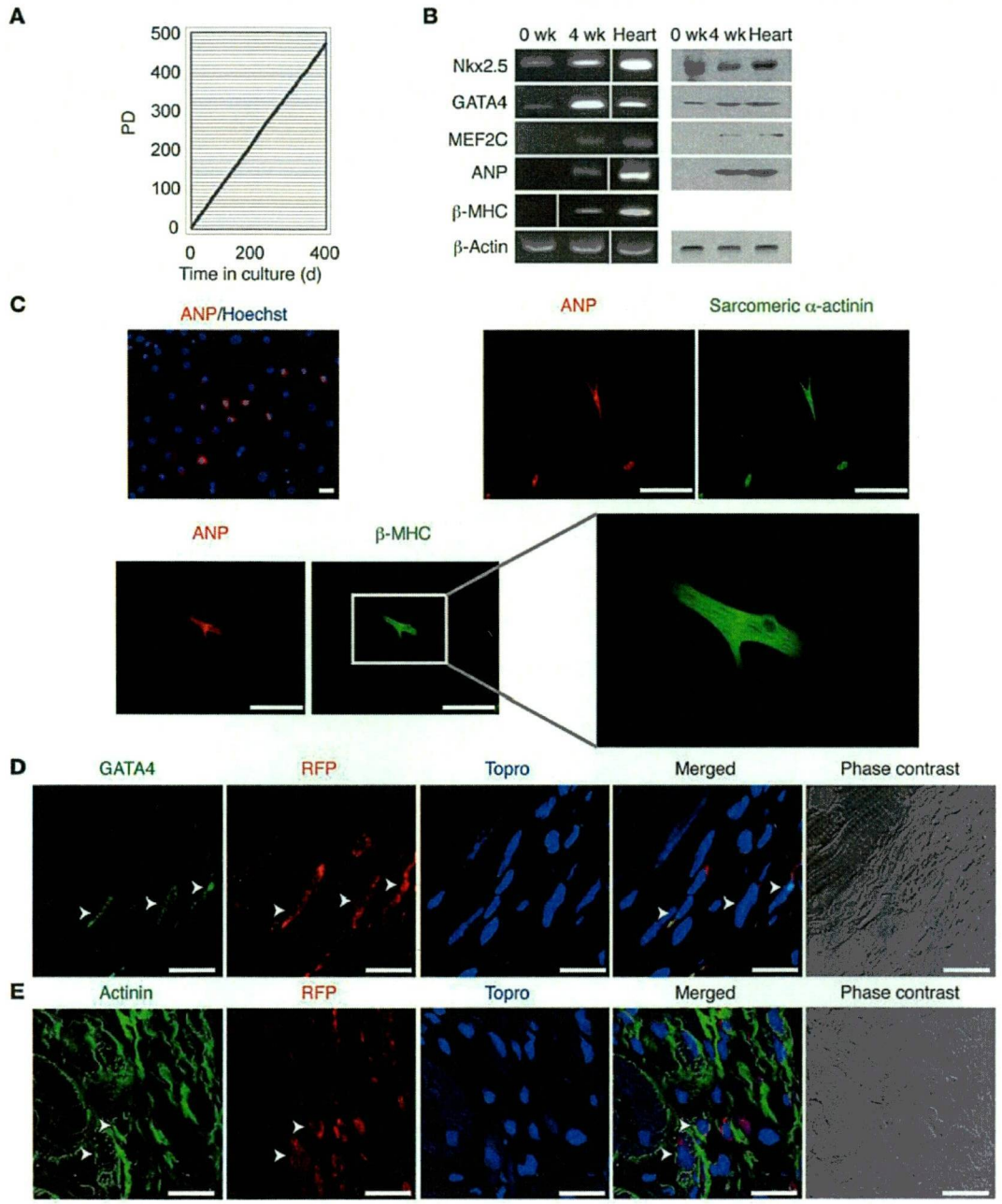


Figure 1
Character of CPCs. (**A**) CPCs were expanded more than 500 population doublings (P.D.) over a 1-year period. (**B** and **C**) Cardiac mRNA and protein expressions in CPCs. (**B**) Left panels show RT-PCR. Noncontiguous lanes from the same gel were spliced together into a composite band. The thin white line indicates the spliced point. Right panels show Western blot. (**C**) Immunofluorescent images of CPCs 4 weeks after starting culture under confluent conditions. Scale bars: 100 μm. (**D** and **E**) Confocal microscopic images of the infarcted heart 4 weeks after direct injection of RFP$^+$ CPCs. (**D**) GATA4-expressing RFP$^+$ cells (arrowheads) were recognized in the infarcted region. (**E**) Some RFP$^+$ cells (arrowheads) expressed sarcomeric α-actinin in the infarcted area. Scale bars: 5 μm.

4.0×10^5 out of approximately 2.0×10^6 transplanted CPCs were thought to survive and undergo engraftment. As approximately 30% of survived CPCs expressed cardiac contractile proteins (Figure 4, C and E), approximately 1.2×10^5 CPCs were estimated to differentiate into cardiomyocytes. Since approximately half of the cardiac protein-expressing cells resulted from cell fusion with

Table 1
The percentage of cell-surface antigens

	Sca-1	CD29	CD31	CD34	CD44	CD45	c-kit
P.D.10	99.8	99.9	0.1	15.6	100	0.5	0.4
P.D.100	95.5	99.7	0.3	42.8	100	0.2	0.3
P.D.200	99.8	100	0.1	45.5	100	0.1	0.1
P.D.300	99.8	99.9	0.2	15.5	99.9	0.4	0.2
P.D.400	100	100	0.3	27.5	100	0.3	0.4
P.D.500	99.9	99.9	0.1	15.4	100	0.5	0.5

existing cardiomyocytes (Supplemental Figure 6E), CPC sheet transplantation was estimated to create approximately 0.6×10^5 new cardiomyocytes in the entire heart. The number of cardiomyocytes in an adult murine heart has been estimated to be 3×10^6 (20). Therefore, approximately 5% of cardiomyocytes were regenerated and might have contributed to improved cardiac function by CPC sheet transplantation.

Secretion of growth factors. Recent reports have suggested that cell transplantation improves cardiac function after MI through the release of humoral factors (8, 9). Analyses of conditioned medium (CM) from CPCs and ATMCs using a cytokine Ab array revealed that sVCAM-1 was more abundant in CPCs, while VEGF was dominantly expressed in ATMCs (Table 2). Western blot analysis of whole-cell lysates and ELISA of CM confirmed altered expressions of VCAM-1/sVCAM-1 and VEGF between CPCs and ATMCs (Figure 5A). VCAM-1 expression was almost identical among the 3 groups at 1 week. However, at 4 weeks after transplantation, expression levels remained high in the CPC group compared with the other 2 groups (Figure 5B). Time course of VCAM-1 expression was consistent with improved cardiac function (Figure 2A). Conversely, VEGF expression was significantly upregulated at 1 week in the ATMC group compared with the other groups. However, at 4 weeks, expression levels were similar among the 3 groups (Figure 5B). This was consistent with observations that transplanted ATMC sheets improved cardiac function at 1 week but not at 4 weeks (Figure 2A). The concentrations of sVCAM-1 and VEGF in peripheral blood remained unchanged in all groups 1 and 4 weeks after transplantation (Supplemental Figure 8).

CPC-derived sVCAM-1–mediated angiogenesis and cardioprotective effects. CPC-derived CM and sVCAM-1 induced greater endothelial cell migration and tube formation compared with control medium (Figure 5, C and D). sVCAM-1–depleted CM, which was obtained from CPCs transfected with VCAM-1–specific microRNA (miRNA) plasmid vector (Supplemental Figure 9, A and B), induced significantly less endothelial cell migration (Figure 5C) and tube formation (Figure 5D) compared with CPC-derived CM. This suggested that angiogenic activity of CPC-derived CM was mediated at least in part by sVCAM-1. Subsequently, the protective

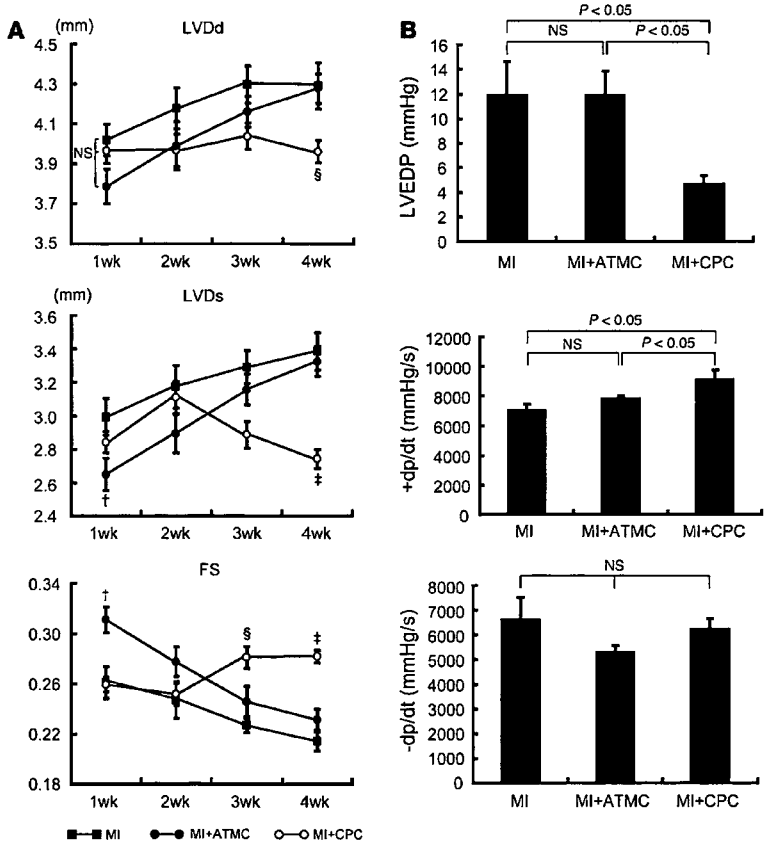


Figure 2
Effects of CPC sheet transplantation on cardiac function after MI. (**A**) Echocardiographic analysis. CPC sheet transplantation inhibited dilatation of LVDd and LVDs and improved FS 3 weeks later. ATMC transplantation inhibited dilatation of LVDs and FS reduction at 1 week, but not afterward. †$P < 0.05$ versus MI or MI plus CPCs ($n = 10$ per group). ‡$P < 0.01$ versus MI or MI plus ATMCs ($n = 10$ per group). §$P < 0.05$ versus MI or MI plus ATMCs ($n = 10$ per group). (**B**) Catheterization analysis at 4 weeks after transplantation. CPC sheet transplantation improved LVEDP and +dp/dt compared with that in the MI or MI plus ATMC groups ($n = 5$). Data are shown as mean ± SEM.

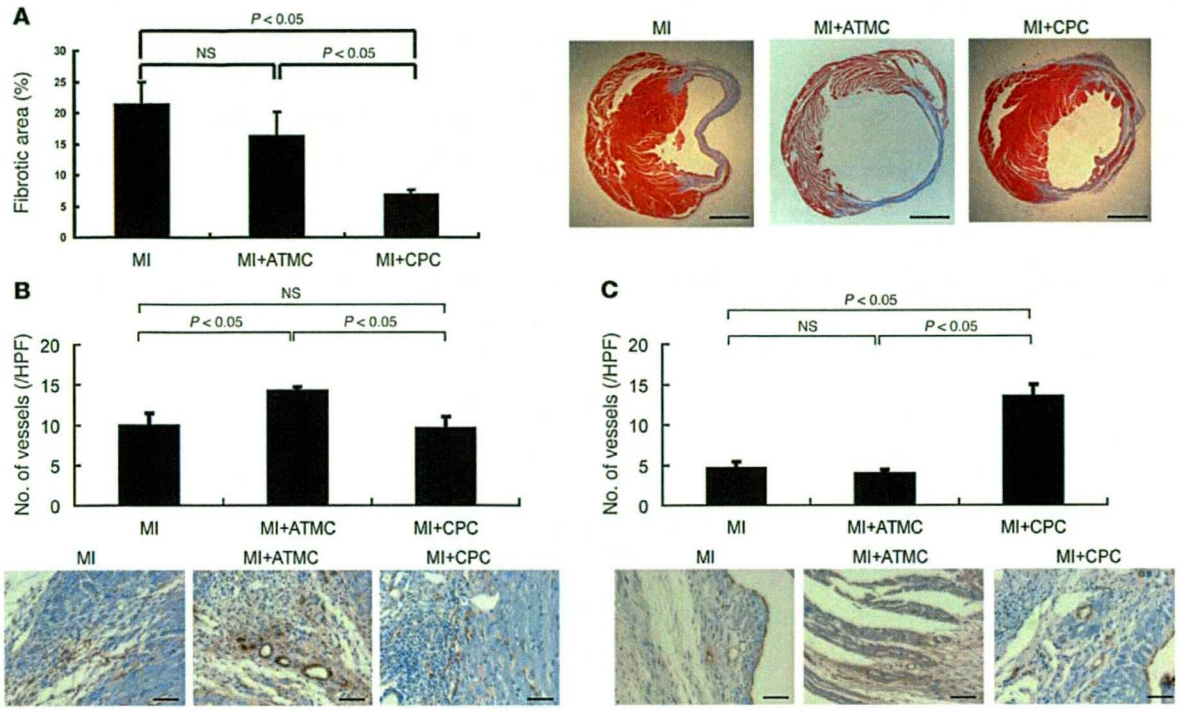


Figure 3

Immunohistochemical analysis of transplanted hearts. (**A**) Masson trichrome staining. The fibrotic area at 4 weeks after transplantation was calculated and is shown in the graph (n = 6). Lower panels show representative images. Scale bars: 1 mm. (**B** and **C**) Endothelial cells were identified by immunohistochemical staining with anti-vWF Ab in the border zone of the infarcted hearts 1 week (**B**) and 4 weeks (**C**) after transplantation. Lower panels show representative images. The vessel number was quantified and is depicted in the graph (n = 6). HPF, high-power field. Scale bars: 100 μm. Data are shown as mean + SEM.

effects of CPC-derived CM and sVCAM-1 on cardiomyocytes were analyzed. When cardiomyocytes were pretreated with CPC-derived CM or sVCAM-1, H_2O_2-induced damage of cardiomyocytes was significantly reduced (Figure 6A). The cardioprotective effects of CPC-derived CM were abolished by pretreatment of cardiomyocytes with Abs against very late antigen-4 (VLA-4, also known as $\alpha_4\beta_1$ integrin), a principal coreceptor of sVCAM-1 (Figure 6A), or sVCAM-1–depleted CM (Figure 6B). These results suggested a crucial role for sVCAM-1/VLA-4 in cardiomyocyte survival.

Integrin-mediated signals influence cardioprotective effects of sVCAM-1. Integrin-mediated signaling induces cell migration and survival by activating various kinases, such as focal adhesion kinase (FAK), Akt, ERK1/2, and p38 MAPK (21, 22). CPC-derived CM and sVCAM-1 induced phosphorylation and activation of FAK, Akt, ERK, and p38 MAPK in neonatal rat cardiomyocytes (Figure 6, C and E). When cardiomyocytes were pretreated with inhibitors of Akt, PI3K (wortmannin), p38 MAPK (SB203580), or ERK (PD98059), the cardioprotective effects of CPC-derived CM and sVCAM-1 were significantly inhibited (Figure 6, D and F). When cardiomyocytes were pretreated with anti–VLA-4 Abs prior to culturing in CPC-derived CM, phosphorylation of FAK, Akt, and ERK but not p38 MAPK was inhibited (Figure 6E). This suggests that the protective effects of CPC-derived CM on cardiomyocytes were achieved through sVCAM-1/VLA-4–mediated activation of Akt and ERK as well as VLA-4–independent activation of p38 MAPK.

sVCAM-1–induced migration of CPCs. Because a large number of transplanted CPCs migrated from the epicardial cell sheet to the ventricular myocardium following transplantation (Figure 4A), the effects of CPC-derived CM and sVCAM-1 on CPC migration were analyzed. When treated with CPC-derived CM or sVCAM-1, CPC migration was promoted, and anti–VLA-4 Abs or sVCAM-1 depletion markedly inhibited CM-induced migration of CPCs (Figure 6G). When CPCs were treated with sVCAM-1, phospho–p38 MAPK expression was significantly increased. However, expression of phospho-Akt and phospho-ERK remained unchanged. Phosphorylation of p38 MAPK was significantly inhibited by anti–VLA-4 Ab treatment (Figure 6H), which suggested that CPCs activated p38 MAPK through VLA-4. SB203580 inhibited CPC-derived CM- and sVCAM-1–induced CPC migration compared with the control (Figure 6I). These findings suggest that CPCs secreted sVCAM-1 and induced CPC migration via the VLA-4/p38 MAPK signaling pathway. Moreover, when VCAM-1 expression was downregulated, CPC viability was significantly decreased and apoptosis increased (Supplemental Figure 10), suggesting that VCAM-1 might be important for CPC survival.

VLA-4 signaling plays a crucial role in the beneficial effects of CPC sheet transplantation. The present findings suggest that CPC-secreted sVCAM-1 induced angiogenesis as well as CPC migration and survival and protected cardiomyocytes via VLA-4 in vitro. Subsequently, VLA-4 signaling was analyzed to determine its role in improved

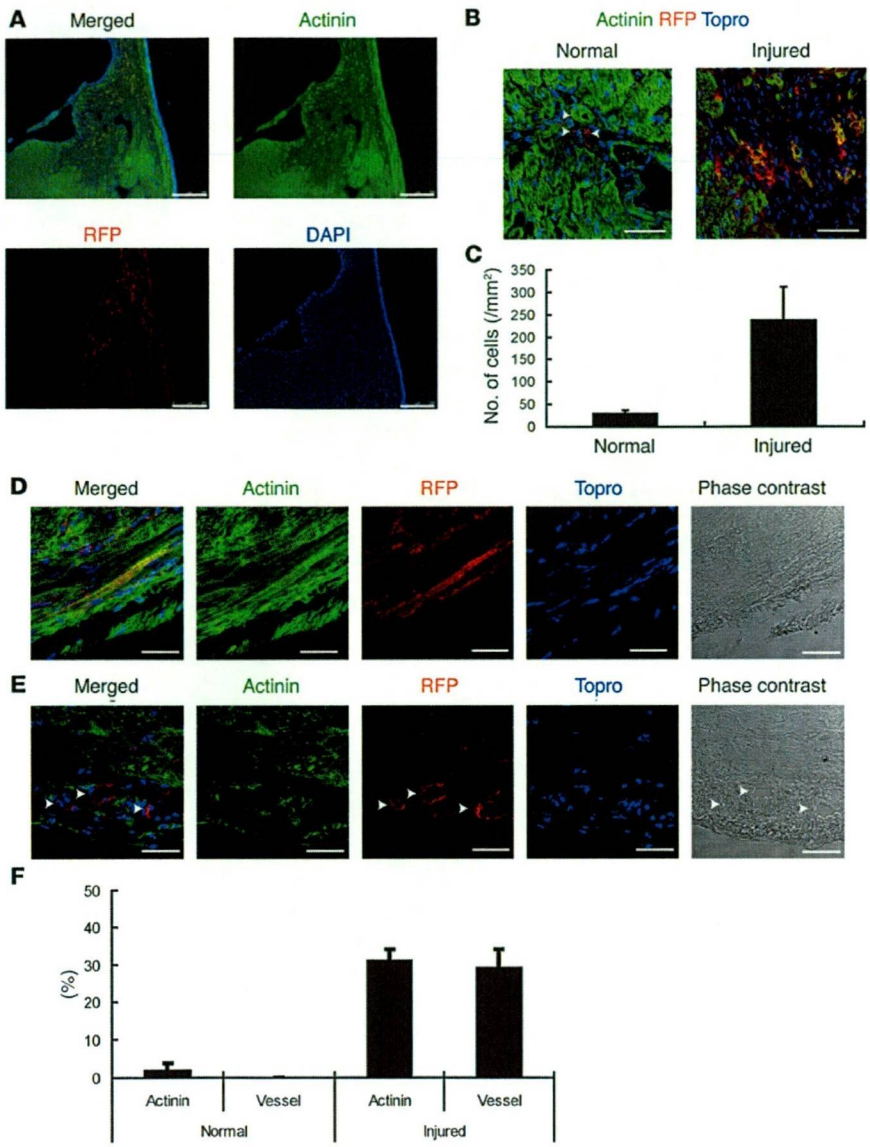


Figure 4
Cell survival and differentiation of transplanted cells. (**A**) Fluorescent microscopic images of infarcted heart 4 weeks after RFP$^+$ CPC sheet transplantation. Left sides of panels show endocardial area. Right sides of panels show epicardial area. Scale bars: 250 μm. (**B**) Confocal microscopic images of infarcted heart 4 weeks after CPC sheet transplantation (sarcomeric α-actinin, green; RFP, red; Topro, blue; yellow in merged images). Left panel shows normal area. Right panel shows injured area. Arrowheads indicate RFP$^+$ cells. Scale bars: 5 μm. (**C**) Number of RFP$^+$ cells were quantified and shown in the graph (n = 5). (**D** and **E**) Transplanted RFP$^+$ cells expressed sarcomeric α-actinin in a fine striated pattern (**D**) and formed vessel structures around α-actinin–positive myocardium (**E**). Nuclei were stained with Topro. Arrowheads indicate vessel structures. Scale bars: 5 μm. (**F**) Percentages of α-actinin–positive cells or vessel structure–forming cells in existing RFP$^+$ cells were calculated and shown in the graph (n = 5). Data are shown as mean + SEM.

cardiac function following transplantation of CPC sheets. Because echocardiographic analysis revealed improved cardiac function 3 weeks after transplantation (Figure 2A), i.p. injection of anti–VLA-4 Abs was performed daily from 2 to 3 weeks after CPC sheet transplantation. At 4 weeks after transplantation, injection of anti–VLA-4 Abs significantly attenuated the beneficial effects of CPC sheet transplantation on cardiac function, fibrosis, and angiogenesis (Figure 7, A–D). The number of RFP$^+$ CPCs in the infarcted area was also markedly decreased following treatment with anti–VLA-4 Abs (Figure 7E). In contrast, anti–VLA-4 Ab treatment did not affect cardiac function, fibrotic area, or blood vessel number in nontransplanted MI mice (Supplemental Figure 11). These findings suggest that CPC sheet transplantation improved cardiac function of infarcted hearts through VLA-4–mediated angiogenesis as well as survival and migration of transplanted CPCs.

Discussion

The present study reports that CPC sheet transplantation inhibited cardiac remodeling and restored cardiac function after MI by increasing the number of blood vessels and cardiomyocytes in the injured area. sVCAM-1 was identified as one of the dominant paracrine factors in CPCs and was shown to induce angiogenesis, cardioprotection, and CPC migration and survival through the VLA-4 signaling pathway. Therefore, sVCAM-1 plays a critical role in improved cardiac function following MI.

CPC transplantation restored cardiac function and angiogenic activity and prevented cardiac remodeling 4 weeks after transplantation. In contrast, ATMC transplantation attenuated cardiac dysfunction and enhanced angiogenesis transiently, and cardiac remodeling progressed at 4 weeks. These findings suggest varying cell survival rates (Supplemental Figure 3) and

Table 2
Results of cytokine Ab array

Cytokine	Fold increase
CPC	
VCAM-1	130.1
MIP-1γ	23.4
TIMP-1	8.0
IL-6	7.0
GM-CSF	6.0
IL-17	5.8
IL-5	5.3
KC	5.2
IFN-γ	5.2
IL-10	5.2
IL-12 p40/p70	4.8
IL-4	4.6
SDF-1α	4.5
IL-2	4.5
IL-12 p70	4.0
TNFα	3.9
MIP-3β	3.8
MIG	3.6
IL-9	3.1
MCP1	3.1
Osteopontin	2.1
ATMC	
MIP-1γ	135.7
KC	43.6
VCAM-1	22.4
RANTES	13.6
TIMP-1	11.9
IL-6	9.8
LIX	6.3
CXCL16	6.1
IL-17	5.6
GM-CSF	5.4
IL-2	4.8
IL-5	4.8
IL-4	4.3
IL-12 p70	4.2
IFN-γ	3.8
IL-10	3.6
IL-12 p40/p70	3.5
IL-9	3.4
Eotaxin	3.4
VEGF	3.2
TNFα	2.9
MCP1	2.9
MIP-3β	2.8
Osteopontin	2.5
MIG	2.4
CRG-2	2.2
SDF-1α	2.1

Each number indicates the fold increase of cytokine expression compared with the negative control. Serum-depleted medium was used as a negative control. SDF-1α, stromal cell–derived factor-1α; MIP-1γ, macrophage inflammatory protein-1γ; KC, keratinocyte-derived chemokine; TIMP-1 tissue inhibitor of metalloproteinase 1; LIX, LPS-induced chemokine; MCP1, monocyte chemotactic protein-1; MIG, monokine induced by gamma; CRT-2, cytokine-responsive gene-2.

distinct protein expression profiles (Figure 5B) in CPCs and ATMCs in the transplanted areas.

Through direct comparison of the protein expression profiles of CPCs and ATMCs, sVCAM-1 was identified as one of the predominantly expressed CPC-derived paracrine factors. VCAM-1, a 110-kDa transmembrane glycoprotein, is detected in various cells, including endothelial and bone marrow stromal cells (23). A soluble form of VCAM-1 has been reported to be shed from VCAM-1 on the cell surface by proteases, including TNF-α–converting enzyme (24). TNF-α–converting enzyme has also been reported to be elevated in the myocardium in heart failure (25) and to be required for fetal murine cardiac development and modeling (26). sVCAM-1 induces migration of endothelial cells through VLA-4 (21, 27, 28). The present study demonstrates that CM from VCAM-1–knocked-down CPCs did not induce endothelial migration, tube formation, cardioprotection, or CPC migration. Anti–VLA-4 Ab treatment abolished the protective effects of CPC-derived CM on cardiomyocytes and migration of CPCs and attenuated improved cardiac function following CPC sheet transplantation. This suggests that sVCAM-1 is a major paracrine factor of cardioprotection. VLA-4 is an integrin dimer that is composed of CD49d (α_4) and CD29 (β_1). Although studies have shown that the β_1 integrin signaling cascade regulates migration, differentiation, and death of various types of cells, such as endothelial cells, cardiomyocytes, and epidermal and hematopoietic stem cells (29–31), the role of sVCAM-1–mediated VLA-4 signaling in stem/progenitor cells remains elusive.

CPC-derived CM and sVCAM-1 phosphorylated several integrin-related downstream signaling molecules, such as Akt, ERK, and p38 MAPK, in cardiomyocytes. Consistent with previous studies, which indicated that Akt and ERK are critical for growth and survival of cardiomyocytes (32, 33), this study showed that promotion of cardiomyocyte survival by CPC-derived CM and sVCAM-1 was regulated through VLA-4–mediated activation of Akt and ERK. Furthermore, migration of CPCs, almost all of which expressed CD29 (β_1 integrin; Supplemental Figure 1), was facilitated via the VLA-4/p38 MAPK signaling pathway. VCAM-1–knockout mice and α_4 integrin–null mice have been shown to exhibit embryonic lethality, which was partly attributed to impaired epicardium formation surrounding the ventricular and atrial chambers, which suggests that VCAM-1/α_4 integrin signaling is critical for heart development (34, 35). In the present study, anti–VLA-4 Ab treatment reduced survival of transplanted CPCs. Furthermore, when VCAM-1 of CPCs was downregulated by specific miRNA, CPC viability was reduced and apoptosis was increased (Supplemental Figure 10), which suggests that VCAM-1–mediated signaling is also important for CPC survival. Since transplanted CPCs are the sources of not only paracrine factors but also newly formed cardiomyocytes, VCAM-1–mediated paracrine effects might also contribute to the cardiomyogenesis of CPCs through improved engraftment. A recent study has suggested that adult cardiac stem cells express α_4 and β_1 integrin in the niches (36). However, the role of α_4 and β_1 integrins is not fully understood. Since adult mammalian cardiomyocytes have been reported to be refreshed by endogenous stem cells after injury (37), our results suggest that sVCAM-1 secreted from transplanted CPCs promoted migration and self renewal of not only transplanted CPCs, but also endogenous cardiac stem/progenitor cells, through α_4 and β_1 integrin.

VCAM-1 is known as an inflammatory mediator, and increased sVCAM-1 in plasma has been reported following acute MI (38). During acute-phase MI, infiltrating leukocytes release cytokines,

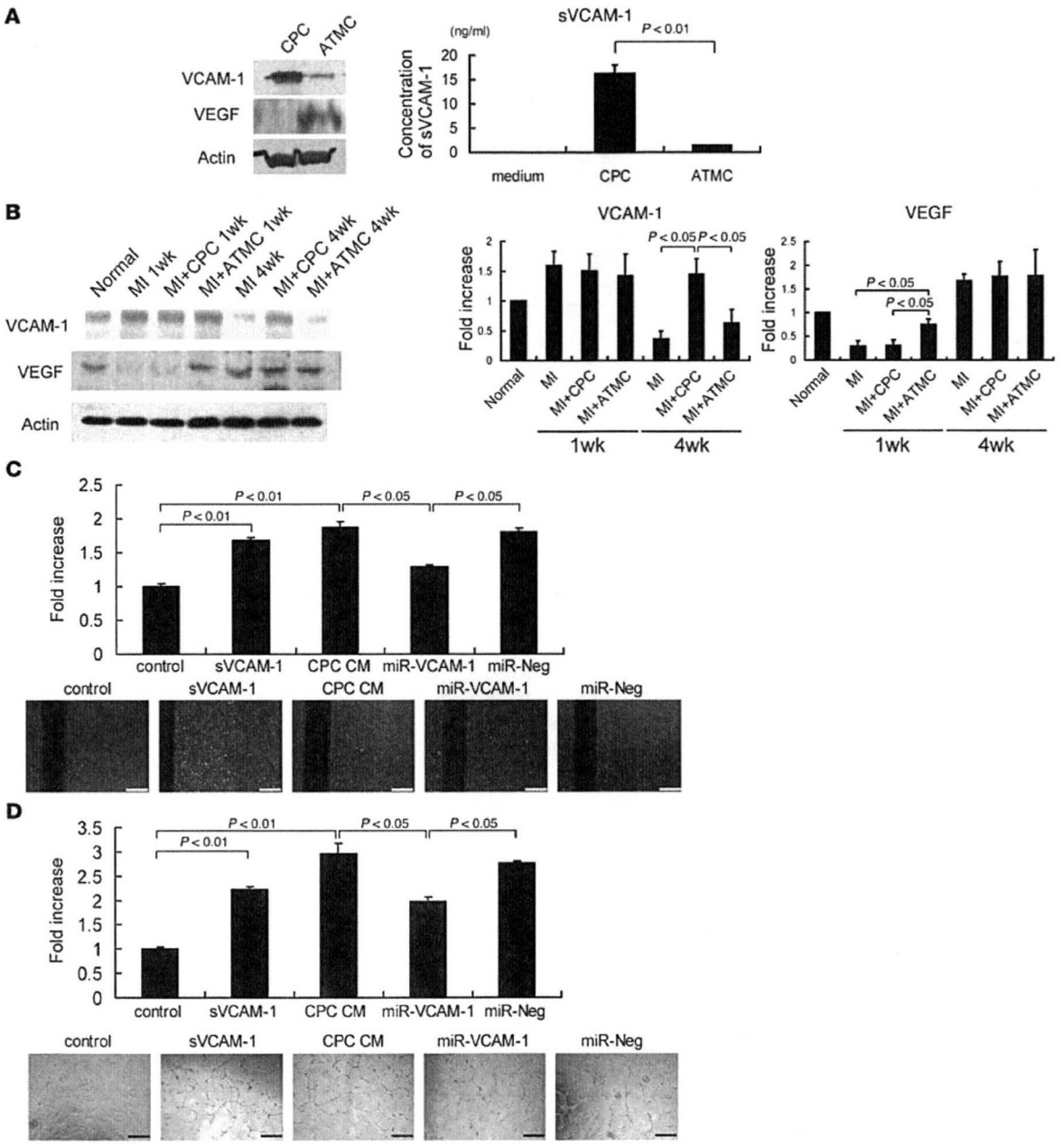
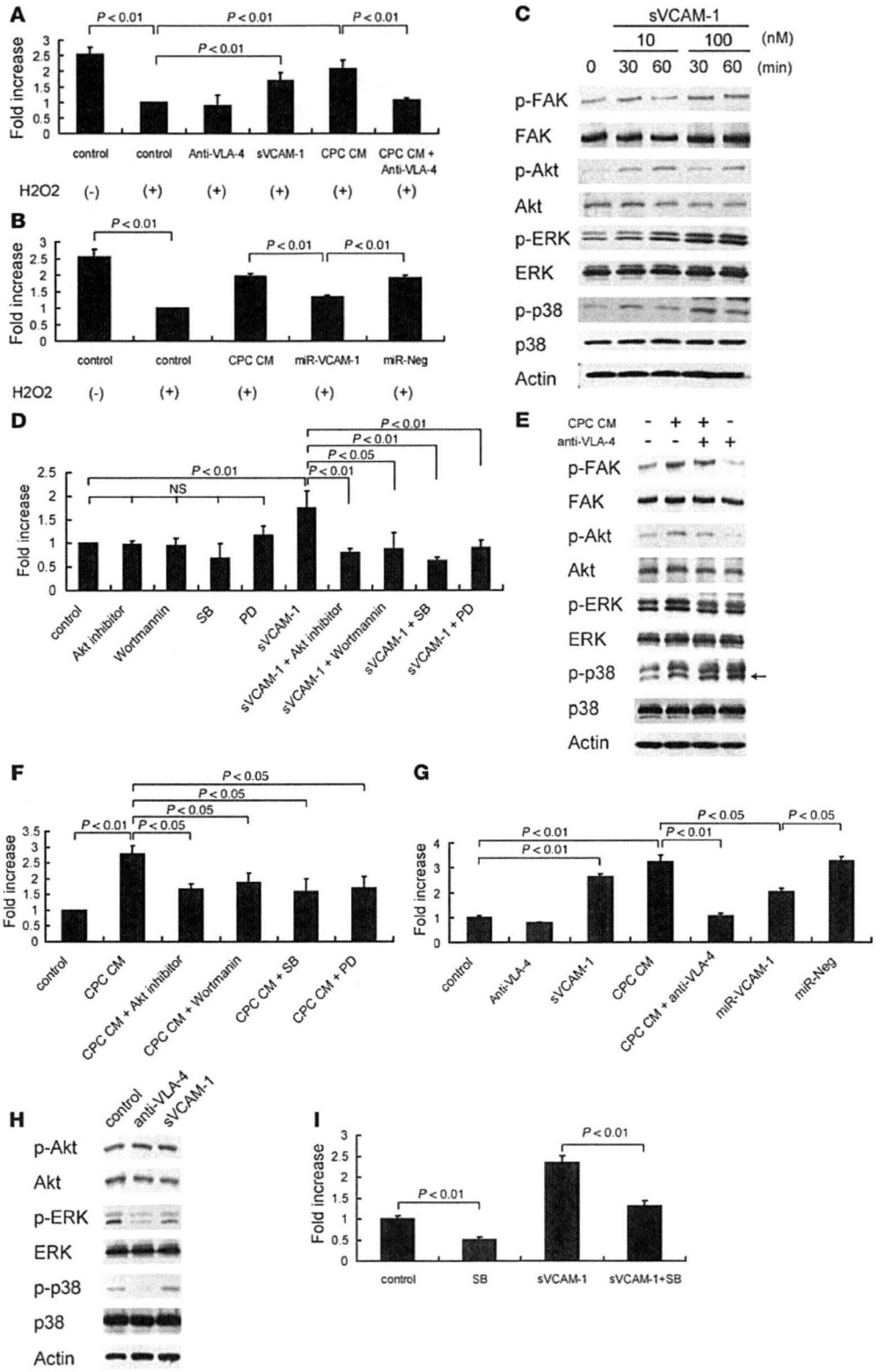


Figure 5

Secreted factor–mediated angiogenesis. (**A**) Left panel shows Western blot analysis results using whole-cell lysates of cultured CPCs and ATMCs. Right panel shows the results of sVCAM-1 ELISA using CM from cultured CPCs and ATMCs ($n = 3$). (**B**) Western blot analysis results of VCAM-1 and VEGF expression in heart after MI. Normal heart was used as a control. Left panel shows representative images. Right panels show quantification results of VCAM-1 and VEGF expression ($n = 3$). (**C**) Scratch-wound assay. CPC-derived CM enhanced endothelial migration ($n = 3$). Lower panels show representative images ($n = 3$). Scale bars: 500 μm. (**D**) CPC-derived CM enhanced endothelial tube formation. Tube length was quantified and is shown in the graph ($n = 3$). Lower panels show representative images. Scale bars: 500 μm. miR, miRNA. Data are shown as mean + SEM.

which activate VCAM-1 expression and promote leukocyte transmigration. In the present study, increased VCAM-1 expression in myocardium was observed in each group 1 week after MI/transplantation, when many inflammatory cells were also observed (data not shown). At 4 weeks, however, VCAM-1 expression remained upregulated in the CPC group despite few inflammatory


A
P < 0.01
P < 0.01
P < 0.01
P < 0.01
Fold increase
control
control
Anti-VLA-4
sVCAM-1
CPC CM
CPC CM + Anti-VLA-4
H2O2 (-) (+) (+) (+) (+) (+)
B
P < 0.01
P < 0.01
P < 0.01
Fold increase
control
control
CPC CM
miR-VCAM-1
miR-Neg
H2O2 (-) (+) (+) (+) (+)
C
sVCAM-1
10
100
(nM)
0 30 60 30 60 (min)
p-FAK
FAK
p-Akt
Akt
p-ERK
ERK
p-p38
p38
Actin
D
P < 0.01
P < 0.01
P < 0.05
P < 0.01
P < 0.01
NS
Fold increase
control
Akt inhibitor
Wortmannin
SB
PD
sVCAM-1
sVCAM-1 + Akt inhibitor
sVCAM-1 + Wortmannin
sVCAM-1 + SB
sVCAM-1 + PD
E
CPC CM - + + -
anti-VLA-4 - - + +
p-FAK
FAK
p-Akt
Akt
p-ERK
ERK
p-p38
p38
Actin
F
P < 0.05
P < 0.05
P < 0.05
P < 0.01
P < 0.05
Fold increase
control
CPC CM
CPC CM + Akt inhibitor
CPC CM + Wortmanin
CPC CM + SB
CPC CM + PD
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P < 0.05
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Fold increase
control
Anti-VLA-4
sVCAM-1
CPC CM
CPC CM + anti-VLA-4
miR-VCAM-1
miR-Neg
H
control
anti-VLA-4
sVCAM-1
p-Akt
Akt
p-ERK
ERK
p-p38
p38
Actin
I
P < 0.01
P < 0.01
Fold increase
control
SB
sVCAM-1
sVCAM-1+SB

Figure 6
sVCAM-1–mediated cardioprotective effects and CPC migration. (**A** and **B**) Cardiomyocyte viability following treatment with H_2O_2 was measured by MTT assay ($n = 3$). IgG isotype Abs were used as a control (**A**). (**C**) sVCAM-1 induced phosphorylation of FAK, Akt, ERK, and p38 MAPK in a dose-dependent manner. (**D**) Cardiomyocyte viability following treatment with H_2O_2 was measured by MTT assay ($n = 4$). SB, SB2035800; PD, PD98059. (**E**) CPC-derived CM induced phosphorylation of FAK, Akt, ERK, and p38 MAPK. Anti–VLA-4 Abs inhibited phosphorylation of FAK, Akt, and ERK induced by CPC-derived CM, but not phosphorylation of p38 MAPK. Arrow indicates appropriate size of phosphorylated p38 MAPK. (**F**) Cardiomyocyte viability following treatment with H_2O_2 was measured by MTT assay ($n = 4$). (**G** and **I**) CPC migration was measured using the scratch wound assay ($n = 3$). IgG isotype Ab was used as a control (**G**). (**H**) Anti–VLA-4 Abs inhibited phosphorylation of ERK and p38 MAPK of CPCs, but not Akt. Activity of p38 MAPK, but not Akt or ERK, was upregulated by sVCAM-1 treatment. Data are shown as mean + SEM.

cells in the infarcted area (Supplemental Figure 5). Furthermore, peripheral blood concentrations of sVCAM-1 were similar between groups 1 and 4 weeks after transplantation, which suggested that VCAM-1 expression in the transplanted heart was derived from CPC sheets, rather than circulating cells in the peripheral blood. VCAM-1 and its receptor, VLA-4, are important for fusion between hematopoietic progenitor cells and cardiomyocytes (39), and Oh et al. have reported that approximately 50% of cardiac protein–expressing transplanted cells arise from fusion with existing cardiomyocytes (3), which suggests that VCAM-1 mediates fusion between CPCs and dormant cardiomyocytes.

The present study compared transplanted cell survival between cell sheet transplantation and direct cell injection (Supplemental Figures 2 and 3). At 1 week after cell sheet transplantation, approximately 40% of cells survived (Supplemental Figure 3), while only 10% of cells survived after direct cell injection (Supplemental Figure 2). Immediately following transplantation, RFP expression in the heart was similar between cell sheet transplantation and direct cell injection, which suggested that the initial transplantation efficiency was the same. These findings indicate that cell sheet transplantation was superior to direct injection into the myocardium.

Many reports have demonstrated that endogenous cardiac stem/progenitor cells or bone marrow–derived cells mobilize to the infarcted area after injury and recruit additional cells through a feedback mechanism (40, 41). As shown in Table 2, CPCs expressed several chemokines, including stromal cell–derived factor-1 (SDF-1), which recruits bone marrow–derived cells to the infarcted myocardium (42). Therefore, CPC sheet transplantation may induce migration of bone marrow–derived cells to the infarcted heart, thereby improving cardiac function. Recently, anti-α_4 integrin Ab treatment was shown to improve cardiac function 2 weeks after MI by inhibiting interactions between bone marrow cells and their niches and promoting bone marrow cell migration and vasculogenesis (43). In the present study, anti–VLA-4 Ab treatment significantly attenuated improved cardiac function and angiogenesis following CPC sheet transplantation. Moreover, anti–VLA-4 Ab treatment did not affect cardiac function, fibrotic area, or blood vessel number in nontransplanted MI mice (Supplemental Figure 11). These findings suggest that bone marrow cells from their niches do not significantly contribute to the beneficial effects of CPC sheet transplantation.

There were a few limitations to the present study. The CPCs used in the experiments exhibited gene expression patterns similar to those of freshly isolated cardiac Sca-1–positive cells. However, profiles associated with proliferation, migration, and cardiomyocyte differentiation may not be the same. HUVECs were employed in endothelial migration and tube formation assays in vitro, and there might be differences between HUVECs and cardiac endothelial cells. Nevertheless, CPC sheet-mediated transplantation might be superior to the combination of other cell sources and tissue engineering methods. Improved survival of transplanted CPCs, in combination with several growth factors (44, 45), or multilayered cell sheets (46) might improve the beneficial effects of CPC sheet transplantation.

The present study identified the VCAM-1/VLA-4 signaling pathway as an important mechanism for CPC transplantation–mediated improved cardiac function. However, other paracrine factors, including thymosin β4 (47), have also been reported to contribute to cardiac repair following MI. Therefore, multiple mechanisms and mutual crosstalk might be involved in cell sheet transplantation to improve cardiac function. It remains to be determined which mechanisms are the most important and should be improved.

Methods

Animals. Wild-type mice (C57BL/6J) were purchased from Japan SLC. Adult GFP transgenic mice (C57BL/6J) were a gift from Masaru Okabe, Osaka University (Osaka, Japan). Neonatal Wistar rats (0 to 1 day old) were purchased from Saitama Experimental Animals Supply. All protocols were approved by the Institutional Animal Care and Use Committee of Tokyo Women's Medical University and Chiba University.

Reagents. FITC-conjugated anti-CD29, PE-conjugated anti-Sca-1, anti-CD44, and anti-c-kit Abs were purchased from eBioscience. PE-conjugated anti-CD31, anti-CD34, and anti-CD45 Abs were purchased from BD Biosciences – Pharmingen. The following Abs were used for immunostaining and Western blot analysis: mouse monoclonal anti-sarcomeric α-actinin, mouse monoclonal anti-β-actin (Sigma-Aldrich), rat monoclonal anti–VLA-4, mouse monoclonal anti-β-myosin heavy chain (anti-β-MHC) (Chemicon; Millipore), goat polyclonal anti-Nkx2.5, goat polyclonal anti-GATA4, rabbit polyclonal anti-atrial natriuretic peptide (anti-ANP), rabbit polyclonal anti-VEGF, goat polyclonal anti-Akt, rabbit polyclonal anti-FAK (Santa Cruz Biotechnology Inc.), rabbit polyclonal anti-GFP, rabbit polyclonal anti-RFP (MBL International Corp.), rabbit polyclonal anti-VCAM-1 (R&D Systems), rabbit polyclonal anti-vWF (Dako), rabbit polyclonal anti–myocyte enhancer factor 2C (anti-MEF2C), rabbit polyclonal anti-phospho Akt (Ser473), rabbit polyclonal anti-phospho p38 MAPK (Thr180/Tyr182), rabbit polyclonal anti-p38 MAPK, rabbit polyclonal anti-phospho ERK1/2, rabbit polyclonal anti-ERK1/2 (Cell Signaling Technology), and rabbit monoclonal anti-phospho FAK (Y397; Invitrogen). Secondary Abs were purchased from Jackson ImmunoResearch Laboratories Inc. Unless otherwise specified, reagents were purchased from Sigma-Aldrich.

Cell isolation. Sca-1–positive cells were isolated from an adult (10 weeks old) wild-type, male mouse, as described previously (4). Isolated cells (1×10^4) were harvested on a 10-cm Primaria dish (BD Falcon) in Iscove's Modified Dulbecco's Medium (IMDM) (Invitrogen) supplemented with 10% FBS and penicillin/streptomycin at 37°C in humid air with 5% CO_2. One month after starting culture, several colonies were recognizable. Each of these colonies was collected using a cloning cup and reseeding to a new 10-cm Primaria dish. After repeating this process 2 more times, a clonal cell line was established.

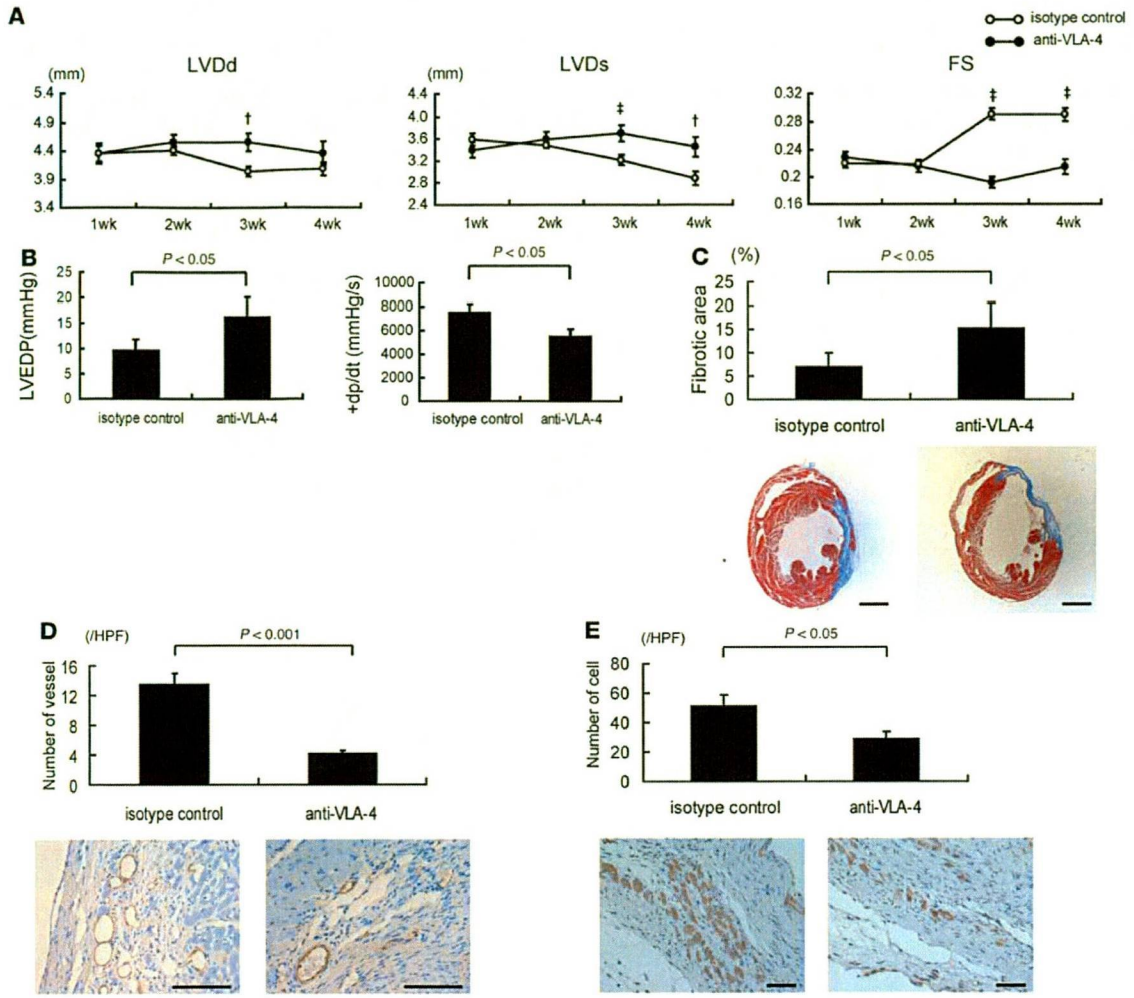

Figure 7

The roles of VLA-4 signaling on CPC sheet transplantation-mediated improved cardiac function. Analysis of cardiac function by echocardiography (**A**, $n = 5$) and catheterization (**B**, $n = 5$). Anti–VLA-4 Ab treatment inhibited the reduction of LVDd, LVDs, and LVEDP and the improvement of FS and +dp/dt by CPC sheet transplantation. Isotype Ab was used as a control. $^{\dagger}P < 0.05$ versus anti–VLA-4 Abs ($n = 5$ per group). $^{\ddagger}P < 0.01$ versus anti–VLA-4 Abs ($n = 5$ per group). (**C**) Masson trichrome staining. The fibrotic area 4 weeks after transplantation was calculated and is shown in the graph ($n = 5$). Anti–VLA-4 Ab treatment inhibited the reduction of fibrotic area following CPC sheet transplantation. Lower panels show representative images. Scale bars: 1 mm. (**D**) vWF staining. The number of vWF-positive vessels in the border area was counted and is shown in the graph ($n = 5$). Anti–VLA-4 Ab treatment inhibited the increased number of vessels in the border area following CPC sheet transplantation. Lower panels show representative images. Scale bars: 100 μm. Nuclei were stained with hematoxylin. (**E**) RFP staining. The number of RFP-positive cells (brown) was counted and is shown in the graph ($n = 5$). Anti–VLA-4 Ab treatment decreased the number of RFP$^+$ cells in the infarcted area following CPC sheet transplantation. Lower panels show representative images. Nuclei were stained with hematoxylin. Scale bars: 100 μm. Data are shown as mean + SEM.

ATMCs were isolated from GFP mice as previously described (48), with a few modifications. In brief, interscapular adipose tissues were digested at 37°C in PBS, which contained 2.5 mg/ml dispase (Invitrogen), for 45 minutes. After filtration through 25-μm filters and centrifugation, isolated ATMCs were suspended in IMDM supplemented with 10% FBS and penicillin/streptomycin/amphotericin B and cultured on 1% gelatin-coated dishes. ATMCs from passages 3–5 were used for cell sheets.

Neonatal rat cardiomyocytes were isolated as previously described (19). Cardiomyocytes were plated at a field density of 1×10^5 cells/cm^2 on 24-well culture dishes (BD Falcon) coated with 1% gelatin and cultured in DMEM supplemented with 10% FBS.

Labeling of cells. Retroviral stocks were generated as previously described (19). CPCs were infected with an RFP-expressing retroviral vector. Infected cells were selected for growth in the presence of neomycin (500 μg/ml) for 2 weeks. Transfection efficiency of RFP was greater than 95%.

Direct cell injection. Within 5 minutes after ligation of the left anterior descending artery, 2.0×10^6 RFP-labeled CPCs were directly injected into the infarcted regions of wild-type mice using a Hamilton syringe.