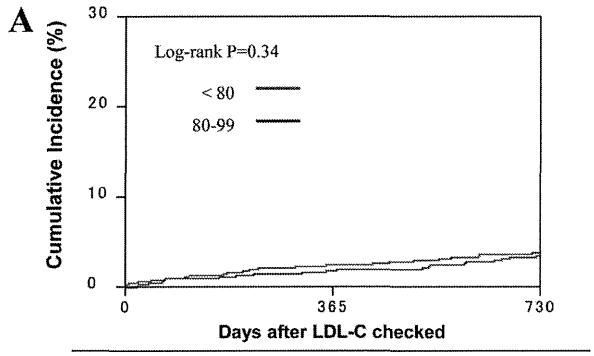
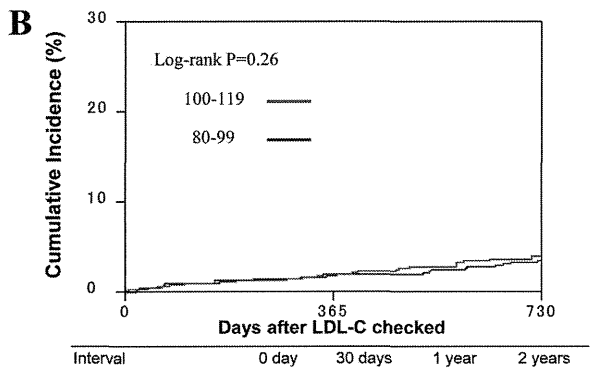
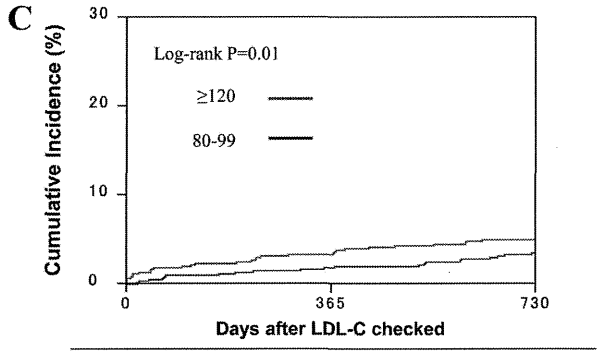


Interval	0 day	30 days	1 year	2 years
LDL-C < 80 mg/dl				
N of events		10	35	44
N of patients at risk	1458	1414	1067	499
Incidence		0.7%	2.6%	3.8%
LDL-C 80-99 mg/dl				
N of events		5	25	37
N of patients at risk	1429	1399	1122	543
Incidence		0.4%	1.9%	3.5%



Interval	0 day	30 days	1 year	2 years
LDL-C 100-119 mg/dl				
N of events		4	18	29
N of patients at risk	970	939	767	370
Incidence		0.4%	2.0%	4.0%
LDL-C 80-99 mg/dl				
N of events		5	25	37
N of patients at risk	1429	1399	1122	543
Incidence		0.4%	1.9%	3.5%



Interval	0 day	30 days	1 year	2 years
LDL-C ≥ 120 mg/dl				
N of events		11	26	35
N of patients at risk	810	776	627	356
Incidence		1.4%	3.4%	5.0%
LDL-C 80-99 mg/dl				
N of events		5	25	37
N of patients at risk	1429	1399	1122	543
Incidence		0.4%	1.9%	3.5%

Figure 3. Incidence of major adverse cardiovascular events (MACE: composite of cardiovascular death, myocardial infarction and stoke) according to the achieved low-density lipoprotein cholesterol (LDL-C) levels. (**A**) Achieved LDL-C <80 mg/dl vs. 80–99 mg/dl, (**B**) achieved LDL-C 100–119 mg/dl vs. 80–99 mg/dl, and (**C**) achieved LDL-C ≥120 mg/dl vs. 80–99 mg/dl.

and target of proximal left anterior descending artery were more prevalent in the achieved LDL-C <80 mg/dl group. Strong statins were more often prescribed in the groups with lower achieved LDL-C level. Baseline levels of total cholesterol and LDL-C were significantly different among the groups stratified by achieved LDL-C level (**Table 3**).

Absolute change in LDL-C level and % LDL-C change were greatest in the achieved LDL-C <80 mg/dl group. In the highest achieved LDL-C category, there was no significant change in LDL-C level from baseline to follow-up (**Table 3**). During the 2-year follow-up after measurement of the on-treatment LDL-C level, the incidence of MACE in the achieved LDL-C <80 mg/dl and 100–119 mg/dl groups was comparable with that in the reference group (achieved LDL-C 80–99 mg/dl) (**Tables 4,5**; **Figure 3A,B**). The incidence of MACE was significantly higher in the achieved LDL-C ≥120 mg/dl group than in the reference group (**Tables 4,5**; **Figure 3C**). After adjusting confounders by multivariable analysis, both the achieved LDL-C <80 mg/dl and 100–119 mg/dl groups had a risk for MACE comparable to that of the reference group (HR 1.15 [95%CI 0.75–1.75], P=0.52, and HR 1.23 [95%CI 0.78–1.94], P=0.38, respectively). An achieved LDL-C level ≥120 mg/dl was independently associated with a higher risk for MACE compared with an achieved LDL-C level of 80–99 mg/dl (HR 1.74 [95%CI 1.11–2.71], P=0.01) (**Table 5**).

The analysis of the association between the achieved LDL-C level and the incidence of MACE were also performed in the strong and standard statin groups. Median % LDL-C change in the <80 mg/dl, 80–99 mg/dl, 100–119 mg/dl and ≥120 mg/dl groups was −42.5%, −30.6%, −15.5% and 1.1% with strong statins and −35.0%, −17.6%, −9.9% and 7.1% with standard statins (**Table S2**). There were no significant differences in the incidence of MACE among those in the strong statin group (≥120 mg/dl 3.8%, log-rank P=0.29; 100–119 mg/dl 4.0%, log-

rank P=0.32; <80 mg/dl 3.8%, log-rank P=0.81 vs. 80–99 mg/dl 3.7%). Within the standard statin group, the incidence of MACE was significantly higher in the ≥120 mg/dl group compared with the 80–99 mg/dl group (6.3% vs. 3.1%, log-rank P=0.009), but was comparable in the 100–119 mg/dl group (3.9% vs. 3.1%, log-rank P=0.51) and <80 mg/dl group (4.0% vs. 3.1%, log-rank P=0.15).

To more directly evaluate the association of LDL-C level achieved with statin therapy and the clinical outcome, the incidence of MACE was compared among statin-naïve patients with different achieved LDL-C levels. The results indicated no significant difference in the incidence of MACE between each achieved LDL-C group and the reference group (≥120 mg/dl 4.7%, log-rank P=0.94; 100–119 mg/dl 1.6%, log-rank P=0.09; <80 mg/dl 3.9%, log-rank P=0.7 vs. 80–99 mg/dl reference group 4.2%).

Discussion

The main findings of the current study are as follows. (1) Treatment with strong statins was associated with a trend toward lower cardiovascular risk compared with treatment with standard statins. (2) The differences in the achieved LDL-C level during statin therapy in the range of LDL-C <120 mg/dl were not associated with significant differences in cardiovascular outcome. (3) An achieved LDL-C level ≥120 mg/dl was associated with a higher risk for cardiovascular events as compared with an achieved LDL-C 80–99 mg/dl.

In the current analysis, which included a large number of stable Japanese CAD patients without prior MI, the effect of strong statins relative to standard statins on cardiovascular outcome was in the same direction and of almost same magnitude as the effects seen in the PROVE-IT[6] and IDEAL[7] trials. Although the effect was statistically non-significant, mainly because of the low rate of cardiovascular events,[17] a 13% risk reduction seems to be clinically meaningful considering the relatively short follow-up period (approximately 3 years) and choice of active control group.

The achieved LDL-C level was significantly lower in the strong statin group compared with the standard statin group, which could be a reason for the lower risk for MACE in the strong statin group. However, the absolute difference in mean achieved LDL-C level between the strong and standard statin groups was relatively small. Effects of strong statins beyond LDL-C lowering could be considered as a possible explanation for the cardiovascular risk reduction in the strong statin group. Known as pleiotropic effects, statins have been reported to have antiinflammatory, antithrombotic and antioxidative effects, and to improve endothelial function.[3,18–20] In the subanalysis of the REVERSAL trial, which compared the percent change in atheroma volume between atorvastatin 80 mg and pravastatin 40 mg, less increase in atheroma volume was observed in the atorvastatin group even with the same percent LDL-C reduction.[21] In the JAPAN-ACS study comparing atorvastatin and pitavastatin in patients with acute coronary syndrome, a marked reduction in the plaque volume was observed in both groups with 1-year treatment with strong statins. The result was more prominent in non-diabetic patients.[22,23] However, there was no correlation between the on-treatment LDL-C level and the degree of reduction in plaque volume in non-diabetic patients, suggesting the presence of mechanisms other than LDL-C lowering in plaque regression. Considering the potential role of the pleiotropic effects of statins in vascular protection, we should further explore whether clinical outcomes differ according to the types of statins with the same or different LDL-C lowering effects. Effects of statins on metabolic homeostasis also differ among statins, which should be considered when the distinctive effects of statins on cardiovascular outcomes are evaluated.[24]

It is obvious that statin therapy with its LDL-C lowering effect provides clinical benefits in the secondary prevention for CAD not only in Western populations but also in the Japanese population,[25,26] which was also suggested in this study. Furthermore, administration of an increased dose of statin with its further LDL-C lowering effect was reported to improve cardiovascular outcomes in the TNT study.[9] Based on the results from the TNT and other randomized controlled studies, "the lower, the better" hypothesis has been widely advocated with regard to the optimal on-treatment LDL-C level in patients with CAD. However, it has not been proven yet whether the lower level of LDL-C itself was the predominant mechanism of the better outcomes in the atorvastatin 80 mg group in the TNT study.

In the current analysis, the differences in the achieved LDL-C level during statin therapy in the range of LDL-C <120 mg/dl were not associated with significant differences in cardiovascular outcome, suggesting that the on-treatment LDL-C level itself might not critically influence the risk for cardiovascular events. The analysis in statin-naïve patients revealed no significant difference in the incidence of MACE between each achieved LDL-C group and the reference group: there was not a significant correlation between achieved LDL-C level and a patient's outcome assessed by MACE. Detailed analysis of the cardiovascular outcomes in each achieved LDL-C group in the strong statin and standard statin groups revealed that the LDL-C ≥120 mg/dl group only had a significantly higher incidence of MACE than the LDL-C 80–99 mg/dl reference group in the standard statin group, but the incidence of MACE was comparable between the LDL-C 80–99 mg/dl reference group and any of the 3 other subgroups in the strong statin group. The % reduction in LDL-C level showed a clear relationship with the achieved LDL-C level within each statin therapy group. The average LDL-C level increased 7.1% from baseline in the LDL-C ≥120 mg/dl group in the standard statin group, whereas the achieved LDL-C was similar (1.1% increase) to the baseline level in the strong statin group. Possible causes of LDL-C increase during follow-up may include poor response and insufficient adherence to statin therapy. These findings suggest that an increase in LDL-C level during statin therapy might be associated with adverse outcomes, although the extent of LDL-C reduction may not have a strong effect on the extent of risk reduction. It also might be possible that the greater pleiotropic effects of the strong statins influenced the risk reduction even in the LDL-C ≥120 mg/dl group with no LDL-C reduction. Results from 2 other Japanese studies were consistent with our result, although the on-treatment LDL-C levels were much higher in those studies than in our study.[10,11] In contrast, in the post-hoc analyses of the PROVE-IT and TNT trials, patients with lower on-treatment LDL-C had significantly better cardiovascular outcoms.[12,13] The apparent discordance of these 2 studies with the Japanese studies might reflect differences in ethnicity. Although we could not fully explain the discordance among the studies, further investigations are obviously warranted to clarify the relation between on-treatment LDL-C level and cardiovascular outcome.

Significant associations between higher total cholesterol and LDL-C levels and a higher incidence of cardiovascular events have been repeatedly shown.[27–29] The current study also indicated that LDL-C ≥120 mg/dl was associated with significantly higher cardiovascular risk than LDL-C 80–99 mg/dl. However,

we do not currently know the specific threshold level of LDL-C above which the high level of on-treatment LDL-C level itself can be an independent risk factor for more cardiovascular events.

Current guidelines for lipid-lowering therapy are generally based on "the target LDL-C level". In the setting of secondary prevention, intensive LDL-C lowering therapy with a goal of LDL-C <70 mg/dl is recommended for very high-risk patients with CAD, based on the evidence from randomized controlled trials in Western countries.[30] It is also recommended in the Japanese guidelines to control LDL-C <100 mg/dl as secondary prevention.[31] However, the current analysis suggests that relatively higher LDL-C levels within the range of <120 mg/dl might not be a risk for more cardiovascular events. Therefore, more intensive LDL-C lowering could not be recommended based solely on the relatively high on-treatment LDL-C level. Thus, "the lower the better" may not be always applicable, but "make it lower with statins" should be always addressed in secondary prevention, even in relatively low-risk patients, including Japanese. A similar conclusion on the significance of statin therapy in secondary prevention in Japanese patients has been drawn in a previous review of Japanese statin studies by Sakamoto and Ogawa.[32] Additional LDL-C reduction with increased doses of statins might be considered in a subgroup of patients with very high risk for CAD, although we do not have data supporting the benefit of higher doses relative to standard doses of statins in reducing cardiovascular events in Japanese CAD patients. A large prospective multicenter randomized controlled trial is ongoing to address the optimal dose of statins in Japanese patients (Randomized Evaluation of Aggressive or Moderate Lipid Lowering Therapy with Pitavastatin in Coronary Artery Disease [REAL-CAD]: Clinical Trials gov. no. NCT01042730).

Study Limitations

This study was an observational study and has the limitations inherent to such studies caused by differences among groups in the patients' background characteristics. As the information about medical therapy was obtained only at hospital discharge, the adherence of the patients to the medications and the crossover of medications have not been considered in this study. The statin-treated patients included both statin-naïve patients and patients being treated with statins before the index hospitalization. Therefore, the statin-treated patients without LDL-C reduction at follow-up may include patients with poor adherence to medical therapies as well as patients who required coronary revascularization despite primary preventive statin therapy. Such patients possibly have a higher risk for MACE, and might be often included in the category of highest achieved LDL-C level. We could not assess the relationships between pre-revascularization medications or adherence to medication and the clinical outcomes. The strong statin group as well as the standard statin group included 3 different statins at different doses. We could not compare the efficacy of different statins at fixed doses in a head-to-head fashion. In addition, the detailed analysis of cardiovascular outcomes according to the achieved LDL-C in each statin therapy group might not have sufficient statistical power, because of the relatively small number of subjects in each subgroup.

In the current study, which focused on the association of LDL-C levels during statin therapy and Japanese patients' outcomes after coronary revascularization, we did not evaluate the association of HDL-C levels and the outcomes in detail. Because a low HDL-C level is associated with the occurrence of cardiac events regardless of the LDL-C level,[33] the HDL-C level should be also considered when an association between an intervention that modifies the lipid profile and the patients' outcomes after coronary revascularization is assessed.

The current study included patients treated by either PCI or CABG. The effect of statins on cardiovascular outcomes might differ according to the type of coronary revascularization. Finally, the doses of the statins and the cardiovascular event rates in the current study were much lower than those in the clinical trials conducted outside Japan. Extrapolation of the results of this study outside Japan must be done very carefully.

Conclusions

The present observational study demonstrated that strong statin therapy was associated with a statistically non-significant trend toward lower cardiovascular risk compared with standard statin therapy in Japanese patients undergoing coronary revascularization. When LDL-C <120 mg/dl was achieved in statin-treated patients, the risk for cardiovascular events was comparable irrespective of the achieved LDL-C level, although LDL-C ≥120 mg/dl was associated with a higher risk for cardiovascular events. Randomized prospective trials that aim to set the adequate therapeutic doses of strong statins for cardiovascular secondary prevention are needed, particularly in Asian populations.

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Disclosures

Takeshi Kimura serves as an advisory board member for Cordis Cardiology, Abbott Vascular and Terumo Company. The remaining authors reported no conflicts of interest.

Sources of Funding

Pharmaceuticals and Medical Devices Agency (PMDA) in Japan.

References

1. Shepherd J, Cobbe SM, Ford I, Isles CG, Lorimer AR, MacFarlane PW, et al. Prevention of coronary heart disease with pravastatin in men with hypercholesterolemia: West of Scotland Coronary Prevention Study Group. *N Engl J Med* 1995; **333:** 1301–1307.
2. Sever PS, Dahlof B, Poulter NR, Wedel H, Beevers G, Caulfield M, et al. Prevention of coronary and stroke events with atorvastatin in hypertensive patients who have average or lower-than-average cholesterol concentrations, in the Anglo-Scandinavian Cardiac Outcomes Trial-Lipid Lowering Arm (ASCOT-LLA): A multicentre randomised controlled trial. *Lancet* 2003; **361:** 1149–1158.
3. Ridker PM, Danielson E, Fonseca FA, Genest J, Gotto AM Jr, Kastelein JJ, et al. Rosuvastatin to prevent vascular events in men and women with elevated C-reactive protein. *N Engl J Med* 2008; **359:** 2195–2207.
4. Randomised trial of cholesterol lowering in 4444 patients with coronary heart disease: The Scandinavian Simvastatin Survival Study (4S). *Lancet* 1994; **344:** 1383–1389.
5. The Long-Term Intervention with Pravastatin in Ischaemic Disease (LIPID) Study Group. Prevention of cardiovascular events and death with pravastatin in patients with coronary heart disease and a broad range of initial cholesterol levels. *N Engl J Med* 1998; **339:** 1349–1357.
6. Cannon CP, Braunwald E, McCabe CH, Rader DJ, Rouleau JL, Belder R, et al. Intensive versus moderate lipid lowering with statins after acute coronary syndromes. *N Engl J Med* 2004; **350:** 1495–1504.
7. Pedersen TR, Faergeman O, Kastelein JJ, Olsson AG, Tikkanen MJ, Holme I, et al. High-dose atorvastatin vs usual-dose simvastatin for secondary prevention after myocardial infarction: The IDEAL study: A randomized controlled trial. *JAMA* 2005; **294:** 2437–2445.
8. Nakamura H, Arakawa K, Itakura H, Kitabatake A, Goto Y, Toyota

T, et al. Primary prevention of cardiovascular disease with pravastatin in Japan (MEGA study): A prospective randomised controlled trial. *Lancet* 2006; **368:** 1155–1163.
9. LaRosa JC, Grundy SM, Waters DD, Shear C, Barter P, Fruchart JC, et al. Intensive lipid lowering with atorvastatin in patients with stable coronary disease. *N Engl J Med* 2005; **352:** 1425–1435.
10. Teramoto T, Nakaya N, Yokoyama S, Ohashi Y, Mizuno K, Nakamura H. Association between lowering low-density lipoprotein cholesterol with pravastatin and primary prevention of cardiovascular disease in mild to moderate hypercholesterolemic Japanese. *J Atheroscler Thromb* 2010; **17:** 879–887.
11. Itakura H, Kita T, Mabuchi H, Matsuzaki M, Matsuzawa Y, Nakaya N, et al. Relationship between coronary events and serum cholesterol during 10 years of low-dose simvastatin therapy: Long-term efficacy and safety in Japanese patients with hypercholesterolemia in the Japan Lipid Intervention Trial (J-LIT) Extension 10 study, a prospective large-scale observational cohort study. *Circ J* 2008; **72:** 1218–1224.
12. Wiviott SD, Cannon CP, Morrow DA, Ray KK, Pfeffer MA, Braunwald E. Can low-density lipoprotein be too low? The safety and efficacy of achieving very low low-density lipoprotein with intensive statin therapy: A PROVE IT-TIMI 22 Substudy. *J Am Coll Cardiol* 2005; **46:** 1411–1416.
13. LaRosa JC, Grundy SM, Kastelein JJ, Kostis JB, Greten H. Safety and efficacy of atorvastatin-induced very low-density lipoprotein cholesterol levels in patients with coronary heart disease (a post hoc analysis of the treating to new targets [TNT] study). *Am J Cardiol* 2007; **100:** 747–752.
14. Kimura T, Morimoto T, Furukawa Y, Nakagawa Y, Kadota K, Iwabuchi M, et al. Long-term safety and efficacy of sirolimus-eluting stents versus bare-metal stents in real world clinical practice in Japan. *Cardiovasc Interv Ther* 2011; **26:** 234–245.
15. Friedewald WT, Levy RI, Fredrickson DS. Estimation of the concentration of low-density lipoprotein cholesterol in plasma, without use of the preparative ultracentrifuge. *Clin Chem* 1972; **18:** 499–502.
16. Serruys PW, Ong AT, van Herwerden LA, Sousa JE, Jatene A, Bonnier JJ, et al. Five-year outcomes after coronary stenting versus bypass surgery for the treatment of multivessel disease: The final analysis of the Arterial Revascularization Therapies Study (ARTS) randomized trial. *J Am Coll Cardiol* 2005; **46:** 575–581.
17. Menotti A, Puddu PE, Lanti M, Kromhout D, Blackburn H, Nissinen A. Twenty-five-year coronary mortality trends in the seven countries study using the accelerated failure time model. *Eur J Epidemiol* 2003; **18:** 113–122.
18. Haramaki N, Ikeda H, Takenaka K, Katoh A, Sugano R, Yamagishi S, et al. Fluvastatin alters platelet aggregability in patients with hypercholesterolemia: Possible improvement of intraplatelet redox imbalance via HMG-CoA reductase. *Arterioscler Thromb Vasc Biol* 2007; **27:** 1471–1477.
19. Zhang M, Zhou SH, Li XP, Shen XQ, Fang ZF, Liu QM, et al. Atorvastatin downregulates BMP-2 expression induced by oxidized low-density lipoprotein in human umbilical vein endothelial cells. *Circ J* 2008; **72:** 807–812.
20. Yasue H, Mizuno Y, Harada E, Itoh T, Nakagawa H, Nakayama M, et al. Effects of a 3-hydroxy-3-methylglutaryl coenzyme A reductase inhibitor, fluvastatin, on coronary spasm after withdrawal of calcium-channel blockers. *J Am Coll Cardiol* 2008; **51:** 1742–1748.
21. Nissen SE, Tuzcu EM, Schoenhagen P, Brown BG, Ganz P, Vogel RA, et al. Effect of intensive compared with moderate lipid-lowering therapy on progression of coronary atherosclerosis: A randomized controlled trial. *JAMA* 2004; **291:** 1071–1080.
22. Hiro T, Kimura T, Morimoto T, Miyauchi K, Nakagawa Y, Yamagishi M, et al. Effect of intensive statin therapy on regression of coronary atherosclerosis in patients with acute coronary syndrome: A multicenter randomized trial evaluated by volumetric intravascular ultrasound using pitavastatin versus atorvastatin (JAPAN-ACS [Japan assessment of pitavastatin and atorvastatin in acute coronary syndrome] study). *J Am Coll Cardiol* 2009; **54:** 293–302.
23. Hiro T, Kimura T, Morimoto T, Miyauchi K, Nakagawa Y, Yamagishi M, et al. Diabetes mellitus is a major negative determinant of coronary plaque regression during statin therapy in patients with acute coronary syndrome: Serial intravascular ultrasound observations from the Japan Assessment of Pitavastatin and Atorvastatin in Acute Coronary Syndrome Trial (the JAPAN-ACS Trial). *Circ J* 2010; **74:** 1165–1174.
24. Koh KK, Sakuma I, Quon MJ. Differential metabolic effects of distinct statins. *Atherosclerosis* 2011; **215:** 1–8.
25. Furukawa Y, Taniguchi R, Ehara N, Ozasa N, Haruna Y, Saito N, et al. Better survival with statin administration after revascularization therapy in Japanese patients with coronary artery disease: Perspectives from the CREDO-Kyoto registry. *Circ J* 2008; **72:** 1937–1945.
26. Sakamoto T, Kojima S, Ogawa H, Shimomura H, Kimura K, Ogata Y, et al. Effects of early statin treatment on symptomatic heart failure and ischemic events after acute myocardial infarction in Japanese. *Am J Cardiol* 2006; **97:** 1165–1171.
27. Dawber TR, Moore FE, Mann GV. Coronary heart disease in the Framingham study. *Am J Public Health Nations Health* 1957; **47:** 4–24.
28. Martin MJ, Hulley SB, Browner WS, Kuller LH, Wentworth D. Serum cholesterol, blood pressure, and mortality: Implications from a cohort of 361,662 men. *Lancet* 1986; **2:** 933–936.
29. Mabuchi H, Kita T, Matsuzaki M, Matsuzawa Y, Nakaya N, Oikawa S, et al. Large scale cohort study of the relationship between serum cholesterol concentration and coronary events with low-dose simvastatin therapy in Japanese patients with hypercholesterolemia and coronary heart disease: Secondary prevention cohort study of the Japan Lipid Intervention Trial (J-LIT). *Circ J* 2002; **66:** 1096–1100.
30. Grundy SM, Cleeman JI, Merz CN, Brewer HB Jr, Clark LT, Hunninghake DB, et al. Implications of recent clinical trials for the National Cholesterol Education Program Adult Treatment Panel III guidelines. *Circulation* 2004; **110:** 227–239.
31. Teramoto T, Sasaki J, Ueshima H, Egusa G, Kinoshita M, Shimamoto K, et al. Executive summary of Japan Atherosclerosis Society (JAS) guideline for diagnosis and prevention of atherosclerotic cardiovascular diseases for Japanese. *J Atheroscler Thromb* 2007; **14:** 45–50.
32. Sakamoto T, Ogawa H. "Just make it lower" is an alternative strategy of lipid-lowering therapy with statins in Japanese patients: LDL-cholesterol: The lower, the better; is it true for Asians? (Con) *Circ J* 2010; **74:** 1731–1741.
33. Mora S, Buring JE, Ridker PM, Cui Y. Association of high-density lipoprotein cholesterol with incident cardiovascular events in women, by low-density lipoprotein cholesterol and apolipoprotein b100 levels: A cohort study. *Ann Intern Med* 2011; **155:** 742–750.

Supplemental Files

Supplemental File 1

Appendix S1. List of Participating Centers and Investigators for the CREDO-Kyoto PCI/CABG Registry Cohort-2

Appendix S2. List of Clinical Research Coordinators

Appendix S3. List of Clinical Event Committee Members

Table S1. Baseline Characteristics and Achieved Lipid Levels in Statin vs. Non-statin Group

Table S2. Achieved Lipid Levels According to the LDL-C Level During Statin Therapy in the Strong and Standard Statin Group

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http://dx.doi.org/10.1253/circj.CJ-11-1356

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CURRENT TOPICS REVIEW ARTICLE

Cardiovascular surgery for realization of regenerative medicine

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Abstract Regenerative medicine is emerging as a new approach to the treatment of severe cardiovascular diseases that are resistant to conventional therapies. Although the type of cell transplanted (e.g., pluripotent stem cells, bone marrow-derived stem cells, skeletal myoblasts, or cardiac stem cells) influences the outcome of stem cell transplantation, the method of transplantation is also important, as the efficiency of engraftment after simple needle injection is poor. Scaffold-free cell sheet transplantation technology is one of the most promising methods in this regard. Although the results of clinical trials of stem cell therapy have been marginal to date, further elucidation of the actual mechanisms of cardiac repair following cell therapy would enhance the potential for full-scale implementation of stem cell therapy. In addition to stem cell therapy, the field of cardiovascular regenerative medicine includes interspecific chimera technology, drug delivery systems using biodegradable materials, and gene therapy. Integration of these new modalities with conventional therapies will be important to realize the goal of cardiovascular regenerative medicine tailored to the condition of each individual patient. Cardiovascular surgery would be an excellent means of carrying out this strategy and could potentially resolve the health problems of the increasing number of advanced cardiovascular patients. Herein, we review the recent basic and clinical research associated with the realization of regenerative medicine in the field of cardiovascular surgery.

Keywords Cardiovascular surgery · Regenerative medicine · Stem cell therapy

Introduction

Cardiovascular disease remains the leading cause of death worldwide. In Japan, over 57,000 cardiovascular surgeries for treatment of advanced cardiovascular diseases are performed annually [1]. However, the surgical treatment of severe heart failure is limited by the shortage of donors for heart transplantation and by the risk for serious complications, such as infection or cerebral thrombosis, after the implantation of ventricular assist devices. This health problem has prompted research into new therapeutic approaches, including cardiac regeneration [2]. Numerous valuable outcomes over more than a decade of basic research are now on the horizon of translation to clinical application ("from bench to clinic"), and the expectations from society are considerable. The field of cardiovascular surgical research, as the practical setting of such translational research, is now gathering significant attention from basic researchers. The results of basic research must be proven by preclinical experiments in animal disease models that mimic human diseases before the techniques can be applied clinically, and cardiovascular surgeons, as experts in human surgical treatments, are therefore the personnel best-suited to make practical contributions to advancing regenerative medicine research.

The review was submitted at the invitation of the editorial committee.

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In this review, we introduce recent basic and clinical research related to the field of cardiovascular surgery, including the different therapeutic approaches to, drawbacks of, and future expectations for new regenerative therapies for cardiovascular diseases.

Cell transplantation

Acute ischemic injury and chronic cardiomyopathies lead to permanent loss of cardiac tissue and, consequently, heart failure. Cell transplantation is thought to be an ideal therapeutic method for replacing lost myocardium [3, 4]. Of the available cell sources, stem cells are now widely preferred for research or clinical trials concerning cardiac cell therapy [2, 5]. The discovery of various stem cell populations possessing cardiogenic potential and the subsequent development of methods to isolate and expand these cells have begun to shape the notion of restorative therapy. Despite the great deal of knowledge gained through numerous basic research studies, significant barriers to true cardiac regeneration remain, and the field still lacks results sufficiently conclusive to support full-scale implementation of such therapies. Very few of the transplanted tissue stem cells seem to differentiate into mature cardiovascular cell types, suggesting that transplanted cells exert paracrine effects by which humoral factors induce or support favorable processes, including angiogenesis, prevention of apoptosis, and promotion of healing, in the injured myocardium rather than differentiating into renewed myocardium [2]. In this section, we introduce the present research achievements in stem cell therapy using various cell types, including clinical trials employing said cell sources, as well as the transplantation technologies that best support effective engraftment of the transplants. The characteristics of each stem cell population with respect to therapeutic potential are summarized in Table 1.

Pluripotent stem cells

Embryonic stem cells (ESCs) can be removed from the inner cell mass of the blastocyst and expanded in vitro practically indefinitely [6]. In culture, ESCs remain undifferentiated and pluripotent. When allowed to differentiate, ESCs can give rise to most somatic cell lineages [7]; their regenerative capacity is thus theoretically limitless. The advantages of these properties of ESC are especially significant for the heart as opposed to other organs, such as endocrine or sensory organs, as the heart functions as an assembly of many types of cells, including cardiomyocytes and others, and as numerous ($>10^8$) heart-composing cells might be required to fully repair a damaged human heart. The differentiation of ESCs can be driven towards cardiomyocytes or other vascular cell types by culture as monolayers or embryoid bodies in various growth media [8–11]. These cells can then be transplanted into the heart. This approach to repairing cardiac tissue has been tested in preclinical studies with encouraging results [10, 12]. In fact, of the various stem cell populations studied so far, ESCs have demonstrated perhaps the greatest capacity for cardiac cell differentiation and long-term cell survival [13].

To date, no human trials of the use of ESCs for cardiac repair have been attempted. There have been 3 main concerns regarding the use of ESC transplantation as a treatment modality. First, the differentiating cell mass contains cells from 3 germ layers, the ectoderm, mesoderm, and endoderm, and therefore possesses the capacity to differentiate along any or all of these lineages. This potential increases the risk of teratoma formation at the transplantation site. Although such teratomas are believed to be largely benign in vivo, some teratoma cells have been found to express markers similar to those found in malignant tumors [14]. We recently reported that the transplantation of cell sheets reassembled with defined mouse ESC-derived cardiovascular populations resulted in functional improvement in a rat sub-acute myocardial infarction (MI) model, and no tumors formed within the 3-month observation period after transplantation [12]. Transplantation of such fully differentiated cells alleviates some of the risk of teratoma formation. Other reports of the transplantation of differentiated cells have shown increased engraftment and functional improvement [10, 13]. Although no long-term studies assessing the real risk of teratoma formation have yet been performed, the theoretical concern remains an important obstacle. The second issue concerns immunity. ESCs have been reported to express specific human leukocyte antigen (HLA) subclasses [15]; this raises worries about graft rejection and might necessitate immunosuppression, which could increase the risk of surgical wound infection after cell transplantation surgery. Finally, the origin of ESCs has raised considerable ethical concerns and led to heated debates among scientists and the wider public.

The discovery that reprogramming adult somatic cells with genes that confer ESC pluripotency generates ESC-like cells, called induced pluripotent stem cells (iPSCs), may resolve the ethical and immunogenic issues associated with the use of ESCs [16, 17]. Mouse iPSCs can be differentiated into cardiovascular cell populations almost identical to those produced from mouse ESCs, indicating that the regenerative capacity of iPSCs is almost equal to that of ESCs [18]. Furthermore, a potent differentiation protocol based on high-density monolayer cultures and chemically defined factors, and modifications thereof, have been reported to produce cardiomyocytes from human iPS

Table 1 Characteristics of stem cell populations used for cardiac cell transplantation therapy

Stem cell type	Origin	Advantages	Disadvantages	Clinical trials
Pluripotent stem cells				
Embryonic stem cells (ESCs)	Inner cell mass of the preimplantation blastocyst	Theoretically unlimited self-renewal capacity; pluripotency	Ethical considerations; teratoma formation; graft-versus-host disease	None
Induced pluripotent stem cells	Most somatic cells (e.g., skin fibroblasts)	Same advantages as ESCs; free from ethical/immunological issues	Potential teratoma formation	None
Bone-marrow derived stem cells				
Hematopoietic stem cells (circulating progenitor cells, bone marrow mononuclear cells)	Bone marrow, peripheral blood	Easy to isolate; safe and feasible to transplant	Limited potential for differentiation into cardiomyocytes and vascular cells	TOPCARE-AMI [25], BOOST [26], REPAIR-AMI [27], LateTIME [28]
Mesenchymal stem cells	Bone marrow (adherent cells), adipose tissue	Easy to isolate and expand in culture; less immunogenic than other lines; multipotent	Great heterogeneity; heterotopic differentiation (e.g., ossification)	Report from Chen et al. (China) [34]
Endothelial progenitor cells	Bone marrow, peripheral blood	Mobilized from bone marrow or present in peripheral blood; important in vasculogenesis	Heterogeneity; small populations; reduced in individuals with cardiovascular comorbidities	REGENT [45]
Skeletal myoblasts	Mature skeletal muscle	Extensive scalability; resistance to ischemia; multipotent; no teratoma formation	Potential for arrhythmias; lack of cardiomyocyte differentiation	MAGIC [58], CAuSMIC [57]
Cardiac stem cells	Special niches in the myocardium	Resident in the recipient heart; robust cardiovascular differentiation potential; reduced tumor formation	Stem cell pool appears to undergo senescence; scalability largely unknown	CADUCEUS [76], SCIPIO [77], ALCADIA [78]

cells with an efficiency of 40–70 % [10, 19]. The application of this technique would strongly promote cardiac regenerative therapy utilizing human iPS cells. Recently, methods for generating human iPS cell lines without genomic integration by using episomal vectors [20] or human artificial chromosome vectors [21] have been reported. These may reduce tumorigenesis due to mutations, which could otherwise limit the clinical application of iPSCs. Based on the results of these basic studies, iPSCs are currently thought to be one of the most promising sources of cells for cardiac regeneration. However, further careful exploration of the feasibility of this new therapeutic modality will be required before its clinical application.

Bone marrow-derived stem cells

There are various cell subsets within the bone marrow niche that possess stem cell properties; these include hematopoietic progenitor/stem cells, mesenchymal stem cells, and endothelial progenitor cells. Each of these cell populations has both advantages and disadvantages for use in cardiac regeneration (Table 1). Although the capacities of these cells to differentiate into cardiomyocytes are rather low compared with those of ESC/iPSCs, their potential for cardiac restoration has been confirmed in many preclinical studies.

Bone marrow hematopoietic stem cells (or circulating peripheral-blood progenitor cells) are a well characterized and abundant source of progenitor cells. A number of remarkable studies have shown that direct transplantation or mobilization from endogenous reservoirs of bone marrow-derived cells significantly improves cardiac function. Some of these studies actually demonstrated regeneration of contracting cardiomyocytes and vascular beds [22, 23]. However, other investigations found limited or no differentiation of bone marrow cells into cardiovascular cell types [24]; this suggests a beneficial effect independent of direct tissue regeneration, such as neovascularization due to paracrine effects. The observed improvements in cardiac function prompted a number of clinical trials using autologous bone marrow cells to treat patients with heart failure or MI. These studies used circulating hematopoietic progenitor cells, or bone marrow mononuclear cells (MNCs), which also contain hematopoietic stem cells. Although the results of small early studies were encouraging, larger, randomized, placebo-controlled and blinded studies have shown mixed results [25, 26]. The results of the REPAIR-AMI trial (the largest of the randomized,

placebo-controlled trials) were positive in that left ventricular function improved and the combined clinical endpoint of death, MI, or revascularization within 2 years was reduced [27]. In contrast to the improved left ventricular function found by the REPAIR-AMI trial, a recent, randomized, double-blind, placebo-controlled study (the LateTIME trial) in which autologous bone marrow MNCs were transplanted into patients with MI 2–3 weeks after successful percutaneous coronary intervention showed no beneficial effect on left ventricular ejection fraction (LVEF) [28]. These apparently conflicting results may be attributable to the cell preparation or the timing of cell administration. Further large-scale trials are clearly necessary to assess the role of infused bone marrow cells in cardiac repair to improve the therapeutic efficacy of this promising technique.

Mesenchymal stem cells (MSCs) are a subset of stem cells found in the stroma of the bone marrow that can differentiate into osteoblasts, chondrocytes, and adipocytes [29] and also into small numbers of cardiomyocytes [30]. MSCs are thought to be either less immunogenic than other lines [31] or inherently immunomodulatory [32], alleviating the need for immunosuppression or autologous therapy. Preclinical studies of transplantation of MSCs into post-infarct animals demonstrated improved left ventricular function, reduced infarct size, and increased survival rate [30, 31, 33]. A clinical study of MSCs in patients with MI demonstrated improvement of left ventricular function [34]. The disadvantage of MSCs for this clinical application is their broad differentiation capacity. MSC populations remain significantly heterogeneous and are therefore less predictable after transplantation. Some studies have indicated that transplanted MSCs had differentiated into osteoblasts inside ventricular tissue [35].

Another bone marrow cell subset is the endothelial progenitor cells (EPCs). In the past, angiogenesis was thought to occur exclusively although the proliferation of mature endothelial cells at injury sites. This concept has changed with the discovery that bone marrow-derived EPCs reach injury sites and incorporate into the microvasculature (vasculogenesis) [36]. This finding drastically altered our understanding of vascular growth and became a new therapeutic approach. EPCs can be identified by their ability to acquire endothelial cell characteristics, i.e., the expression of cell surface makers, such as cluster of differentiation molecule 133 (CD133), CD34, the vascular endothelial growth factor receptor 2 kinase (VEGFR-2; also designed as KDR), and vascular endothelial cadherin (VE-cadherin), both in vitro and in vivo. Of these, $CD34^+$ and $CD133^+$ cells are the most widely recognized and utilized [37]. EPCs are mobilized from the bone marrow during states of injury, such as trauma, MI, or cancer [38–40]. The research into their use began with attempts to enhance their mobilization or incorporate EPCs directly into the vasculature of injured sites. VEGF, granulocyte colony stimulating factor (G-CSF), and statins (3-hydroxy-3-methylglutaryl coenzyme-A reductase inhibitors) have been reported to increase the mobilization of EPCs from the bone marrow [41–43]. Preclinical studies of the use of EPCs to treat experimental hind-limb ischemia demonstrated significant improvements in blood-flow recovery and limb salvage [36]. Furthermore, injection of EPCs into infarcted myocardium improved left ventricular function [44]. The results of a randomized multicenter clinical trial in patients with acute ischemia (REGENT trial) showed no significant improvement in left ventricular function after treatment with EPCs. However, there was a trend in favor of EPC therapy in the patients with the most severely impaired left ventricular function and in those with longer delays between the onset of symptoms and revascularization [45]. EPCs have already been used in the field of interventional cardiology. Drug-eluting stents (impregnated with various chemicals that inhibit neointimal thickening) reduce the restenosis rate, but increase the risk of in-stent thrombosis, a potentially fatal event. GENOUS stents are coated with anti-CD34 antibodies, which work to trap circulating EPCs and augment the process of luminal endothelialization; this may prevent restenosis [46]. A prospective study showed that implantation of the EPC-capture stent is safe and effective, with satisfactory immediate results and mid-term outcomes and no evidence of stent thrombosis [47]. However, EPCs have several disadvantages as a therapeutic material. First, this cell population is heterogeneous: EPCs circulating in the peripheral blood span the full range of differentiation from angioblasts to mature endothelial cells. Second, the stem cell pool of EPCs is quite limited, and ex vivo expansion would be the only way to attain sufficient numbers of EPCs for the treatment of a significant injury or ischemic event [48]. Finally, the circulating pool of EPCs is reduced in patients with such common comorbidities of cardiac ischemic disease as diabetes mellitus, hypertension, and hypercholesterolemia [49]. Although further research is required to overcome these problems and to enhance the therapeutic efficiency of EPCs in ischemic tissue, these cells remain promising as a potential therapeutic material.

Skeletal myoblasts

In adult skeletal muscle, a stem cell population called "satellite cells" exists beneath the basal membrane of muscle tissue, where they lie dormant until stimulated by muscle injury to proliferate [50]. Skeletal myoblasts (SMs) are derived from the satellite cells. SMs have been considered an attractive source for cardiac repair for several

reasons. First, these cells are further differentiated than ESCs and therefore less prone to teratoma formation. Second, they can be harvested from the host, expanded ex vivo, and autologously re-transplanted, thus avoiding the need for immunosuppression [51]. Third, SMs are resistant to ischemia, an obstacle to the function of stem cells in injured myocardium, and are therefore especially appropriate for cardiac repair [52]. Finally, SMs have the capacity to differentiate into non-muscle cell types in vitro [53]. Most transplantation experiments in animal cardiac disease models produced improved left ventricular function and decreased remodeling [51, 52, 54, 55]. However, skeletal myoblasts do not fully differentiate into cardiomyocytes in vivo after transplantation, and the myotubules generated do not operate in synchrony with the surrounding myocardium [55], possibly due to a lack of connexin activity and electrical coupling with the surrounding myocardial cells. However, the improvement in left ventricular function in animal models prompted a series of clinical investigations. Early clinical studies were aimed at assessing the feasibility and safety of SM implantation [52, 56, 57]. Although these studies proved the therapy feasible and showed that SMs survive in the heart, only marginal benefit was recognized. Large scale clinical trials were undertaken to assess the benefit of myoblast therapy. The most notable to date was the Myoblast Autologous Grafting in Ischemic Cardiomyopathy (MAGIC) trial, which randomized patients to receive injection of either SMs or culture medium. The results of this trial have been disappointing in that no significant benefit of SM transplantation has been observed [58]. Further clinical studies are ongoing and may show different results. Several barriers to the use of SMs still remain. The first limitation is the arrhythmogenic potential of the engrafted SMs. The MAGIC trial showed a higher number of arrhythmic events in the myoblast-treated patients [58]. Animal experiments have shown that the electrical coupling of SMs to host cardiomyocytes increases when the SMs are induced to overexpress connexin-43, indicating that their arrhythmogenicity may be a surmountable obstacle [59]. The second limitation is the relatively poor engraftment of the injected cells into the host myocardium. Cellular mortality of over 90 % within the first few days after injection has been reported in mice [60]. Some studies in humans have shown similarly high rates of cell death, with only scarce surviving cells [52]. Clinical research on autologous myoblast-sheet transplantation for the treatment of severe heart failure is now ongoing in Japan [61]. As the cell sheet transplantation method is thought to improve the survival of the transplants over needle injection (mentioned below), this strategy is a promising means of resolving the survival problem, and better clinical outcomes would be expected. The third limitation of SM use is that the engrafted cells differentiate into myotubules rather than cardiomyocytes and therefore do not constitute a true regenerative therapy.

Cardiac stem cells

The modest (rather unsatisfactory) functional effects of the transplantation of bone marrow cells or SMs in human studies have stimulated further research into the natural and endogenous regenerative mechanisms of cardiac tissue. The heart has traditionally been considered a post-mitotic organ, and mature cardiomyocytes withdraw from the proliferative cell cycle. However, contradictory data have accumulated, as cardiomyocyte proliferation and cell cycling have been observed under pathological conditions such as hypertension or MI [62, 63] and even in the healthy heart [64]. Moreover, estimates of the death rates of adult cardiomyocytes suggested the existence of a pool of cardiac progenitor cells [65]. This evidence prompted a search for such resident cardiac cells. Several different cell populations with stem cell characteristics were subsequently discovered in the adult heart. The first cell population with stem cell properties is called the side population (SP); these cells have also been identified in various other organs, including bone marrow, skeletal muscle, and adipose tissue [66]. Isolated cardiac SP cells can differentiate into cardiomyocytes, endothelial cells, or smooth muscle cells, suggesting that they represent cardiac and vascular progenitor cells [67]. The second putative resident progenitor population constitutes cells expressing the stem cell factor receptor c-Kit (also designed as CD117), which are located in small clusters within the adult heart. c-Kit$^+$ cells have regenerative potential after transplantation and give rise to cardiomyocytes, endothelial cells, and smooth muscle cells. c-Kit$^+$ cell transplantation after ischemic injury significantly improves cardiac function [68]. The third cell type in the heart with stem cell properties consists of cells expressing stem cell antigen 1 (Sca-1). Sca-1$^+$ cells home to infarcted myocardium and differentiate into cardiomyocytes around the injured area [69]. Finally, enzymatic digestion of heart tissue obtained via endomyocardial biopsy yields round cardiac progenitor cells that form so-called cardiospheres in suspension [70]. Cardiosphere-derived cells (CDCs) can also differentiate into cardiomyocytes, endothelial cells, and smooth muscle cells and exhibit remarkable capacities for proliferation and differentiation [70]. Once isolated, this cell population can be induced to differentiate into spontaneously contracting aggregates of cardiomyocytes that can then be transplanted into injured myocardium [71]. The injection of CDCs has produced functional improvement in preclinical studies [71]. Small numbers of CDCs have been observed to integrate into the injured myocardium, but this extent of

cardiac tissue regeneration is insufficient to explain the functional improvement.

Cardiac stem cells appear to exist in specialized niches that support the growth and maintenance of the stem cell pool [72]. Putative niches have been thought to be localized throughout the myocardium [68]. However, there is recent evidence that the adult heart contains a resident stem cell population that originates from the epicardium and has the potential to differentiate into cardiomyocytes after MI [73]. Although the different cardiac stem cell pools are small relative to the number of mature resident cardiomyocytes, they are believed to be the source of the new cells in damaged myocardium [74]. It is unclear whether the various cardiac stem cells are distinct types or represent different stages of a single cell lineage. Furthermore, the cardiac stem cell pool appears to diminish with age, possibly contributing to the poor efficiency of regeneration in elderly people [74]. This highlights the need to discover how to rejuvenate this senescent stem cell population, as it is largely the elderly who suffer increased mortality from cardiac ischemia.

Several phase-1 clinical trials using cardiac stem cells have been performed to date [75]. The CArdiosphere-Derived aUtologous stem CElls to reverse ventricULar dySfunction (CADUCEUS) study was a proof-of-principle study of intracoronary delivery of CDCs in patients with recent MI and left ventricular dysfunction [76]. In CADUCEUS, patients underwent endomyocardial biopsy sampling about 4 weeks after MI to generate autologous CDCs that were subsequently injected into the coronary artery. The rates of serious adverse effects and arrhythmias did not differ between patients in the CDC group and controls receiving conventional medical therapy. MRI analysis of patients treated with CDCs showed reduced scar mass and increased viable heart mass and regional contractility. However, changes in end-diastolic volume, end-systolic volume, and LVEF 6 months after transplantation did not differ between the groups. The Stem Cell Infusion in Patients with Ischemic cardiOmyopathy (SCIPIO) trial was performed in patients with post-infarction left ventricular dysfunction before coronary artery bypass grafting (CABG). Intracoronary administration of autologous c-Kit$^+$ cardiac stem cells about 4 months after surgery improved LVEF, and no adverse effects related to cell administration were reported [77]. In Japan, the results of the AutoLogous Human CArdiac-Derived Stem Cell to Treat Ischemic cArdiomyopathy (ALCADIA) trial, which tested combined therapy with autologous CDC transplantation and topical administration of basic fibroblast growth factor (bFGF), are now under evaluation [78]. Further investigations incorporating larger numbers of patients, longer follow-up times, and a true placebo arm will be needed to confirm the safety and efficacy of cardiac stem cell therapy.

Effective cell delivery: beyond needle injection

The results of this research have led to the conclusion that stem cells may be beneficial in the treated hearts but act primarily through paracrine mechanisms rather than through direct differentiation as initially expected. However, the low rates of grafted cell survival and engraftment diminish their potential and are serious technical limitations of stem cell therapy [79]. Over 70 % of injected cells have been reported to die progressively during the first 48 h after needle injection due to the hypoxic, inflammatory, and/or fibrotic environment [80]. Another report indicates that only 5.4–8.8 % of microspheres directly injected into the beating myocardium remain shortly after the injection due to massive mechanical loss [81]. Therefore, new strategies, such as combining the cells with bioengineering techniques have been developed and are the subjects of intense research, and early results suggest that these new strategies may improve the efficiency of stem cell therapies. Initial experiments were performed by combining the cells with injectable biomaterials, such as collagen, fibrin, or gelatin. Matrigel and other factors that provide a favorable environment rich in cytokines and growth factors were also tested. In general, these early studies showed increased survival of the transplanted cells and greater improvements in the cardiac function of the treated hearts [82]. However, these approaches did not assure complete cell retention or adequate distribution of the grafted cells. New techniques such as the creation of microtissues, i.e., cell sheets or patches, are now being developed in order to enhance both cell survival and the homogeneous and organized distribution of the cells [83].

Cellular patches are created using biomaterials that act as delivery platforms or scaffolds for the cells, assuring their engraftment and interaction with the tissue. One class of materials used is hydrogel/extracellular matrix-based matrices, in which the cells are embedded in a soluble hydrogel matrix that condenses in response to a temperature change to form a cellularized patch that can be applied to the pericardium. Patches of MSCs entrapped in a collagen-I matrix have been created and implanted into infarcted rat hearts, where they increased cell engraftment and functional improvement; these results appear to have been due to potentiation of the trophic effect of the MSCs by their increased survival in the tissue in the patch format [84]. Three-dimensional contractile loops of mixed collagen and neonatal cardiomyocytes, a more-sophisticated approach, have been successfully created. Implantation of these loops could support the contractile function of damaged heart [85].

Another promising approach is the creation of cell sheets with no scaffold support. This approach would avoid inflammatory reactions against the biomaterials constituting the scaffolds. Scaffold-free sheets have been

constructed using culture dishes covalently grafted with the temperature-responsive polymer poly (*N*-isopropylacrylamide) (PIPAAm), which enables the preparation of cell sheets without enzymatic digestion [86]. The beneficial potential of this technique has been demonstrated by the transplantation of monolayers of adipose tissue-derived MSCs [33] or triple-layer cardiac tissue sheets bioengineered with ESC-derived defined cardiac cell populations (our recent report) [12] into infarcted hearts. Both methods demonstrated increased tissue neovascularization and positive attenuation of heart remodeling, resulting in improved cardiac function. The direct mechanical support of the transplanted cell sheets would be desirable for more effective cardiac regeneration. However, no evidence of the reinforcement of contraction by the physical integration of the cell sheet and host myocardium was reported to date. To realize that, more increased survival of cell sheets would be essential, and supplemental strategies together with current cell sheet transplantation, such as vascularization of cell sheet, might be promising [12].

Transplantation of in vitro-created 3D cardiospheres has recently been shown to improve both engraftment of cardiac progenitors and their in vivo differentiation towards cardiac and vascular cells [87]. De-cellularized tissues have also been explored as scaffolds for cell transplantation. Tissues such as bovine pericardium [88] and omentum [89] have also been used to support cell types such as mesenchymal cells to enhance their paracrine effects. However, obtaining sufficient cardiac cells with no immunological risk and creating patches/organs that mimic the structure and function of the heart remain challenging.

Future directions for cardiac cell therapy

Despite the knowledge described above, the application of stem cells have been much less studied in chronically than in acutely infarcted hearts [90]. Indeed, stem cell therapy for chronic MI is of capital importance due to the growing patient population. Although the inhibition of tissue degeneration in the acute stage of the disease through paracrine effects, such as angiogenesis is a main goal of cell therapy, the implementation of this strategy in hearts with chronic MI, in which the inflammation has receded and the angiogenic processes mostly ended, could also have therapeutic effects, such as avoiding progression toward heart failure, reducing fibrosis, or regenerating the myocardium as a new contractile muscle mass. To date, cell therapy for chronic MI has been effective in both small and large animal models [33, 55, 89]. However, despite some exceptions, the effect on cardiac contractility is indirect rather than through true cardiac regeneration.

One future direction is the combination of stem cell therapy and conventional surgical procedures. Concomitant CABG and stem cell administration has been studied in patients with chronic myocardial ischemia, but the results were too marginal to justify full-scale therapeutic implementation [58, 77]. We have previously reported that combined rat fetal cardiomyocyte injection and plication of left ventricular aneurysm resulted in sufficient functional improvement in a rat chronic MI model [4]. The combination of cell therapy and various surgical procedures other than CABG, such as left ventricular reconstruction or mitral repair for ischemic mitral insufficiency, might be a promising strategy in the future and could provide hope for many patients, especially those with severe chronic heart failure who are ineligible for heart transplantation.

Another direction of future research is the further elucidation of the mechanisms of cardiac repair through cell therapy. Previous studies of stem cell therapy relied on injections of heterogeneous cell populations, which limited the insights they could provide into the cellular and molecular behaviors and mechanisms of action of transplanted cells. An understanding of the roles of each cell population as well as the various complex intercellular interactions in the heterogeneous populations transplanted would be a breakthrough in the improvement of cardiac cell therapy. Utilizing the mouse ES cell differentiation system to obtain defined cardiovascular populations, we recently found a major cellular mechanism, that is, cardiomyocytes are essential for sufficient cardiac restoration after sub-acute MI mainly through angiogenesis [12]. This approach to the elucidation of the regenerative mechanisms could be especially important in the context of chronic MI.

The last direction of future research that we introduce in this section is the "bioartificial heart." Replacing the injured area with a cardiac sheet or patch might be useful when a relatively small area of the heart is affected. However, this approach would not suffice when the heart has become nonfunctional and organ transplantation is indicated. Bioartificial hearts would be the ideal option in these cases, as they would theoretically avoid the problems of both organ transplants, such as donor shortages and immune rejection, and those of mechanical hearts, such as thromboembolism formation. In vitro heart "reconstruction" using decellularized cadaveric hearts has been demonstrated to be feasible. It is possible to re-vascularize and re-muscularize a heart "skeleton" of which only the extracellular matrix has been preserved to create a new heart. Cardiomyocytes and endothelial and fibroblastic cells are perfused using a bioreactor, which provides a pulsatile flow and pacing that mimics physiological conditions. Surprisingly, such newly formed hearts can contract spontaneously [91]. Of course, many issues, such as methods for isolating a sufficient volume of cardiovascular cell populations without the risk of immune rejection

(in this regard, iPS cells would be the ideal cell source) and preventing fatal arrhythmias, remain to be resolved before these bioartificial organs are developed to the point of clinical use. In any case, the demonstration of the possibility of creating such organs represents a great step forward in the treatment of cardiac diseases.

Other research related to cardiovascular regeneration

Interspecific chimera technology

There are many promising approaches to cardiac regeneration besides the cell therapies discussed above. The generation of human hearts from other animals using interspecific chimera technology with blastocyst complementation is one such approach. The injection of rat wild-type iPSCs into $Pdx1^{-/-}$ (pancreatogenesis-disabled) mouse blastocysts was recently reported to result in the generation of normally functioning rat pancreases in $Pdx1^{-/-}$ mice [92]. This result indicates that organs derived from donor iPSCs can be generated in vivo in a xenogenic environment. The development of this technology for the generation of human hearts within size-matched animals, such as pigs, is ongoing in Japan ("NAKAUCHI Stem Cell and Organ Regeneration" project [93]).

However, several problems remain to be overcome. The first of these concerns xenotransplantation. As the vessels (including the aorta and other large vessels), blood cells, nervous system, and other associated tissues would be derived from animals, while the organ itself would be of human origin, the transplantation of these chimeric organs into humans would be partial xenotransplantation rather than full allo/auto-transplantation. The generation of animals with genetically human blood vessels and nervous systems would be one solution to the potential for immunological rejection of xenotransplanted organs. The second problem is that in the abovementioned experiment, the iPSC-derived cells were found not only in the pancreas but also in all organs and tissues, including the brain and gonads [92]. Without proper control of the differentiation of iPSCs, the generation of human organs in livestock animals will pose ethical issues. One approach to address this problem is the use of lineage-committed stem or progenitor cells in place of iPSCs. The introduction of such cells into an appropriate microenvironment at the appropriate developmental time point might allow restriction of differentiation to a particular organ.

Biomaterials for efficient drug delivery

The technology for realizing the beneficial effects of cell therapy must be further advanced before it can attain its full potential. The combination of cell therapy and local administration of proteins that induce paracrine effects such as angiogenesis is one possible method of enhancing the therapeutic potential of cell therapy. Tabata et al. [94] have developed a system for sustained release of angiogenic cytokines, such as bFGF, from a biodegradable material, gelatin hydrogel; this system enables us to control the release of cytokines over the periods required for efficient clinical outcomes. The addition of such sustained release of bFGF enhanced the functional benefit of the transplantation of CDCs in a porcine MI model [71] and is being used in the ongoing clinical trial ALCADIA mentioned above [78].

In addition to its use in cardiac regeneration, the sustained–release system is also applicable to the treatment of critical limb ischemia. We found that the sustained release of bFGF improved the resolution of foot ulcers or other clinical symptoms in patients with severe limb ischemia in an initial phase 1–2a study [95], and an advanced clinical trial is now ongoing. Drug delivery systems using biodegradable biomaterials could thus be a promising strategy for the advancement of cardiovascular regeneration.

Gene therapy

Another avenue of regenerative medicine is gene therapy, which is emerging as a potential treatment option in patients suffering from a wide spectrum of cardiovascular diseases, including coronary artery disease, peripheral vascular disease, vein graft failure, and in-stent restenosis [96]. Gene therapy, which is the direct introduction of transgenes into the vasculature or myocardium, may contribute in controlling the symptoms of cardiovascular disease and may also be able to reverse the pathological processes involved. However, before these objectives can be achieved, three goals must be accomplished: suitable vectors must be generated, a suitable gene or group of genes must be identified, and an appropriate delivery system must be developed. The optimal characteristics of these components may vary depending on the disease being targeted.

Gene therapy to induce calcium upregulation in patients with advanced heart failure was recently attempted. The Calcium Upregulation by Percutaneous Administration of Gene Therapy in Cardiac Disease (CUPID), a phase-2 trial, decreased the frequency of cardiovascular events in the patient group, who received intracoronary administration of adeno-associated virus type 1/sarcoplasmic reticulum Ca^{2+}-ATPase [97]. MicroRNAs (miRNAs), which are small, non-coding RNAs that regulate gene expression in a sequence-dependent manner, are also being investigated as a new modality of gene therapy for ischemic heart disease or vascular diseases [98].

In 2010, Ieda et al. [99] reported that a combination of 3 developmental transcription factors (Gata4, Mef2c, and

Tbx5) rapidly, efficiently, and directly reprogrammed postnatal cardiac or dermal fibroblasts into differentiated cardiomyocyte-like cells in vitro (direct reprogramming). This technology was recently applied to an in vivo mouse MI model in which the 3 genes were delivered by a retroviral vector, resulting in the direct reprogramming of cardiac fibroblasts within the infarction site into cardiomyocyte-like cells and the attenuation of cardiac dysfunction [100]. Therefore, despite concerns over the ethics and safety of gene therapy (the latter related to the potential unexpected side effects of genomic integration), it is a promising segment of the broad field of cardiovascular disease research.

Conclusion

Herein, we introduced the status quo and future directions of stem cell therapy for treatment of cardiac disease and, more briefly, other approaches to cardiac regenerative research. We emphasize that we should not discuss which of these therapeutic modalities is to be preferred but rather consider them as components of an integrated medicine that would, as the summation of the new therapies introduced in this review and others that were not discussed, constitute a step towards the realization of cardiac regenerative therapy as a realistic option. In this context, the power of cardiovascular surgery as the integrator of basic research and clinical practice is virtually immeasurable.

Although much more work remains to be done, cardiac regenerative medicine, in conjunction with current treatment modalities, may help to further reduce the mortality and improve the quality of life of cardiovascular disease patients.

Conflict of interest None.

References

1. Sakata R, Fujii Y, Kuwano H. Thoracic and cardiovascular surgery in Japan during 2009: annual report by the Japanese Association for Thoracic Surgery. Gen Thorac Cardiovasc Surg. 2011;59:636–67.
2. Joggerst SJ, Hatzopoulos AK. Stem cell therapy for cardiac repair: benefits and barriers. Expert Rev Mol Med. 2009;11:e20.
3. Ohno N, Fedak PW, Weisel RD, Komeda M, Mickle DA, Li RK. Cell transplantation in non-ischemic dilated cardiomyopathy: a novel biological approach for ventricular restoration. Jpn J Thorac Cardiovasc Surg. 2002;50:457–60.
4. Sakakibara Y, Tambara K, Lu F, Nishina T, Nagaya N, Nishimura K, et al. Combined procedure of surgical repair and cell transplantation for left ventricular aneurysm: an experimental study. Circulation. 2002;106:I193–7.
5. Chien KR. Regenerative medicine and human models of human disease. Nature. 2008;453:302–5.
6. Thomson JA, Itskovitz-Eldor J, Shapiro SS, Waknitz MA, Swiergiel JJ, Marshall VS, et al. Embryonic stem cell lines derived from human blastocysts. Science. 1998;282:1145–7.
7. Murry CE, Keller G. Differentiation of embryonic stem cells to clinically relevant populations: lessons from embryonic development. Cell. 2008;132:661–80.
8. Yamashita J, Itoh H, Hirashima M, Ogawa M, Nishikawa S, Yurugi T, et al. Flk1-positive cells derived from embryonic stem cells serve as vascular progenitors. Nature. 2000;408:92–6.
9. Yamashita JK, Takano M, Hiraoka-Kanie M, Shimazu C, Yan P, Yanagi K, et al. Prospective identification of cardiac progenitors by a novel single cell-based cardiomyocyte induction. FASEB J. 2005;19:1534–6.
10. Laflamme MA, Chen KY, Naumova AV, Muskheli V, Fugate JA, Dupras SK, et al. Cardiomyocytes derived from human embryonic stem cells in pro-survival factors enhance function of infarcted rat hearts. Nat Biotechnol. 2007;25:1015–24.
11. Sone M, Itoh H, Yamahara K, Yamashita JK, Yurugi-Kobayashi T, Nonoguchi A, et al. Pathway for differentiation of human embryonic stem cells to vascular cell components and their potential for vascular regeneration. Arterioscler Thromb Vasc Biol. 2007;27:2127–34.
12. Masumoto H, Matsuo T, Yamamizu K, Uosaki H, Narazaki G, Katayama S, et al. Pluripotent stem cell-engineered cell sheets re-assembled with defined cardiovascular populations ameliorate reduction in infarct heart function through cardiomyocyte-mediated neovascularization. Stem Cells. 2012;30:1196–205.
13. van Laake LW, Passier R, Monshouwer-Kloots J, Verkleij AJ, Lips DJ, Freund C, et al. Human embryonic stem cell-derived cardiomyocytes survive and mature in the mouse heart and transiently improve function after myocardial infarction. Stem Cell Res. 2007;1:9–24.
14. Blum B, Benvenisty N. The tumorigenicity of human embryonic stem cells. Adv Cancer Res. 2008;100:133–58.
15. Draper JS, Pigott C, Thomson JA, Andrews PW. Surface antigens of human embryonic stem cells: changes upon differentiation in culture. J Anat. 2002;200:249–58.
16. Takahashi K, Yamanaka S. Induction of pluripotent stem cells from mouse embryonic and adult fibroblast cultures by defined factors. Cell. 2006;126:663–76.
17. Takahashi K, Tanabe K, Ohnuki M, Narita M, Ichisaka T, Tomoda K, et al. Induction of pluripotent stem cells from adult human fibroblasts by defined factors. Cell. 2007;131:861–72.
18. Narazaki G, Uosaki H, Teranishi M, Okita K, Kim B, Matsuoka S, et al. Directed and systematic differentiation of cardiovascular cells from mouse induced pluripotent stem cells. Circulation. 2008;118:498–506.
19. Uosaki H, Fukushima H, Takeuchi A, Matsuoka S, Nakatsuji N, Yamanaka S, et al. Efficient and scalable purification of cardiomyocytes from human embryonic and induced pluripotent stem cells by VCAM1 surface expression. PLoS One. 2011;6:e23657.
20. Okita K, Matsumura Y, Sato Y, Okada A, Morizane A, Okamoto S, et al. A more efficient method to generate integration-free human iPS cells. Nat Methods. 2011;8:409–12.
21. Hiratsuka M, Uno N, Ueda K, Kurosaki H, Imaoka N, Kazuki K, et al. Integration-free iPS cells engineered using human artificial chromosome vectors. PLoS One. 2011;6:e25961.
22. Orlic D, Kajstura J, Chimenti S, Jakoniuk I, Anderson SM, Li B, et al. Bone marrow cells regenerate infarcted myocardium. Nature. 2001;410:701–5.
23. Orlic D, Kajstura J, Chimenti S, Limana F, Jakoniuk I, Quaini F, et al. Mobilized bone marrow cells repair the infarcted heart, improving function and survival. Proc Natl Acad Sci USA. 2001;98:10344–9.

24. Murry CE, Soonpaa MH, Reinecke H, Nakajima H, Nakajima HO, Rubart M, et al. Haematopoietic stem cells do not transdifferentiate into cardiac myocytes in myocardial infarcts. Nature. 2004;428:664–8.
25. Leistner DM, Fischer-Rasokat U, Honold J, Seeger FH, Schächinger V, Lehmann R, et al. Transplantation of progenitor cells and regeneration enhancement in acute myocardial infarction (TOPCARE-AMI): final 5-year results suggest long-term safety and efficacy. Clin Res Cardiol. 2011;100:925–34.
26. Meyer GP, Wollert KC, Lotz J, Steffens J, Lippolt P, Fichtner S, et al. Intracoronary bone marrow cell transfer after myocardial infarction: eighteen months' follow-up data from the randomized, controlled BOOST (BOne marrOw transfer to enhance ST-elevation infarct regeneration) trial. Circulation. 2006;113:1287–94.
27. Assmus B, Rolf A, Erbs S, Elsässer A, Haberbosch W, Hambrecht R, et al. Clinical outcome 2 years after intracoronary administration of bone marrow-derived progenitor cells in acute myocardial infarction. Circ Heart Fail. 2010;3:89–96.
28. Traverse JH, Henry TD, Ellis SG, Pepine CJ, Willerson JT, Zhao DX, et al. Effect of intracoronary delivery of autologous bone marrow mononuclear cells 2 to 3 weeks following acute myocardial infarction on left ventricular function: the LateTIME randomized trial. JAMA. 2011;306:2110–9.
29. Jiang Y, Jahagirdar BN, Reinhardt RL, Schwartz RE, Keene CD, Ortiz-Gonzalez XR, et al. Pluripotency of mesenchymal stem cells derived from adult marrow. Nature. 2002;418:41–9.
30. Tomita S, Li RK, Weisel RD, Mickle DA, Kim EJ, Sakai T, et al. Autologous transplantation of bone marrow cells improves damaged heart function. Circulation. 1999;100:II247–56.
31. Dai W, Hale SL, Martin BJ, Kuang JQ, Dow JS, Wold LE, et al. Allogeneic mesenchymal stem cell transplantation in postinfarcted rat myocardium: short- and long-term effects. Circulation. 2005;112:214–23.
32. Le Blanc K, Ringdén O. Immunomodulation by mesenchymal stem cells and clinical experience. J Intern Med. 2007;262:509–25.
33. Miyahara Y, Nagaya N, Kataoka M, Yanagawa B, Tanaka K, Hao H, et al. Monolayered mesenchymal stem cells repair scarred myocardium after myocardial infarction. Nat Med. 2006;12:459–65.
34. Chen SL, Fang WW, Ye F, Liu YH, Qian J, Shan SJ, et al. Effect on left ventricular function of intracoronary transplantation of autologous bone marrow mesenchymal stem cell in patients with acute myocardial infarction. Am J Cardiol. 2004;94:92–5.
35. Yoon YS, Park JS, Tkebuchava T, Luedeman C, Losordo DW. Unexpected severe calcification after transplantation of bone marrow cells in acute myocardial infarction. Circulation. 2004;109:3154–7.
36. Asahara T, Murohara T, Sullivan A, Silver M, van der Zee R, Li T, et al. Isolation of putative progenitor endothelial cells for angiogenesis. Science. 1997;275:964–7.
37. Hristov M, Weber C. Endothelial progenitor cells in vascular repair and remodeling. Pharmacol Res. 2008;58:148–51.
38. Gill M, Dias S, Hattori K, Rivera ML, Hicklin D, Witte L, et al. Vascular trauma induces rapid but transient mobilization of VEGFR2(+)AC133(+) endothelial precursor cells. Circ Res. 2001;88:167–74.
39. Shintani S, Murohara T, Ikeda H, Ueno T, Honma T, Katoh A, et al. Mobilization of endothelial progenitor cells in patients with acute myocardial infarction. Circulation. 2001;103:2776–9.
40. Patenaude A, Parker J, Karsan A. Involvement of endothelial progenitor cells in tumor vascularization. Microvasc Res. 2010;79:217–23.
41. Kalka C, Tehrani H, Laudenberg B, Vale PR, Isner JM, Asahara T, et al. VEGF gene transfer mobilizes endothelial progenitor cells in patients with inoperable coronary disease. Ann Thorac Surg. 2000;70:829–34.
42. Fukuhara S, Tomita S, Nakatani T, Ohtsu Y, Ishida M, Yutani C, et al. G-CSF promotes bone marrow cells to migrate into infarcted mice heart, and differentiate into cardiomyocytes. Cell Transplant. 2004;13:741–8.
43. Llevadot J, Murasawa S, Kureishi Y, Uchida S, Masuda H, Kawamoto A, et al. HMG-CoA reductase inhibitor mobilizes bone marrow-derived endothelial progenitor cells. J Clin Invest. 2001;108:399–405.
44. Kocher AA, Schuster MD, Szabolcs MJ, Takuma S, Burkhoff D, Wang J, et al. Neovascularization of ischemic myocardium by human bone-marrow-derived angioblasts prevents cardiomyocyte apoptosis, reduces remodeling and improves cardiac function. Nat Med. 2001;7:430–6.
45. Tendera M, Wojakowski W, Ruzyłło W, Chojnowska L, Kepka C, Tracz W, et al. Intracoronary infusion of bone marrow-derived selected CD34+CXCR4+ cells and non-selected mononuclear cells in patients with acute STEMI and reduced left ventricular ejection fraction: results of randomized, multicentre Myocardial Regeneration by Intracoronary Infusion of Selected Population of Stem Cells in Acute Myocardial Infarction (REGENT) Trial. Eur Heart J. 2009;30:1313–21.
46. den Dekker WK, Houtgraaf JH, Onuma Y, Benit E, de Winter RJ, Wijns W, et al. Final results of the HEALING IIB trial to evaluate a bio-engineered CD34 antibody coated stent (Genous^{TM}Stent) designed to promote vascular healing by capture of circulating endothelial progenitor cells in CAD patients. Atherosclerosis. 2011;219:245–52.
47. Miglionico M, Patti G, D'Ambrosio A, Di Sciascio G. Percutaneous coronary intervention utilizing a new endothelial progenitor cells antibody-coated stent: a prospective single-center registry in high-risk patients. Catheter Cardiovasc Interv. 2008;71:600–4.
48. Jujo K, Ii M, Losordo DW. Endothelial progenitor cells in neovascularization of infarcted myocardium. J Mol Cell Cardiol. 2008;45:530–44.
49. Vasa M, Fichtlscherer S, Aicher A, Adler K, Urbich C, Martin H, et al. Number and migratory activity of circulating endothelial progenitor cells inversely correlate with risk factors for coronary artery disease. Circ Res. 2001;89:E1–7.
50. Buckingham M, Montarras D. Skeletal muscle stem cells. Curr Opin Genet Dev. 2008;18:330–6.
51. Murry CE, Wiseman RW, Schwartz SM, Hauschka SD. Skeletal myoblast transplantation for repair of myocardial necrosis. J Clin Invest. 1996;98:2512–23.
52. Pagani FD, DerSimonian H, Zawadzka A, Wetzel K, Edge AS, Jacoby DB, et al. Autologous skeletal myoblasts transplanted to ischemia-damaged myocardium in humans. Histological analysis of cell survival and differentiation. J Am Coll Cardiol. 2003;41:879–88.
53. Arsic N, Mamaeva D, Lamb NJ, Fernandez A. Muscle-derived stem cells isolated as non-adherent population give rise to cardiac, skeletal muscle and neural lineages. Exp Cell Res. 2008;314:1266–80.
54. Taylor DA, Atkins BZ, Hungspreugs P, Jones TR, Reedy MC, Hutcheson KA, et al. Regenerating functional myocardium: improved performance after skeletal myoblast transplantation. Nat Med. 1998;4:929–33.
55. Farahmand P, Lai TY, Weisel RD, Fazel S, Yau T, Menasché P, et al. Skeletal myoblasts preserve remote matrix architecture and global function when implanted early or late after coronary ligation into infarcted or remote myocardium. Circulation. 2008;118:S130–7.
56. Siminiak T, Fiszer D, Jerzykowska O, Grygielska B, Rozwadowska N, Kałmucki P, et al. Percutaneous trans-coronary-venous transplantation of autologous skeletal myoblasts in the

treatment of post-infarction myocardial contractility impairment: the POZNAN trial. Eur Heart J. 2005;26:1188–95.
57. Dib N, Dinsmore J, Lababidi Z, White B, Moravec S, Campbell A, et al. One-year follow-up of feasibility and safety of the first US, randomized, controlled study using 3-dimensional guided catheter-based delivery of autologous skeletal myoblasts for ischemic cardiomyopathy (CAuSMIC study). JACC Cardiovasc Interv. 2009;2:9–16.
58. Menasché P, Alfieri O, Janssens S, McKenna W, Reichenspurner H, Trinquart L, et al. The Myoblast Autologous Grafting in Ischemic Cardiomyopathy (MAGIC) trial: first randomized placebo-controlled study of myoblast transplantation. Circulation. 2008;117:1189–200.
59. Roell W, Lewalter T, Sasse P, Tallini YN, Choi BR, Breitbach M, et al. Engraftment of connexin 43-expressing cells prevents post-infarct arrhythmia. Nature. 2007;450:819–24.
60. Suzuki K, Murtuza B, Beauchamp JR, Smolenski RT, Varela-Carver A, Fukushima S, et al. Dynamics and mediators of acute graft attrition after myoblast transplantation to the heart. FASEB J. 2004;18:1153–5.
61. Development of New Strategy for Severe Heart Failure Using Autologous Myoblast Sheets. UMIN Clinical Trials Registry (UMIN-CTR). http://www.umin.ac.jp/ctr/index-j.htm UMIN000 003273 (Accessed 8 April 2012).
62. Anversa P, Palackal T, Sonnenblick EH, Olivetti G, Capasso JM. Hypertensive cardiomyopathy. Myocyte nuclei hyperplasia in the mammalian rat heart. J Clin Invest. 1990;85:994–7.
63. Beltrami AP, Urbanek K, Kajstura J, Yan SM, Finato N, Bussani R, et al. Evidence that human cardiac myocytes divide after myocardial infarction. N Engl J Med. 2001;344:1750–7.
64. Bergmann O, Bhardwaj RD, Bernard S, Zdunek S, Barnabé-Heider F, Walsh S, et al. Evidence for cardiomyocyte renewal in humans. Science. 2009;324:98–102.
65. Ellison GM, Torella D, Karakikes I, Nadal-Ginard B. Myocyte death and renewal: modern concepts of cardiac cellular homeostasis. Nat Clin Pract Cardiovasc Med. 2007;4:S52–9.
66. Challen GA, Little MH. A side order of stem cells: the SP phenotype. Stem Cells. 2006;24:3–12.
67. Oyama T, Nagai T, Wada H, Naito AT, Matsuura K, Iwanaga K, et al. Cardiac side population cells have a potential to migrate and differentiate into cardiomyocytes in vitro and in vivo. J Cell Biol. 2007;176:329–41.
68. Beltrami AP, Barlucchi L, Torella D, Baker M, Limana F, Chimenti S, et al. Adult cardiac stem cells are multipotent and support myocardial regeneration. Cell. 2003;114:763–76.
69. Oh H, Bradfute SB, Gallardo TD, Nakamura T, Gaussin V, Mishina Y, et al. Cardiac progenitor cells from adult myocardium: homing, differentiation, and fusion after infarction. Proc Natl Acad Sci USA. 2003;100:12313–8.
70. Messina E, De Angelis L, Frati G, Morrone S, Chimenti S, Fiordaliso F, et al. Isolation and expansion of adult cardiac stem cells from human and murine heart. Circ Res. 2004;95:911–21.
71. Takehara N, Tsutsumi Y, Tateishi K, Ogata T, Tanaka H, Ueyama T, et al. Controlled delivery of basic fibroblast growth factor promotes human cardiosphere-derived cell engraftment to enhance cardiac repair for chronic myocardial infarction. J Am Coll Cardiol. 2008;52:1858–65.
72. Moore KA, Lemischka IR. Stem cells and their niches. Science. 2006;311:1880–5.
73. Smart N, Bollini S, Dubé KN, Vieira JM, Zhou B, Davidson S, et al. De novo cardiomyocytes from within the activated adult heart after injury. Nature. 2011;474:640–4.
74. Torella D, Ellison GM, Méndez-Ferrer S, Ibanez B, Nadal-Ginard B. Resident human cardiac stem cells: role in cardiac cellular homeostasis and potential for myocardial regeneration. Nat Clin Pract Cardiovasc Med. 2006;3:S8–13.
75. Siu CW, Tse HF. Cardiac regeneration: messages from CADUCEUS. Lancet. 2012;379:870–1.
76. Makkar RR, Smith RR, Cheng K, Malliaras K, Thomson LE, Berman D, et al. Intracoronary cardiosphere-derived cells for heart regeneration after myocardial infarction (CADUCEUS): a prospective, randomised phase 1 trial. Lancet. 2012;379:895–904.
77. Bolli R, Chugh AR, D'Amario D, Loughran JH, Stoddard MF, Ikram S, et al. Cardiac stem cells in patients with ischaemic cardiomyopathy (SCIPIO): initial results of a randomised phase 1 trial. Lancet. 2011;378:1847–57.
78. AutoLogous human CArdiac-Derived stem cell to treat Ischemic cArdiomyopathy (ALCADIA) trial. http://www.clinicaltrials.gov/ct2/show/ NCT00981006 (Accessed 8 April 2012).
79. Pelacho B, Mazo M, Gavira JJ, Prósper F. Adult stem cells: from new cell sources to changes in methodology. J Cardiovasc Transl Res. 2011;4:154–60.
80. Müller-Ehmsen J, Whittaker P, Kloner RA, Dow JS, Sakoda T, Long TI, et al. Survival and development of neonatal rat cardiomyocytes transplanted into adult myocardium. J Mol Cell Cardiol. 2002;34:107–16.
81. Teng CJ, Luo J, Chiu RC, Shum-Tim D. Massive mechanical loss of microspheres with direct intramyocardial injection in the beating heart: implications for cellular cardiomyoplasty. J Thorac Cardiovasc Surg. 2006;132:628–32.
82. Cortes-Morichetti M, Frati G, Schussler O, Duong Van Huyen JP, Lauret E, Genovese JA. Association between a cell-seeded collagen matrix and cellular cardiomyoplasty for myocardial support and regeneration. Tissue Eng. 2007;13:2681–7.
83. Christman KL, Lee RJ. Biomaterials for the treatment of myocardial infarction. J Am Coll Cardiol. 2006;48:907–13.
84. Simpson D, Liu H, Fan TH, Nerem R, Dudley SC Jr. A tissue engineering approach to progenitor cell delivery results in significant cell engraftment and improved myocardial remodeling. Stem Cells. 2007;25:2350–7.
85. Zimmermann WH, Melnychenko I, Wasmeier G, Didié M, Naito H, Nixdorff U, et al. Engineered heart tissue grafts improve systolic and diastolic function in infarcted rat hearts. Nat Med. 2006;12:452–8.
86. Okano T, Yamada N, Sakai H, Sakurai Y. A novel recovery system for cultured cells using plasma-treated polystyrene dishes grafted with poly (*N*-isopropylacrylamide). J Biomed Mater Res. 1993;27:1243–51.
87. Bartosh TJ, Wang Z, Rosales AA, Dimitrijevich SD, Roque RS. 3D-model of adult cardiac stem cells promotes cardiac differentiation and resistance to oxidative stress. J Cell Biochem. 2008;105:612–23.
88. Wei HJ, Chen CH, Lee WY, Chiu I, Hwang SM, Lin WW, et al. Bioengineered cardiac patch constructed from multilayered mesenchymal stem cells for myocardial repair. Biomaterials. 2008;29:3547–56.
89. Shudo Y, Miyagawa S, Fukushima S, Saito A, Shimizu T, Okano T, et al. Novel regenerative therapy using cell-sheet covered with omentum flap delivers a huge number of cells in a porcine myocardial infarction model. J Thorac Cardiovasc Surg. 2011;142:1188–96.
90. Mazo M, Pelacho B, Prósper F. Stem cell therapy for chronic myocardial infarction. J Cardiovasc Transl Res. 2010;3:79–88.
91. Ott HC, Matthiesen TS, Goh SK, Black LD, Kren SM, Netoff TI, et al. Perfusion-decellularized matrix: using nature's platform to engineer a bioartificial heart. Nat Med. 2008;14:213–21.
92. Kobayashi T, Yamaguchi T, Hamanaka S, Kato-Itoh M, Yamazaki Y, Ibata M, et al. Generation of rat pancreas in mouse

by interspecific blastocyst injection of pluripotent stem cells. Cell. 2010;142:787–99.
93. Exploratory Research for Advanced Technology (ERATO), Japan Science and Technology Agency (JST). http://www.jst.go.jp/erato/project/nks_P/nks_P.html.
94. Tabata Y. Tissue regeneration based on growth factor release. Tissue Eng. 2003;9:S5–15.
95. Marui A, Tabata Y, Kojima S, Yamamoto M, Tambara K, Nishina T, et al. A novel approach to therapeutic angiogenesis for patients with critical limb ischemia by sustained release of basic fibroblast growth factor using biodegradable gelatin hydrogel: an initial report of the phase I–IIa study. Circ J. 2007;71:1181–6.
96. Gaffney MM, Hynes SO, Barry F, O'Brien T. Cardiovascular gene therapy: current status and therapeutic potential. Br J Pharmacol. 2007;152:175–88.
97. Jessup M, Greenberg B, Mancini D, Cappola T, Pauly DF, Jaski B, et al. Calcium Upregulation by Percutaneous Administration of Gene Therapy in Cardiac Disease (CUPID): a phase 2 trial of intracoronary gene therapy of sarcoplasmic reticulum Ca^{2+}-ATPase in patients with advanced heart failure. Circulation. 2011;124:304–13.
98. Frost RJ, van Rooij E. miRNAs as therapeutic targets in ischemic heart disease. J Cardiovasc Transl Res. 2010;3:280–9.
99. Ieda M, Fu JD, Delgado-Olguin P, Vedantham V, Hayashi Y, Bruneau BG, et al. Direct reprogramming of fibroblasts into functional cardiomyocytes by defined factors. Cell. 2010;142:375–86.
100. Qian L, Huang Y, Spencer CI, Foley A, Vedantham V, Liu L, et al. In vivo reprogramming of murine cardiac fibroblasts into induced cardiomyocytes. Nature. 2012;485:593–8.

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Pluripotent Stem Cell-Engineered Cell Sheets Reassembled with Defined Cardiovascular Populations Ameliorate Reduction in Infarct Heart Function Through Cardiomyocyte-Mediated Neovascularization

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ABSTRACT

Although stem cell therapy is a promising strategy for cardiac restoration, the heterogeneity of transplanted cells has been hampering the precise understanding of the cellular and molecular mechanisms. Previously, we established a cardiovascular cell differentiation system from mouse pluripotent stem cells, in which cardiomyocytes (CMs), endothelial cells (ECs), and mural cells (MCs) can be systematically induced and purified. Combining this with cell sheet technology, we generated cardiac tissue sheets reassembled with defined cardiovascular populations. Here, we show the potentials and mechanisms of cardiac tissue sheet transplantation in cardiac function after myocardial infarction (MI). Transplantation of the cardiac tissue sheet to a rat MI model showed significant and sustained improvement of systolic function accompanied by neovascularization. Reduction of the infarct wall thinning and fibrotic length indicated the attenuation of left ventricular remodeling. Cell tracing with species-specific fluorescent in situ hybridization after transplantation revealed a relatively early loss of transplanted cells and an increase in endogenous neovascularization in the proximity of the graft, suggesting an indirect angiogenic effect of cardiac tissue sheets rather than direct CM contributions. We prospectively dissected the functional mechanisms with cell type-controlled sheet analyses. Sheet CMs were the main source of vascular endothelial growth factor. Transplantation of sheets lacking CMs resulted in the disappearance of neovascularization and subsequent functional improvement, indicating that the beneficial effects of the sheet were achieved by sheet CMs. ECs and MCs enhanced the sheet functions and structural integration. Supplying CMs to ischemic regions with cellular interaction could be a strategic key in future cardiac cell therapy. STEM CELLS *2012;30:1196–1205*

Disclosure of potential conflicts of interest is found at the end of this article.

INTRODUCTION

Cardiovascular disease remains the leading cause of death worldwide, and this has prompted researches into new therapeutic approaches including cardiac regeneration. With discoveries of various stem/progenitor cell populations possessing cardiogenic or cardioprotective potential, the notion of restorative stem cell therapies has begun to take shape [1–8]. Several clinical trials and preclinical reports have demonstrated that intracoronary or intramyocardial injection of bone marrow (BM)-derived cells (hematopoietic stem/progenitor cells, mesenchymal stem cells, and endothelial progenitor cells) [9–14], skeletal myoblasts [15, 16], or cardiac stem/

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progenitor cells [17, 18] ameliorate cardiac function following acute and chronic myocardial infarction (MI), even though there is still a deficiency in conclusive results for a full-scale therapy [19–21]. There seems to be little differentiation of transplanted stem cells into mature cardiovascular cell types, suggesting paracrine effects of transplanted cells in which humoral factors favorably affect the injured myocardium, including angiogenesis, apoptosis prevention, and promotion of healing [18, 22–27]. Nevertheless, as previous studies are consequences of injections of heterogeneous cell populations, insights to the cellular and molecular behavior and mechanisms after cell transplantation are still restricted. It would be a breakthrough for further improvement of cardiac cell therapy to understand the role of each cell population as well as the various cellular interactions in the chaos of heterogeneity.

In this regard, it is rational to use defined cell populations, which can be prepared from pluripotent stem cells, to analyze the mechanism of cardiac regeneration. Previously, we have developed a novel embryonic stem (ES) and induced pluripotent stem cell (iPSC) differentiation system that exhibits early cardiovascular developmental processes using Flk1 (also designed as vascular endothelial cell growth factor [VEGF] receptor-2)-positive cells as common cardiovascular progenitors. Three cardiovascular cell types, namely cardiomyocytes (CMs), endothelial cells (ECs), and vascular mural cells (MCs), can be systematically induced and purified with this system [28–31]. These defined cell populations with directed differentiation from ESCs/iPSCs are valuable experimental tools toward cell type-oriented understanding of the underlying mechanisms and the exploration of novel cardiac restoration strategies.

Another factor that has been hampering the elucidation of the mechanisms involved is the poor engraftment of injected cells to the heart [32]. Alternative strategies for cell transplantation are required to facilitate survival of grafted cells. We have developed a culture surface covalently grafted with temperature-responsive polymer poly(*N*-isopropylacrylamide) that enables the preparation of cell sheets without enzymatic digestion [6, 33–36]. Combining this cell sheet system and our unique ESC/iPSC differentiation system, we can reconstitute and transplant various cell type-controlled sheets with combinations of defined cardiovascular cell populations. This new experimental system enables us to prospectively elucidate the effects, roles, and interactions of each cell type in vitro and in vivo.

This study demonstrates that pluripotent stem cell-derived cell sheets as cardiac tissue have a distinct potential to ameliorate cardiac dysfunction following MI. We also demonstrate the possible mechanisms for cardiac functional improvement following transplantation with cell tracing and cell type-controlled sheet analyses. We indicate novel roles of CMs and the valuable cellular interactions in functional amelioration after cell transplantation, suggesting a strategic key role for future cell therapy.

Materials and Methods

Detailed methods and associated references are provided in Supporting Information.

Mouse ESC Culture

Two mouse ESC sublines from E14tg2a cell line were used. EMG7 mouse ESC line that carries mouse α-myosin heavy chain (MHC) promoter-driven EGFP gene was used for differentiation of CMs [28, 30]. EStTA-ROSA mouse ESC line was used for differentiation of ECs and MCs [29].

Cell Differentiation

Induction of and sorting for $Flk1^+$ cells were performed as previously described [28, 30]. In brief, mouse ESCs were cultured in differentiation medium (DM) [alpha minimum essential medium (GIBCO, Grand Island, NY, http://www.invitrogen.com/site/us/en/home/brands/Gibco.html) supplemented with 10% fetal bovine serum (FBS) and 5.5 mmol/l 2-mercaptoethanol] without leukemia inhibitory factor (LIF) on gelatin-coated dishes for 96–112 hours. For the differentiation of CMs, purified $Flk1^+$ cells were plated onto mitomycin-C-treated confluent OP9 cell sheets (MMC-OP9) and cultured in DM to induce CM differentiation (Supporting Information Fig. S1A). Cyclosporin-A (3 μg/ml) was added to $Flk1^+$ cell culture to promote CM differentiation [37]. For the differentiation of ECs or MCs, purified $Flk1^+$ cells were plated onto gelatin-coated dishes and then cultured with DM in the presence of $VEGF_{165}$ (50 ng/ml) and 8-bromoadenosine-3′: 5′-cyclic monophosphate sodium salt (8bromo-cAMP) (0.5 mmol/l) (Supporting Information Fig. S1B) [38].

Flow Cytometry Analysis and Cell Sorting

After 4 days culture of $Flk1^+$ cells on MMC-OP9, cultured cells were harvested and subjected to cell sorting with FACS (fluorescence-activated cell sorting) (Aria II, BD Biosciences, Franklin Lakes, NJ, http://www.bdbiosciences.com/home.jsp). Viable green fluorescent protein (GFP)-positive cell population was evaluated and sorted as differentiated CMs (Supporting Information Fig. S1A) [30]. ESC-derived ECs and MCs were selectively induced and harvested on the third day of $Flk1^+$ cell culture on gelatin-coated dishes with VEGF and 8bromo-cAMP (Flk1-d3) [28, 38].

Mouse ESC-Derived Tissue Sheet Formation

$Flk1^+$ cells induced from EStTA-ROSA cells were plated onto a gelatin-coated 12-multiwell UpCell at $2.5–4.0 \times 10^4$ cells per well with 1 ml of DM containing $VEGF_{165}$ (50 ng/ml) and 8bromo-cAMP (0.5 mmol/l) to selectively induce ECs and MCs on UpCell. After 3 days of culture, purified cell suspension [5.0×10^5 CMs and 5.0×10^5 bulk harvest of Flk1-d3 (ECs and MCs)] was plated onto vascular cells on UpCell (i.e., purified C + E + M cells onto E + M cells) (Supporting Information Fig. S1D, S1E). After 4 days of culture, cells were moved to room temperature. Within 15–30 minutes, cells detached spontaneously and floated in the media as monolayer cell sheets (C+E+M sheet) (C: *C*Ms, E: *E*Ms, M: *M*Cs).

Animals

Male athymic nude rats aged between 10 and13 weeks were used for transplantation. All protocols were performed in accordance with the guidelines for Animal Experiments of Kyoto University, which conforms to Japanese law and the Guide for the Care and Use of Laboratory Animals.

Animal Model Preparation and Transplantation

MI model rats were generated as previously described [39, 40]. The rats whose hearts revealed less than 40% of left ventricular (LV) fractional shortening (FS) with echocardiogram on the sixth day of MI induction were enrolled in further experiments. One week after MI induction, each rat was randomly assigned to one of the two groups: transplantation (Tx) group and sham group (Supporting Information Fig. S2C, S2D; Supporting Information Video 3). In Tx group, three-layered cell sheet was put on the area and spread manually to make whole MI area covered by the sheet. The sheet could be stably placed onto the epicardium without sutures. The chest was closed 15 minutes after transplantation. In sham group, the chest was closed 15 minutes after thoracotomy without cell sheet transplantation.

Echocardiogram and Catheterization

Transthoracic echocardiogram was performed with a Vivid 7 system (GE Healthcare, Waukesha, WI, http://www3.gehealthcare.com/en/Global_Gateway) provided with an 11-MHz imaging transducer (GE 10S ultrasound probe, GE Healthcare). Echocardiographic measurements were performed as previously described [39, 40]. LV pressure-volume loop analysis with cardiac catheterization was performed as previously described [41]. In brief, right internal carotid artery was exposed and cannulated with a conductance- and pressure-measuring catheter transducer (SPR-869; Millar instruments, Houston, TX, http://millar.com/), which was then advanced into the LV. Pressure-volume loops were recorded with or without preload reduction by inferior vena cava compression via midline laparotomy.

Species-Specific Fluorescent In Situ Hybridization Analysis

The fluorescent in situ hybridization (FISH) probes that recognize and hybridize with sequence repeats specific for each animal species were arranged by Chromosome Science Labo (Sapporo, Japan, http://www.chromoscience.ip/index_e.html) [42, 43]. The nucleotide probes were applied to the fixed and pretreated sections, denatured, followed by hybridization. Additional immunofluorescent staining for cardiac troponin-T (cTnT) was performed to the FISH samples.

RNA Extraction and Quantitative Reverse-Transcription Polymerase Chain Reaction

Total RNA was extracted from cell sheets using RNeasy (QIAGEN, Venlo, The Netherlands, http://www.qiagen.com/default.aspx), according to the manufacturer's instructions. Reverse transcription was performed with the SuperScript III first-strand synthesis system (Invitrogen, Eugene, OR, http://www.invitrogen.com/site/us/en/home.html). Quantitative PCR was performed using Power SYBR Green PCR Master Mix (Applied Biosystems, Foster City, CA, http://www.appliedbiosystems.com/absite/us/en/home.html) and StepOnePlus system (Applied Biosystems).

Statistical Analysis

The data were processed using Dr. SPSS II software for windows (version 11.0.1J, SPSS Inc, Chicago, IL, http://www-01.ibm.com/software/analytics/spss/). Comparisons between two groups were made with unpaired or paired t test. Comparisons between more than two groups were made with one-way analysis of variance followed by Tukey's test as post hoc. Values are shown in mean $\pm$ SD. p values $<.05$ were considered significant.

RESULTS

Construction and Characterization of Cardiac Tissue Sheet

First, we attempted to fabricate cardiac tissue consisting of mouse ESC-derived CMs, ECs, and MCs. CMs were differentiated and purified as previously reported [30, 37]. Briefly, when Flk1$^+$ cells derived from mouse ESCs carrying cardiac-specific GFP gene are cultured on OP9 stroma cells, spontaneously beating CMs (GFP$^+$) can be induced and subsequently purified by FACS. The purity of sorted GFP$^+$ CMs was 96.0% $\pm$ 2.0% in this study (Supporting Information Fig. S1A). On the other hand, ECs and MCs were selectively induced as previously reported [28, 29]. When Flk1$^+$ cells are cultured on gelatin-coated dishes with VEGF, almost all of the cells that appear are either ECs or MCs. Vascular endothelial (VE)-cadherin$^+$ ECs were 31.8% $\pm$ 14.8% on average in this study. The remainder indicated MC population (approximately 68%) (Supporting Information Fig. S1B, S1C). We reassembled these defined cardiovascular populations on 12-multiwell temperature-responsive culture dishes (UpCell; CellSeed, Tokyo, Japan) (Fig. 1; see detailed protocol in Methods; Supporting Information Fig. S1). Temperature reduction successfully provided reassembled cardiac tissue sheets that consisted of *C*Ms (40.2% $\pm$ 8.5% of total cells), *E*Cs (8.4% $\pm$ 5.7%), and *M*Cs (51.4% $\pm$ 9.2%) (C + E + M sheets; $n = 24$, FACS analyzed) (Fig. 1A, 1B; Supporting Information Fig. S1, Supporting Information Video 1). Total cell count of the sheet was 2.62 $\pm$ 0.74 $\times$ 10^5 on average ($n = 24$).

The sheet consisted of three to four cell layers with intact stratified structure of collagen adjacent to the cell component (Fig. 1C). Immunostaining for CMs (cTnT$^+$) and ECs (VE-cadherin$^+$) showed an almost even distribution of CMs, ECs, and MCs (*c*TnT$^-$/VE-cadherin$^-$) in the sheet (Fig. 1D–1F). Cell surface potentials showed continuous, regular, and unidirectional electrical propagation in the sheet without ectopic foci (Fig. 1G–1I; Supporting Information Video 2).

Cardiac Tissue Sheet Transplantation Ameliorates Cardiac Function

Three cardiac cell sheets were piled up to form a three-layered cell sheet and transplanted to rat subacute MI model (Supporting Information Fig. S2; Supporting Information Video 3). Cell sheet transplantation (sham operation, $n = 9$; C + E + M sheet transplantation, $n = 9$) was performed 1 week after MI induction. All rats survived for 4 weeks of observation periods after transplantation. We evaluated cardiac functions with echocardiogram ($n = 9$ each) (Fig. 2A–2E; Supporting Information Video 4) [39, 40] and LV pressure-volume loop study with catheterization ($n = 8$ each) (Fig. 2F, 2G) [41]. Transplantation group revealed restoration of anterior wall contraction (Fig. 2A). Transplantation significantly increased FS and fractional area change compared to those of pretreatment phase as well as sham operation group (Fig. 2B, 2C). The systolic thickening of infarct wall significantly recovered to almost normal value (1.6 $\pm$ 0.2 vs. 1.6 $\pm$ 0.3 [normal value ($n = 26$) vs. sheet transplantation (4 weeks, $n = 9$)]) (Fig. 2D). Increase in diastolic area of LV showed a tendency to be decreased (Fig. 2E). LV pressure-volume loop study can measure LV performance independent from loading conditions or heart rate [41]. End-systolic elastance (Ees) reflecting systolic function was significantly higher at 4 weeks after transplantation (Fig. 2F, 2G). Time constant (Tau), an index for diastolic function, was, however, not significantly improved (Fig. 2G). Transplantation limited the extent of fibrosis and thinning of the infarct wall at 4 weeks after transplantation (Fig. 2H, 2I). The improvement of systolic function in echocardiogram was stably maintained at 3 months after transplantation (sham operation, $n = 3$; C + E + M sheet transplantation, $n = 3$) (Supporting Information Figs. S2D, S3). No tumor formation was observed after the cardiac tissue sheet transplantation throughout all experimental periods ($n = 12$). All these findings indicate that cardiac cell sheet transplantation successfully improves LV systolic function and attenuates LV remodeling following MI.

Survival of Transplanted CMs In Vivo

Next, to precisely evaluate the cellular and molecular mechanisms of transplantation, we tried to trace the fate and distribution of transplanted cell sheets using species-specific (SS)-FISH analysis, which can distinguish mouse donor cells from rat recipient cells [42, 43]. A pilot study, just after transplantation of a single-layered cell sheet to normal rat heart, evidently demonstrated a single array of mouse nuclei on the very surface of rat heart, certifying the validity of this method

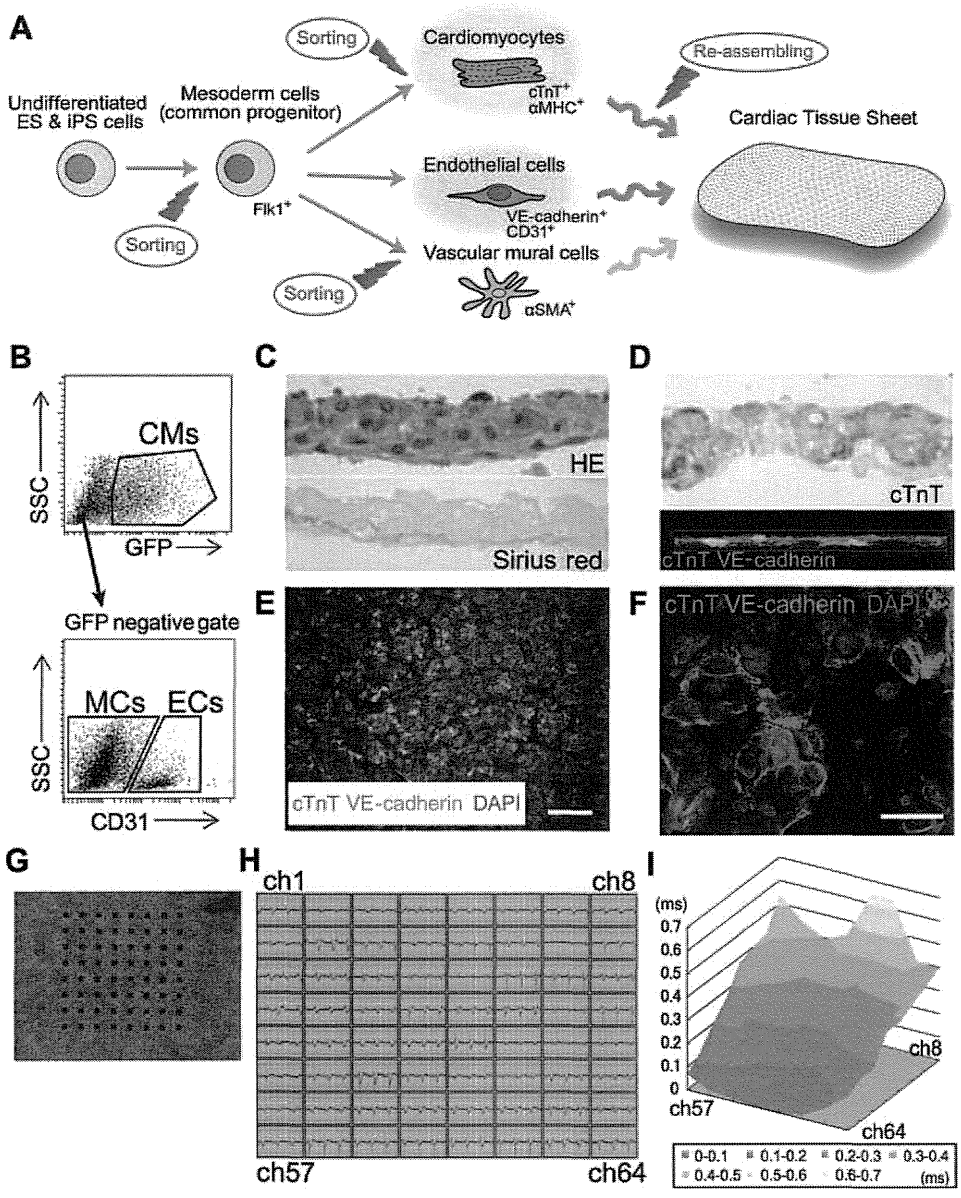

Figure 1. Construction and characterization of cardiac tissue sheets. (**A**): Schematic diagram of cardiac tissue sheet formation. Defined cardiac cell populations (CMs, ECs, and vascular MCs) are systematically differentiated from pluripotent stem cells, sorted, and reassembled to construct tissue sheets. (**B–F**): Tissue sheet components. (B): Flow cytometry analysis (n = 24). Upper panel: α-MHC-GFP$^+$ CMs in total cells from sheets. Lower panel: CD31$^+$ ECs in GFP$^-$ non-CM population. GFP$^-$/CD31$^-$ cells correspond to MCs. Representative plot data are shown. (C, D) Cross-sections. (C): Upper panel: H&E staining showing cell appearance of the sheet. Lower panel: Sirius red staining showing intact extracellular matrix. (D): Upper panel: cTnT staining (brown). Lower panel: two-photon laser imaging microscopy. Double staining for cTnT (CMs, red) and VE-cadherin (ECs, green). (E, F) Immunostaining of sheets for cTnT (red), VE-cadherin (green), and DAPI. Low-magnification view with full-focused fluorescent imaging microscopy (E) and high-magnification view with two-photon laser imaging microscopy (F) showing distribution of CMs (cTnT$^+$), ECs (VE-cadherin$^+$), and MCs (cTnT$^-$/VE-cadherin$^-$). (**G–I**): Electrophysiological study for extracellular potentials. (G): Tissue sheet set on the electrodes (black dots). (H): Extracellular potentials of each microelectrode. (I): Propagation map. Electrical propagations are visualized as a contour image. Regular and unidirectional electrical conduction is observed. Scale bars = 200 μm in (E) and 50 μm in (F). Abbreviations: αMHC, α-myosin heavy chain; αSMA, α-smooth muscle actin; ch, channel; CM, cardiomyocyte; cTnT, cardiac troponin-T; DAPI, 4,6-diamidino-2-phenylindole; ECs, endothelial cells; ES, embryonic stem; GFP, green fluorescent protein; H&E, Hematoxylin and Eosin; iPS, induced pluripotent stem; MC, mural cell; SSC, side scatter; VE-cadherin, vascular-endothelial cadherin.

(Fig. 3A). We performed quantification of the engrafted CMs with double staining of cTnT (immunostaining; for mouse and rat CMs) and SS-FISH after cell sheet transplantation to MI model. Whereas obvious cTnT$^+$ CM graft area with mouse nuclei on the surface of the infarct heart was observed 1 day after the transplantation, the grafted CM area started to diminish within several days. Very little CM clusters remained at 4 weeks after transplantation (Fig. 3B, 3C). No mouse-nuclei-positive cells were observed outside of these cluster areas, suggesting that most of the engrafted mouse cell populations had disappeared. The discrepancy between functional improvement and CM engraftment suggests that the improvement of cardiac function is not mainly mediated by direct contribution of CMs but other indirect roles, possibly