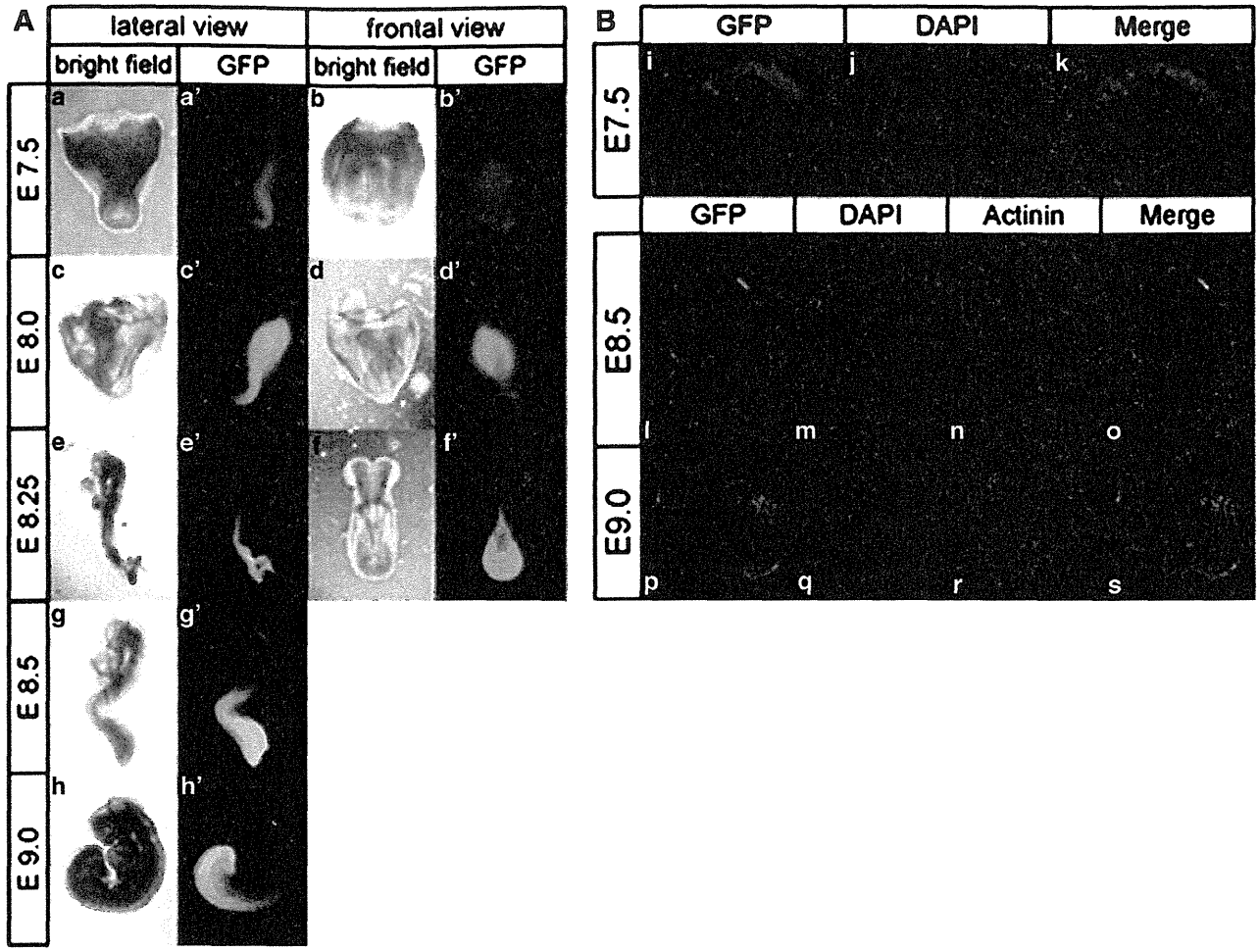

Fig. 5. Analysis of ins-TOPEGFP mice (E7.5–9.0) shows that the Wnt/β-catenin pathway is not activated in heart forming regions. (A) Whole-mount view of ins-TOPEGFP mice under bright-field and fluorescent light. (a–d) EGFP signals are present in the primitive streak but not in the anterior regions of the E7.5 and E8.0 embryos. (e, f) EGFP signals are expressed in the posterior side of the embryo, as well as in the midbrain at E8.25. (g) At E8.5, EGFP signals are detected in the posterior side of the embryo, midbrain, and paraxial mesoderm but not in the heart. (h) The E9.0 mouse embryo shows no EGFP expression in the heart. EGFP expression is detected in the dorsal domain of the dermomyotome and the anterior somites. (B) The ins-TOPEGFP murine embryo (E7.5, 8.5 and 9.0) sections. (i–k) The sections of ins-TOPEGFP mouse (E7.5). EGFP signals are detected in the primitive streak but not in the precardiac mesoderm. (l–s) Immunostaining of ins-TOPEGFP mouse (E8.5 and E9.5) sections for GFP and α-Actinin. EGFP signals are evident in the central nervous system and the newly formed somites.

previous report showed that *Wnt2* knockout mice had impaired vascularization of extra-embryonic tissues, which resulted in incomplete penetrant [30]. And loss of *Wnt2* results in defective development of the posterior pole of the heart, resulting in a phenotype resembling the human congenital heart syndrome complete common atrioventricular canal [32]. Although genetic knockout using homologous recombination is a reliable system, complete and continuous deletion may lead to effective compensation. But, sudden gene abrogation using siRNA may reveal significant phenotypes, since compensation for sudden gene disappearance is less likely to occur. Complete *Wnt2* deletion in ES cells also decreased cardiomyocyte differentiation and increased hematopoietic differentiation, whereas a complete knockout system could not address when and how Wnt2 acts on cardiomyocyte differentiation [46]. Therefore, in further investigations to determine the effective time window for Wnt2, we showed that Wnt2 promotes cardiac development not in the primitive development of the mesodermal progenitor, but after determination of the cardiac mesoderm. This knockdown phase variation advances our understanding of how Wnt2 acts in cardiogenesis.

Wnts have various subtypes and redundancy, as well as several signal transmission pathways thorough β-catenin, c-Jun N-terminal kinase (JNK) and protein kinase C (PKC) [6,7]. These signal transduction pathways are associated with particular Wnts, receptor, co-receptor and recipient cell types. For example, Wnt1 and Wnt3a, which were thought to activate canonical pathways, can also activate non-canonical pathways based on cell or tissue types. In order to clarify whether Wnt2 acts during cardiac development through canonical pathway in vivo, we analyzed the ins-TOPEGFP mice. *Wnt2* was clearly expressed in the cardiac crescent at E7.5, whereas there was no signal which suggested canonical Wnt signal activation in the cardiac developing region at this stage. We also elucidated that JNK/AP-1 pathway is necessary for Wnt2 dependent cardiomyocyte differentiation from ES cells. For non-canonical Wnt, Wnt11 was expressed in the primitive mouse heart at E8.0 and E8.5 and it promoted cardiogenesis [22, 47]. Considering their similarities, Wnt2 and Wnt11 may be redundant or may compensate for each other via the non-canonical pathway.

From the viewpoint of clinical applications, the most important issue is the efficient regulation of ES cell differentiation into cardiomyocytes. However, this is not easy to achieve, as cardiomyocyte development is complex both in vivo and in vitro. In the present study, we demonstrate that Wnt2 can increase the number of murine ES cell-derived cardiomyocytes. Furthermore, we now have another

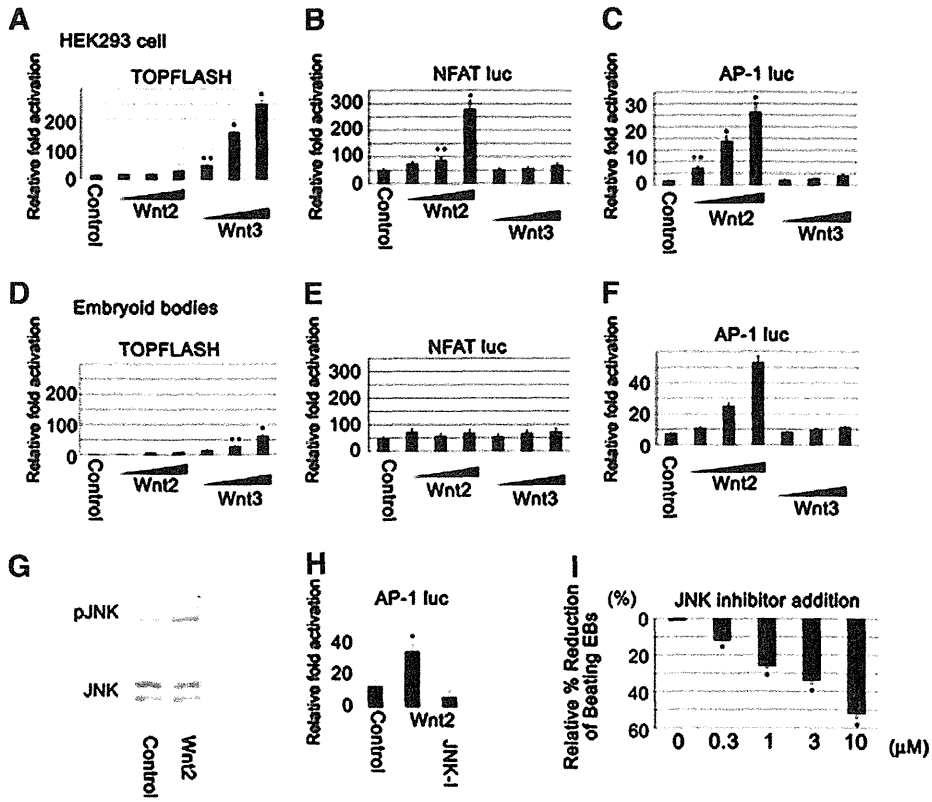

Fig. 6. JNK/AP-1 pathway is activated by Wnt2 in differentiating ES cells. (A) Relative fold activation of luciferase activity of TOPFLASH by Wnt2 or Wnt3 addition in HEK293 cells. (B) Relative fold activation of luciferase activity of NFAT reporter by Wnt2 or Wnt3 addition in HEK293 cells. (C) Relative fold activation of luciferase activity of AP-1 reporter by Wnt2 or Wnt3 addition in HEK293 cells. (D) Relative fold activation of luciferase activity of TOPFLASH by Wnt2 or Wnt3 addition in EBs. (E) Relative fold activation of luciferase activity of NFAT reporter by Wnt2 or Wnt3 addition in EBs. (F) Relative fold activation of luciferase activity of AP-1 reporter by Wnt2 or Wnt3 addition in EBs. (G) Western blotting for JNK and pJNK using differentiating EBs, in the presence or absence of Wnt2. (H) JNK inhibitor addition in EBs decreases luciferase activity of AP-1 reporter in the presence of Wnt2. (I) JNK inhibitor inhibits the formation of beating EBs by dose dependent manner. *P<0.01, **P<0.05 versus control.

stem cell candidate, iPS cells, which can be used in further developmental studies and in clinical applications for the treatment of various diseases [2,48]. Next we have to generate human cardiomyocyte differentiation systems using Wnt2. And the functional properties of Wnt2 induced-cardiomyocyte should be examined to confirm that those cardiomyocytes have normal function for future cell replacement therapy. To advance regenerative medicine using human ES cell-derived or iPS-derived cardiomyocytes, Wnt2 treatment for cardiomyocyte induction is easy to implement and is valuable, and may be used in conjunction with other cardiomyocyte differentiation protocols. The present results indicate that Wnt2 plays a positive stage-specific role in cardiogenesis.

Supplementary materials related to this article can be found online at doi:10.1016/j.yjmcc.2011.11.010.

Disclosure of potential conflicts of interest

The authors indicate no potential conflicts of interest.

Acknowledgments

This study was supported in part by research grants from the Ministry of Education, Science and Culture, Japan, the Program for Promotion of Fundamental Studies in Health Science of the National Institute of Biomedical Innovation, Japan, and by the Keio Gijuku Academic Development Funds. We are grateful to Hitoshi Niwa for providing the ES cell line EB3, to Andrew P McMahon for the cDNAs of mouse *Wnt2*, and to Jan Kitajewski for the Wnt2 expression plasmid.

References

[1] Laflamme MA, Murry CE. Regenerating the heart. Nat Biotechnol 2005;23:845–56.
[2] Takahashi K, Yamanaka S. Induction of pluripotent stem cells from mouse embryonic and adult fibroblast cultures by defined factors. Cell 2006;126:663–76.
[3] Seki T, Yuasa S, Oda M, Egashira T, Yae K, Kusumoto D, et al. Generation of induced pluripotent stem cells from human terminally differentiated circulating T cells. Cell Stem Cell 2010;7:11–4.
[4] Yuasa S, Fukuda K. Recent advances in cardiovascular regenerative medicine: the induced pluripotent stem cell era. Expert Rev Cardiovasc Ther 2008;6:803–10.
[5] Shimoji K, Yuasa S, Onizuka T, Hattori F, Tanaka T, Hara M, et al. G-CSF promotes the proliferation of developing cardiomyocytes in vivo and in derivation from ESCs and iPSCs. Cell Stem Cell 2010;6:227–37.
[6] Fukuda K, Yuasa S. Stem cells as a source of regenerative cardiomyocytes. Circ Res 2006;98:1002–13.
[7] Cohen ED, Tian Y, Morrisey EE. Wnt signaling: an essential regulator of cardiovascular differentiation, morphogenesis and progenitor self-renewal. Development 2008;135:789–98.
[8] Marvin MJ, Di Rocco G, Gardiner A, Bush SM, Lassar AB. Inhibition of Wnt activity induces heart formation from posterior mesoderm. Genes Dev 2001;15:316–27.
[9] Schneider VA, Mercola M. Wnt antagonism initiates cardiogenesis in *Xenopus laevis*. Genes Dev 2001;15:304–15.
[10] Foley AC, Mercola M. Heart induction by Wnt antagonists depends on the homeodomain transcription factor Hex. Genes Dev 2005;19:387–96.
[11] Manisastry SM, Han M, Linask KK. Early temporal-specific responses and differential sensitivity to lithium and Wnt-3A exposure during heart development. Dev Dyn 2006;235:2160–74.
[12] Tzahor E, Lassar AB. Wnt signals from the neural tube block ectopic cardiogenesis. Genes Dev 2001;15:255–60.
[13] Yamashita JK, Takano M, Hiraoka-Kanie M, Shimazu C, Peishi Y, Yanagi K, et al. Prospective identification of cardiac progenitors by a novel single cell-based cardiomyocyte induction. FASEB J 2005;19:1534–6.

[14] Lickert H, Kutsch S, Kanzler B, Tamai Y, Taketo MM, Kemler R. Formation of multiple hearts in mice following deletion of beta-catenin in the embryonic endoderm. Dev Cell 2002;3:171–81.
[15] Nakamura T, Sano M, Songyang Z, Schneider MD. A Wnt- and beta-catenin-dependent pathway for mammalian cardiac myogenesis. Proc Natl Acad Sci U S A 2003;100:5834–9.
[16] Naito AT, Shiojima I, Akazawa H, Hidaka K, Morisaki T, Kikuchi A, et al. Developmental stage-specific biphasic roles of Wnt/beta-catenin signaling in cardiomyogenesis and hematopoiesis. Proc Natl Acad Sci U S A 2006;103:19812–7.
[17] Ueno S, Weidinger G, Osugi T, Kohn AD, Golob JL, Pabon L, et al. Biphasic role for Wnt/beta-catenin signaling in cardiac specification in zebrafish and embryonic stem cells. Proc Natl Acad Sci U S A 2007;104:9685–90.
[18] Kwon C, Arnold J, Hsiao EC, Taketo MM, Conklin BR, Srivastava D. Canonical Wnt signaling is a positive regulator of mammalian cardiac progenitors. Proc Natl Acad Sci U S A 2007;104:10894–9.
[19] Eisenberg CA, Gourdie RG, Eisenberg LM. Wnt-11 is expressed in early avian mesoderm and required for the differentiation of the quail mesoderm cell line QCE-6. Development 1997;124:525–36.
[20] Eisenberg CA, Eisenberg LM. WNT11 promotes cardiac tissue formation of early mesoderm. Dev Dyn 1999;216:45–58.
[21] Pandur P, Lasche M, Eisenberg LM, Kuhl M. Wnt-11 activation of a non-canonical Wnt signalling pathway is required for cardiogenesis. Nature 2002;418:636–41.
[22] Terami H, Hidaka K, Katsumata T, Iio A, Morisaki T. Wnt11 facilitates embryonic stem cell differentiation to Nkx2.5-positive cardiomyocytes. Biochem Biophys Res Commun 2004;325:968–75.
[23] Moriyama A, Kii I, Sunabori T, Kurihara S, Takayama I, Shimazaki M, et al. GFP transgenic mice reveal active canonical Wnt signal in neonatal brain and in adult liver and spleen. Genesis 2007;45:90–100.
[24] Yuasa S, Onizuka T, Shimoji K, Ohno Y, Kageyama T, Yoon SH, et al. Zac1 is an essential transcription factor for cardiac morphogenesis. Circ Res 2010;106:1083–91.
[25] Niwa H, Miyazaki J, Smith AG. Quantitative expression of Oct-3/4 defines differentiation, dedifferentiation or self-renewal of ES cells. Nat Genet 2000;24:372–6.
[26] Nagy A, Rossant J, Nagy R, Abramow-Newerly W, Roder JC. Derivation of completely cell culture-derived mice from early-passage embryonic stem cells. Proc Natl Acad Sci U S A 1993;90:8424–8.
[27] Hooper M, Hardy K, Handyside A, Hunter S, Monk M. HPRT-deficient (Lesch-Nyhan) mouse embryos derived from germline colonization by cultured cells. Nature 1987;326:292–5.
[28] Hara M, Yuasa S, Shimoji K, Onizuka T, Hayashiji N, Ohno Y, et al. G-CSF influences mouse skeletal muscle development and regeneration by stimulating myoblast proliferation. J Exp Med 2011;208:715–27.
[29] Yuasa S, Itabashi Y, Koshimizu U, Tanaka T, Sugimura K, Kinoshita M, et al. Transient inhibition of BMP signaling by Noggin induces cardiomyocyte differentiation of mouse embryonic stem cells. Nat Biotechnol 2005;23:607–11.
[30] Monkley SJ, Delaney SJ, Pennisi DJ, Christiansen JH, Wainwright BJ. Targeted disruption of the Wnt2 gene results in placentation defects. Development 1996;122:3343–53.
[31] Alexandrovich A, Arno M, Patient RK, Shah AM, Pizzey JA, Brewer AC. Wnt2 is a direct downstream target of GATA6 during early cardiogenesis. Mech Dev 2006;123:297–311.
[32] Tian Y, Yuan L, Goss AM, Wang T, Yang J, Lepore JJ, et al. Characterization and in vivo pharmacological rescue of a Wnt2-Gata6 pathway required for cardiac inflow tract development. Dev Cell 2010;18:275–87.
[33] Kispert A, Herrmann BG. Immunohistochemical analysis of the Brachyury protein in wild-type and mutant mouse embryos. Dev Biol 1994;161:179–93.
[34] Saga Y, Miyagawa-Tomita S, Takagi A, Kitajima S, Miyazaki J, Inoue T. MesP1 is expressed in the heart precursor cells and required for the formation of a single heart tube. Development 1999;126:3437–47.
[35] Recillas-Targa F, Pikaart MJ, Burgess-Beusse B, Bell AC, Litt MD, West AG, et al. Position-effect protection and enhancer blocking by the chicken beta-globin insulator are separable activities. Proc Natl Acad Sci U S A 2002;99:6883–8.
[36] West AG, Gaszner M, Felsenfeld G. Insulators: many functions, many mechanisms. Genes Dev 2002;16:271–88.
[37] DasGupta R, Fuchs E. Multiple roles for activated LEF/TCF transcription complexes during hair follicle development and differentiation. Development 1999;126:4557–68.
[38] Maretto S, Cordenonsi M, Dupont S, Braghetta P, Broccoli V, Hassan AB, et al. Mapping Wnt/beta-catenin signaling during mouse development and in colorectal tumors. Proc Natl Acad Sci U S A 2003;100:3299–304.
[39] Mohamed OA, Clarke HJ, Dufort D. Beta-catenin signaling marks the prospective site of primitive streak formation in the mouse embryo. Dev Dyn 2004;231:416–24.
[40] Yamaguchi TP, Takada S, Yoshikawa Y, Wu N, McMahon AP. T (Brachyury) is a direct target of Wnt3a during paraxial mesoderm specification. Genes Dev 1999;13:3185–90.
[41] Arnold SJ, Stappert J, Bauer A, Kispert A, Herrmann BG, Kemler R. Brachyury is a target gene of the Wnt/beta-catenin signaling pathway. Mech Dev 2000;91:249–58.
[42] Lindsley RC, Gill JG, Kyba M, Murphy TL, Murphy KM. Canonical Wnt signaling is required for development of embryonic stem cell-derived mesoderm. Development 2006;133:3787–96.
[43] Ishitani T, Kishida S, Hyodo-Miura J, Ueno N, Yasuda J, Waterman M, et al. The TAK1-NLK mitogen-activated protein kinase cascade functions in the Wnt-5a/Ca^{2+} pathway to antagonize Wnt/{beta}-catenin signaling. Mol Cell Biol 2003;23:131–9.
[44] Tzahor E. Wnt/[beta]-catenin signaling and cardiogenesis: timing does matter. Dev Cell 2007;13:10–3.
[45] Logan CY, Nusse R. The Wnt signaling pathway in development and disease. Annu Rev Cell Dev Biol 2004;20:781–810.
[46] Wang H, Gilner JB, Bautch VL, Wang D-Z, Wainwright BJ, Kirby SL, et al. Wnt2 coordinates the commitment of mesoderm to hematopoietic, endothelial, and cardiac lineages in embryoid bodies. J Biol Chem 2007;282:782–91.
[47] Kispert A, Vainio S, Shen L, Rowitch DH, McMahon AP. Proteoglycans are required for maintenance of Wnt-11 expression in the ureter tips. Development 1996;122:3627–37.
[48] 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.

Heart Vessels (2012) 27:528–531
DOI 10.1007/s00380-011-0208-3

CASE REPORT

A case of severe aortic stenosis with severe coronary artery disease that was successfully treated by balloon aortic valvuloplasty and percutaneous coronary intervention

Yuichiro Maekawa · Akio Kawamura · Akira Furuta · Shinsuke Yuasa · Keiichi Fukuda

Received: 27 June 2011 / Accepted: 14 October 2011 / Published online: 11 November 2011

Abstract We describe an 85-year-old woman with severe aortic stenosis, who also had severe coronary artery disease. She suffered from dyspnea on exertion and frequent syncope. Echocardiography revealed an immobile and heavily calcified aortic valve, and coronary angiography revealed two-vessel disease including chronic total occlusion. Open-heart surgery was refused and she was referred to our department. She underwent percutaneous coronary intervention (PCI) for the right coronary artery and left anterior descending artery. Following PCI, percutaneous balloon aortic valvuloplasty (BAV) was performed on the same day. We chose balloons of 15 × 60 mm, 18 × 60 mm, and 20 × 60 mm, respectively. Improvement in the mean aortic valve pressure gradient (PG) and calculated aortic valve area (mean PG 48–23 mmHg, 0.8–1.2 cm^2, respectively) was observed after the final balloon dilatation. No significant complications occurred. The combination of BAV with PCI may be a useful treatment for relief of the associated symptoms of severe aortic stenosis and coronary artery disease, though it does not improve the long-term prognosis.

Keywords Valvuloplasty · Percutaneous coronary intervention · Aortic stenosis · Chronic total occlusion

Introduction

The aging population is rapidly increasing in developed countries. The number of elderly patients with cardiovascular disease is growing, and coronary artery disease and aortic stenosis are frequently observed in elderly patients [1, 2]. Elderly patients also have more concomitant diseases, including peripheral artery disease, cerebrovascular disease, and chronic kidney disease. These diseases strongly affect the perioperative risk for elderly patients and contribute to the prognosis of elderly patients. In inoperable patients with severe aortic stenosis (AS), transcatheter aortic valve implantation (TAVI) is an effective treatment modality [3]. High-risk inoperable patients with severe AS as well as significant coronary artery disease (CAD) requiring revascularization, however, are not eligible for TAVI. In these patients, balloon aortic valvuloplasty (BAV) is currently the only method used to alleviate symptoms associated with severe AS, such as dyspnea and syncope, though long-term survival after BAV is poor. Here we report the case of an elderly female patient with severe AS and CAD including chronic total occlusion, who underwent BAV and percutaneous coronary intervention (PCI) on the same day.

Case report

An 85-year-old woman was admitted to our hospital with dyspnea (New York Heart Association functional class III) and frequent syncope. A transthoracic echocardiogram revealed AS with a heavily calcified trileaflet valve area of 0.76 cm^2, mean pressure gradient of 48 mmHg, and normal left ventricular function. Coronary angiography revealed an occluded right coronary artery (RCA) and severe stenosis of the left anterior descending (LAD) coronary artery (Fig. 1a, b). Open-heart surgery was refused because of old age and multiple comorbidities, and the patient was referred to our department. The predicted mortality estimated by the logistic

Y. Maekawa (✉) · A. Kawamura · A. Furuta · S. Yuasa · K. Fukuda
Department of Cardiology, Keio University School of Medicine, Shinanomahi 35, Shinjuku-ku, Tokyo 160-8582, Japan
e-mail: ymaekawa@gmail.com

EuroSCORE was 17.4%, and she suffered from rheumatoid arthritis, interstitial pneumonia, anemia, chronic kidney disease (stage III), diabetes mellitus, and hypertension.

Percutaneous coronary intervention procedure

After providing informed consent, the patient underwent PCI of the RCA and LAD. A 6-Fr sheath was inserted into the right femoral artery and a 6-Fr guiding catheter (AL 0.75; Medtronic, Minneapolis, MN, USA) engaged in the RCA. A 0.010-inch guide wire (Wizard 3 g; Japan Lifeline, Tokyo, Japan), supported by a microcatheter (Finecross, Terumo, Japan) was successfully advanced to the distal end of the occluded RCA. Balloon catheters (1.5 and 2.0 mm) were used to expand the occluded artery and a drug-eluting stent (DES) (Promus 2.5 × 28 mm; Boston Scientific, Natick, MA, USA) was implanted. Intravascular ultrasound revealed the satisfactory result that the stent was fully expanded (Fig. 1c). Following the RCA, we performed PCI of the LAD, which showed severe stenosis. A 6-Fr guiding catheter (JL 4.0; Medtronic) was engaged in the LAD and a DES (Promus 2.5 × 23 mm; Boston Scientific) was implanted after balloon predilation. Intravascular ultrasound was then performed to confirm the stent apposition (Fig. 1d).

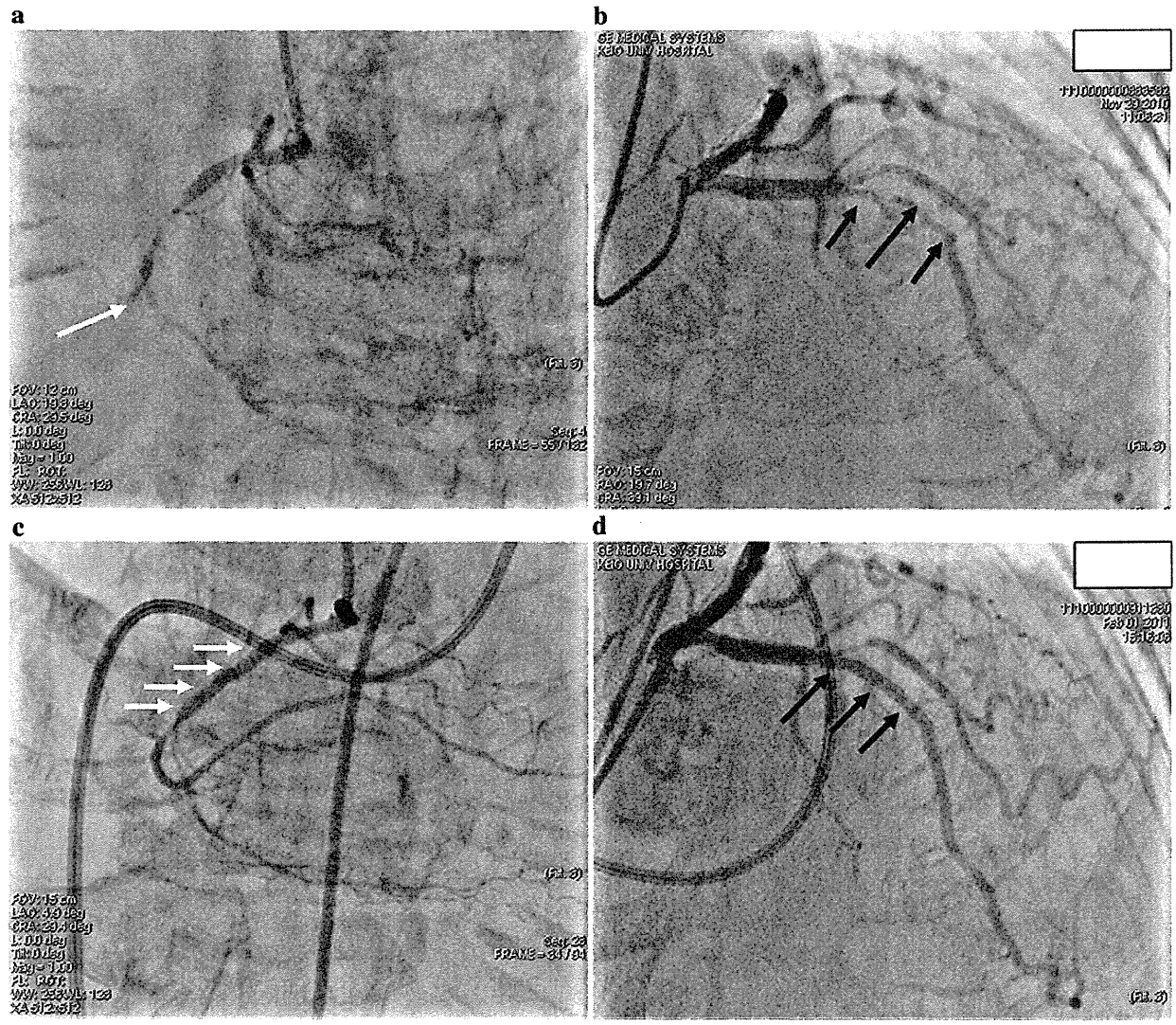

Fig. 1 Coronary angiograms of **a** the right coronary artery (RCA) and **b** the left anterior descending artery (LAD) before percutaneous coronary intervention (PCI). Left anterior oblique cranial view showing the occluded mid-RCA (*white arrow*). Right anterior oblique cranial view showing severe stenosis of the mid-LAD (*black arrows*). Coronary angiograms of **c** the RCA and **d** the LAD after PCI. A drug-eluting stent was successfully implanted in the RCA (*white arrows*) and the left coronary artery (*black arrows*), respectively

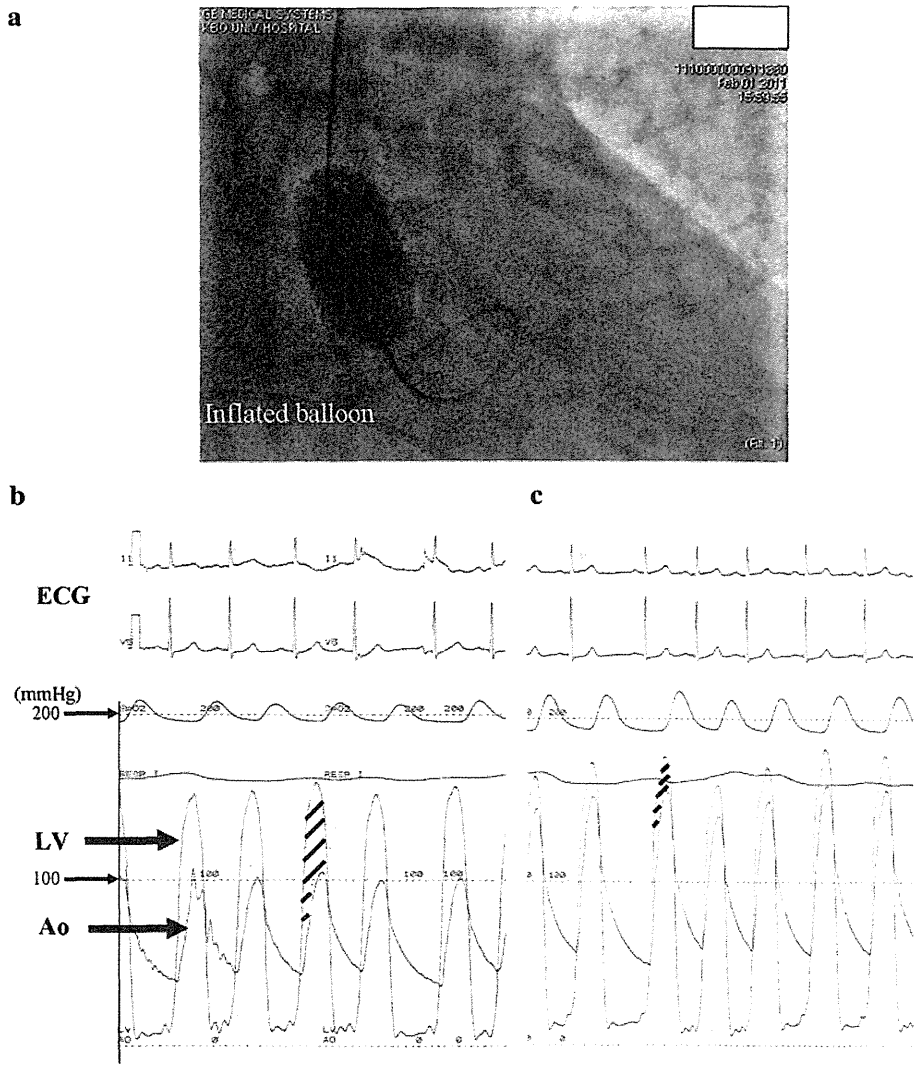

Fig. 2 **a** Balloon aortic valvuloplasty (BAV) was performed. The heart was paced at a high rate until the blood pressure fell to 50 mmHg before inflation. The left ventricular (*LV*) and aortic (*Ao*) pressure tracing **b** before BAV and **c** after BAV. The LV to aortic mean pressure gradient dramatically decreased after BAV (*dashed area*). *ECG* electrocardiogram

Balloon aortic valvuloplasty procedure

A 14-Fr sheath was inserted into the right femoral artery. Balloon aortic valvuloplasty was performed using the retrograde femoral approach. To stabilize the balloon position across the valve, the heart was paced at a high rate (200 beats/min) until the blood pressure fell to 50 mmHg before inflation. Pacing was continued until the balloon was fully deflated. Tyshak® (NuMED, Hopkinton, NY, USA) balloons of 15 × 60 mm, 18 × 60 mm, and 20 × 60 mm, respectively, were placed across the aortic valve (Fig. 2a). After inflation of each balloon, the left ventricle to aortic pressure gradient was markedly reduced (Fig. 2b, c), and there was no increase in aortic regurgitation. Finally the aortic valve area was 1.2 cm^2 and the mean pressure gradient was 23 mmHg. She was discharged to her home 4 days after the procedure. One month later, her dyspnea had greatly improved (New York Heart Association functional class II).

Discussion

We describe an inoperable high-risk patient with severe AS and CAD treated with BAV and PCI.

It is not uncommon to improve symptomatic status in hospital and within 30 days of the BAV in AS patients [4, 5]. However, long-term outcome of patients with AS after BAV alone is poor [6–8]. Nowadays aortic valve replacement can be performed in octogenarians, with acceptable mortality [9]. Transcatheter aortic valve implantation is a possible alternative treatment for inoperable high-risk patients with AS [10], but some older patients are not eligible candidates for TAVI based on strict inclusion criteria of the PARTNER

trial [3]. Although BAV should be used as a bridge to percutaneous or surgical valve replacement therapy [11], it effectively alleviates the patient's symptoms in selected cases of AS, especially in inoperable high-risk patients with significant CAD. However, improvement in symptomatic status is observed only for a short period [4]. In the present case, surgical or percutaneous aortic valve replacement was not considered feasible due to the patient's low respiratory function, higher age, and severe CAD. Therefore, we selected BAV to relieve the associated symptoms of AS, although the restenosis rate after BAV is high. In addition, in some patients with severe AS and severe CAD, performing BAV and PCI on the same day can dramatically improve the patient's symptoms.

The 0.010-inch system is safe and practical for the treatment of chronic total occlusions [12]. Consistent with the results reported in the PIKACHU registry, the success rate of the initial 0.010-inch guide-wire passage is high in our institute. We used the 0.010-inch system for chronic total occlusion of the RCA, and the guide wire easily passed through the target lesion.

In patients with severe AS and CAD, there is no definite answer to the question of whether BAV or PCI should be performed first. Although symptoms associated with AS or CAD may be important aspects in answering this question, it is very difficult to distinguish the symptoms of AS from those of CAD. In patients with severe AS and unstable angina or myocardial infarction, PCI should be performed first, whereas in patients with severe AS accompanied by dyspnea and chest pain but no new ECG changes or serum troponin T elevation, BAV should be performed first.

To our knowledge, this is the first reported case of BAV and PCI performed on the same day for severe AS and CAD, including chronic total occlusion. Therefore, in selected patients, the combination of BAV with PCI may be a useful treatment for relief of the associated symptoms of severe AS and CAD, though it does not improve the long-term prognosis.

References

1. Nkomo VT, Gardin JM, Skelton TN, Gottdiener JS, Scott CG, Enriquez-Sarano M (2006) Burden of valvular heart diseases: a population-based study. Lancet 368:1005–1011
2. Shirasawa K, Hwang MW, Sasaki Y, Takeda S, Inenaga-Kitaura K, Kitaura Y, Kawai C (2011) Survival and changes in physical ability after coronary revascularization for octa-nonagenerian patients with acute coronary syndrome. Heart Vessels 26:385–391
3. Leon MB, Smith CR, Mack M, Miller DC, Moses JW, Svensson LG, Tuzcu EM, Webb JG, Fontana GP, Makkar RR, Brown DL, Block PC, Guyton RA, Pichard AD, Bavaria JE, Herrmann HC, Douglas PS, Petersen JL, Akin JJ, Anderson WN, Wang D, Pocock S (2010) Transcatheter aortic-valve implantation for aortic stenosis in patients who cannot undergo surgery. N Engl J Med 363:1597–1607
4. Cribier A, Savin T, Saoudi N, Rocha P, Berland J, Letac B (1986) Percutaneous transluminal valvuloplasty of acquired aortic stenosis in elderly patients: an alternative to valve replacement? Lancet 1:63–67
5. Percutaneous balloon aortic valvuloplasty. Acute and 30-day follow-up results in 674 patients from the NHLBI Balloon Valvuloplasty Registry (1991) Circulation 84:2383–2397
6. Lewin RF, Dorros G, King JF, Mathiak L (1989) Percutaneous transluminal aortic valvuloplasty: acute outcome, follow-up of 125 patients. J Am Coll Cardiol 14:1210–1217
7. Otto CM, Mickel MC, Kennedy JW, Alderman EL, Bashore TM, Block PC, Brinker JA, Diver D, Ferguson J, Holmes DR Jr, Lambrew CT, McKay CR, Palacios IF, Powers ER, Rahimtoola SH, Weiner BH, Davis KB (1994) Three-year outcome after balloon aortic valvuloplasty. Insights into prognosis of valvular aortic stenosis. Circulation 89:642–650
8. Lieberman EB, Bashore TM, Hermiller JB, Wilson JS, Pieper KS, Keeler GP, Pierce CH, Kisslo KB, Harrison JK, Davidson CJ (1995) Balloon aortic valvuloplasty in adults: failure of procedure to improve long-term survival. J Am Coll Cardiol 26:1522–1528
9. Aoyagi S, Fukunaga S, Arinaga K, Tomoeda H, Akasu K, Ueda T (2010) Heart valve surgery in octogenarians: operative and long-term results. Heart Vessels 25:522–528
10. Vavuranakis M, Vrachatis DA, Toutouzas K, Economopoulos G, Kalogeras KI, Stefanadis C (2010) Successful percutaneous aortic valve implantation via a stenotic left subclavian artery access. Heart Vessels 25:359–362
11. Ben-Dor I, Pichard AD, Satler LF, Goldstein SA, Syed AI, Gaglia MA Jr, Weissman G, Maluenda G, Gonzalez MA, Wakabayashi K, Collins SD, Torguson R, Okubagzi P, Xue Z, Kent KM, Lindsay J, Waksman R (2010) Complications and outcome of balloon aortic valvuloplasty in high-risk or inoperable patients. JACC Cardiovasc Interv 3:1150–1156
12. Matsukage T, Masutani M, Yoshimachi F, Takahashi A, Katsuki T, Saito S, Terai H, Katahira Y, Uehara Y, Tohara S, Ohba Y, Shinohara S, Asano H, Matsumura T, Hata T, Ikari Y (2010) A prospective multicenter registry of 0.010-inch guidewire and compatible system for chronic total occlusion: the PIKACHU registry. Catheter Cardiovasc Interv 75:1006–1012

SAGE-Hindawi Access to Research
Stem Cells International
Volume 2011, Article ID 348960, 7 pages
doi:10.4061/2011/348960

Review Article

Induced Pluripotent Stem Cells in Cardiovascular Medicine

Toru Egashira,[1] Shinsuke Yuasa,[1,2] and Keiichi Fukuda[1]

[1] *Department of Cardiology, Keio University School of Medicine, 35 Shinanomachi, Shinjuku, Tokyo 160-8582, Japan*
[2] *Center for Integrated Medical Research, Keio University School of Medicine, 35 Shinanomachi, Shinjuku, Tokyo 160-8582, Japan*

Correspondence should be addressed to Keiichi Fukuda, kfukuda@sc.itc.keio.ac.jp

Received 22 December 2010; Revised 25 May 2011; Accepted 18 July 2011

Academic Editor: Randall J. Lee

Induced pluripotent stem (iPS) cells are generated by reprogramming human somatic cells through the forced expression of several embryonic stem (ES) cell-specific transcription factors. The potential of iPS cells is having a significant impact on regenerative medicine, with the promise of infinite self-renewal, differentiation into multiple cell types, and no problems concerning ethics or immunological rejection. Human iPS cells are currently generated by transgene introduction principally through viral vectors, which integrate into host genomes, although the associated risk of tumorigenesis is driving research into nonintegration methods. Techniques for pluripotent stem cell differentiation and purification to yield cardiomyocytes are also advancing constantly. Although there remain some unsolved problems, cardiomyocyte transplantation may be a reality in the future. After those problems will be solved, applications of human iPS cells in human cardiovascular regenerative medicine will be envisaged for the future. Furthermore, iPS cell technology has generated new human disease models using disease-specific cells. This paper summarizes the progress of iPS cell technology in cardiovascular research.

1. Introduction

Cardiovascular disease remains a major cause of mortality in developed countries, with severe heart failure being the leading cause of cardiac death [1]. There is no fundamental therapy for refractory heart failure other than heart transplantation, which is not regarded as a robust option because of the associated problems such as too few donors and immunological rejection [2]. A clear need, therefore, exists for novel therapies for severe cardiovascular disease, and recent advances in stem cell biology have indicated that regenerative medicine might meet that need in the future.

Stem cells are defined by the characteristics of self-renewal and differentiation capability to multiple tissues. These cells thus hold great promise as a source for cell transplantation therapy. Embryonic organs rapidly develop, and embryonic cells have a strong proliferation potential. In particular, embryonic stem (ES) cells that are established from the inner cell mass of the morula stage of mammalian embryos are regarded as a powerful cell source for clinical application because of their promising growth potential and pluripotency. However, there are major obstacles with the use of human ES cells such as bioethical issues surrounding the destruction of a fertilized egg and immune rejection due to alloantigens following transplantation. Adult organs are also seen as a potential source of somatic stem cells with the ability to proliferate and differentiate into the specific cells in each tissue and thus help to maintain tissue homeostasis and organ healing after injury. Adult heart, for instance, contains cardiac stem cells that can be differentiated into mature cardiomyocytes and vascular cells, and is, therefore, a candidate source for cardiac cell transplantation therapy [3, 4]. However, there are not sufficient numbers of somatic stem cells in adult organs and the proliferation and differentiation ability of somatic stem cells is limiting compared to that of ES cells.

The recent generation of induced pluripotent stem (iPS) cells has created new hope in the field of regenerative medicine for overcoming the dilemma of ES cells. iPS cells can be generated from somatic cells by introducing defined reprogramming factors (Oct3/4, Sox2, Klf4, c-Myc, Nanog, and Lin28) in various mammals including mouse, rat, pig, monkey, and human [5–10]. These cells have the two crucial stem cell characteristics of self-renewal and pluripotency. In addition, the ethical issues associated with generating iPS cells are trivial compared to those for ES cells because

the sacrifice of an embryo is dispensable, and the use of the recipient's own cells eliminates the risk of immune rejection generally associated with transplantation. Because of these tremendous benefits, iPS cells hold great promise as a novel cell source for regenerative medicine. Moreover, the unique characteristics harbored in the genetic information of almost every adult cell could allow the creation of patient-specific pluripotent stem cells, which can be then dedicated to any cell type in vitro. Consequently, iPS cell technology has raised concerns in the fields of disease pathogenesis and drug discovery [11–13].

2. iPS Induction Methods

ES cells have the ability to proliferate infinitely and can differentiate into the cell types of all three germ layers with germ line transmission [14]. The first human ES cells were successfully generated in 1998 [15]. Human iPS cells were established in 2007 by the transduction of various gene cocktails [6, 7]. The characteristics of iPS cells are quite similar to those of ES cells in terms of morphology, proliferation ability, patterning of global gene expression, and the epigenetic status of promoter regions for stem-cell-specific transcription factors. As a matter of course, these iPS cells also can differentiate into cells of the three germ layers in vitro and in vivo.

For iPS cell technology to realize its significant promise for advanced regenerative medicine, several difficult problems must be addressed. The original method of generating iPS cells involves the transduction of core transcription factors into somatic cells by retroviral vectors with the accompanying insertion of transgenes into the host genome. These transgenes could theoretically disrupt the native genes and/or alter normal gene expression. Although the expression of such viral-mediated exogenous genes are virtually silenced once the cells are fully reprogrammed into iPS cells, small but sustained leakage of transgene expression or the reactivation of those transgenes occasionally disturbs the differentiation and maintenance of an undifferentiated state [16]. In particular, oncogenic transgene reactivation such as that of *c-Myc* could increase the risk of tumor formation after transplantation of the iPS-derived cells [17]. In addition, the detail mechanism of reprogramming is not yet elucidated sufficiently, and the reprogramming efficiency of somatic cells is still low. These problems need to be at least minimized before the application of iPS technology in the clinical setting can proceed.

To establish safer iPS cells, several novel methods are being investigated that do not involve transgene insertions into the host genome. Various gene-delivery systems have been developed for the reprogramming of human somatic cells into iPS cells [18–24]. In addition, some chemical compounds can raise the reprogramming efficiency and reduce the required number of the transducing factors to generate iPS cells [25–28]. There are many reports of somatic cells being reprogrammed to iPS cells, although most cases involve dermal fibroblasts obtained from a dermal punch biopsy, which leaves a small scar on the donor's skin. Such skin biopsies should be avoided to generate human iPS cells. Recently, we and several other groups reported novel methods for generating iPS cells less invasively from human circulating peripheral blood cells [29–32]. Our method uses Sendai viral systems to deliver the reprogramming factors [33]. Sendai virus is categorized as a negative-strand RNA virus that cannot integrate into the host genome, but can highly infect activated T cells [34]. It is very important for human clinical usage to obtain the nonintegration human iPS cell easily, stably, and efficiently and attenuate the physical invasion for donors to obtain somatic cells. These improved methods can be helpful to extend the indication of generating iPS cells and to be easily accessible to realize the future clinical application of the iPS technology.

3. Differentiation into Cardiomyocytes from Pluripotent Stem Cells

ES cells and iPS cells can give rise to all cell types of all three germ layers, using quite similar methodologies to control the differentiation. In the conventional method, ES cell differentiation is performed through embryoid bodies (EBs), which are aggregates of ES cells maintained in suspension cultures. In this section, we focus on the differentiation systems for generating cardiomyocytes from ES cells and iPS cells.

A recent report indicated no significant difference in the fundamental characteristics of cardiomyocytes differentiated from either ES or iPS cells [35–38]. However, the efficiency of cardiogenesis from ES and iPS cells is still too low and not sufficiently stable to realize the goals of cardiac regenerative medicine. Generally considered, the mechanisms of in vitro differentiation from pluripotent stem cells are similar to the regulatory mechanisms of normal early development. To improve the efficiency of in vitro cardiogenesis, various screens for essential signalling molecules in normal heart development have been performed. Among several signal proteins associated with cardiac development, canonical Wnt/beta-catenin [39–42], activin/nodal [43, 44], and BMP signaling [45–51] have the crucial roles in normal heart development, and supplementation of those molecules into ES/iPS cell differentiation systems could significantly boost the efficiency of cardiogenesis. Moreover, it also seems that bidirectional stimulation in these pathways is necessary at different developmental stages [52, 53].

We previously reported that transit inhibition of BMP signaling very early during differentiation is crucial for cardiogenesis in murine ES cells [54]. Whole-mount in situ hybridization for various BMP antagonists on mouse embryos at different developmental stages revealed that noggin, a BMP antagonist, was expressed only briefly in the heart-forming area. This phenomenon was also observed in early embryos of chick and *Xenopus*, suggesting a conserved mechanism in heart development [55, 56]. We proposed that administration of noggin before the EB formation stage would mimic its transient and strong expression during early gastrulation. Indeed, noggin administration around EB formation day led to a marked increase in cardiogenesis

from murine ES cells. Other BMP antagonists also act to increase cardiomyocyte differentiation efficiency, indicating that transient relief from the intrinsic BMP signal is critical for cardiomyocyte induction.

We also identified growth factors crucial for embryonic premature cardiomyocytes through the screening of global gene expressions in noggin-treated mouse ES cell-derived cardiomyocytes by microarray analysis [57]. We found that the expression level of *csf3r*, which encodes the granulocyte colony-stimulating factor receptor (G-CSFR), was significantly higher in cardiomyocyte-differentiating ES cells [58], and confirmed that both G-CSFR and G-CSF were specifically but transiently expressed in embryonic mouse heart at the midgestational stage. When extrinsic G-CSF was administered to ES/iPS cell-derived cardiomyocytes, it markedly augmented their proliferation. These findings indicated that G-CSF is critical for cardiomyocyte proliferation and could be used to boost the yield of cardiomyocytes from ES/iPS cells for their potential application in regenerative medicine.

To some extent, we can manage to differentiate pluripotent stem cells into cardiomyocytes effectively and control the proliferation of premature cardiomyocytes. However, human heart is a large organ, and huge numbers of cardiomyocytes would be needed for human cardiac regenerative therapy, requiring even more sophisticated methods for cardiomyocyte generation. To this end, prominent differences have been demonstrated in the ability to differentiate into specific lineage cells among iPS cell [59]. Moreover, their response to various growth factor and cytokine stimulations also varied markedly among cell lines. Recent studies have also demonstrated that the characteristics of iPS cells are influenced by the derived somatic cells according to their epigenetic memory [60–62]. It would, therefore, be preferable to ascertain which cell lines are the best sources of iPS cells for each lineage cell differentiation and what is the best way to select such iPS cell lines before starting the differentiation.

4. Stem Cell-Based Cardiac Regeneration

ES and iPS cells both have the potential to be a definite cell source for regenerative therapy because they can proliferate infinitely. In the cardiovascular field, regenerative cell therapy using pluripotent cells is expected to complement rather than replace heart transplantation in the future [12, 63, 64]. Molecular biological and physiological studies have demonstrated that ES and iPS cell-derived cardiomyocytes have the required cardiovascular function [65–67]. iPS cells can differentiate into several types of cardiomyocytes including atrial, nodal, and ventricular cells, with similar properties to native cardiomyocytes. Cardiomyocytes derived from iPS cells properly express typical ion channels with the expected functional responses to several ion channel blockers [68]. There are also many reports that the transplantation of either cardiomyocytes or cardiac progenitor cells derived from human ES cells into infarcted rodent heart could improve cardiac function [50, 69, 70]. Transplanted ES/iPS cell-derived cardiomyocytes were integrated into the infarcted host heart and supplied working muscles functioning cooperatively, while the molecules secreted in a paracrine or autocrine fashion from transplanted cells are also important. It was also reported that the transplantation of iPS cells into the postinfarcted heart of immunocompetent mouse significantly recovered the cardiac function of failing heart and transplanted cells were successfully differentiated into cardiomyocytes, smooth muscles, and endothelial cells in the heart [71]. The precise mechanisms underpinning these successful cell transplantations remain unclear. However, accumulating evidence indicates that the transplantation of iPS cell-derived cardiomyocytes will be a viable future alternative for treating diseased heart.

One of the most critical issues in these efforts is how to eliminate undifferentiated ES/iPS cells from the transplanting cells. Undifferentiated pluripotent stem cells implanted into the recipient body increase concerns about tumors like teratoma emerging from the transplanted cells [72]. Although it is not yet determined whether terminally differentiated cells or progenitor cells are better for cell transplants, undifferentiated pluripotent stem cells need to be eliminated from the equation before transplantation in any case to relieve the risk of tumorigenesis. A variety of purification methods for ES/iPS cell-derived differentiated cells have been developed, with most involving gene manipulation to label the selection marker such as GFP regulated under a specific promoter [73, 74]. However, gene modification in itself could also induce tumorigenesis. The ideal method to purify the cells would, therefore, not use genetic manipulation. We recently obtained highly pure (>99% purity) human ES/iPS cell-derived cardiomyocytes by fluorescence-activated cell sorting with the fluorescent dye that labels mitochondria [75]. This method involves no gene modification and could be used in the clinical setting. In addition, cell surface proteins in nascent cardiomyocytes and cardiomyogenic progenitors have been defined as non genetic selection markers for cardiomyocytes in the mouse ES/iPS cells [43, 76–80]. Such methodology to purify the objective cells is constantly advancing the goals for cardiac regenerative therapy. Long-term followup is necessary to analyze recipient safety and prognosis, and all cell transplantation should be tested in a large animal model before clinical usage.

5. Genetic and Epigenetic Profile of iPS Cell and iPS Cell-Derived Cardiomyocyte

There are several concerns about using iPS cell for regenerative medicine, because accumulating genetic and epigenetic profiles proved there remain important problems. Initial study about epigenetic memory showed that iPS cells derived from adult murine tissues harbour residual DNA methylation signatures characteristic of their somatic tissue of origin, which favours their differentiation along lineages related to the donor cell, which suggest an epigenetic memory of the tissue of origin that may have influence on directed differentiation for applications in disease modelling or treatment [61]. In terms of pluripotent stem cell-derived cardiomyocyte, iPS cell-derived cardiomyocytes are

transcriptionally highly similar to ES cell-derived cardiomyocytes, but iPS cell-derived cardiomyocyte also still possesses the same somatic signature [81]. Exome analysis revealed that hiPS cells acquire genetic modifications in addition to epigenetic modifications which suggest extensive genetic screening should become a standard procedure to ensure hiPS cell safety before clinical usage [82]. Surprisingly latest research showed that abnormal gene expression in some cells differentiated from iPSCs can induce T-cell-dependent immune response in syngeneic recipients in contrast to derivatives of ESCs [83]. The expression of these abnormal minor antigens also may be due to epigenetic difference between iPS cells and ES cells. These data suggests that extensive genetic and epigenetic screening should become a standard procedure to ensure hiPS cell safety before clinical use.

6. Investigation of Patient-Specific iPS Cells

Another expectation for iPS cells is the generation of human disease-specific pluripotent stem cells, since established iPS cells have identical genetic mutations to the source tissue. Patient-specific iPS cells can differentiate into any type of cell, allowing us to directly and repetitively analyse the diseased cells in vitro. We now need to establish a revolutionary assay system to elucidate the disease pathogenesis and to use these patient-specific cells as a novel tool for drug screening in combination with personalized medicine. Generally, it is difficult or impossible to take large samples from specific patient tissues like neurons and cardiomyocytes and, even if possible, such terminally differentiated cells cannot proliferate sufficiently in vitro. For that reason, researchers in this field eagerly await this kind of promising stem cell.

To date, many kinds of patient-specific iPS cells have been generated to diseases such as adenosine deaminase deficiency, the Schwachman-Bodian-Diamond syndrome, Gaucher's disease, the Duchenne and Becker muscular dystrophy, Parkinson's disease, Huntington's disease, type 1 diabetes mellitus, Down's syndrome, the Lesch-Nyhan syndrome, amyotrophic lateral sclerosis, spinal muscular atrophy, and Fanconi's anemia [84–88]. According to these previous reports, iPS cells generated from a patient suffering from early-onset disease are more likely to closely reproduce the disease phenotypes. Recently, cardiac disease-specific iPS cells were also reported. One line is specific for the LEOPARD syndrome, which is an autosomal dominant developmental disorder belonging to a relatively prevalent class of inherited RAS-mitogen-activated protein kinase signalling diseases with pleomorphic effects on several tissues and organ systems [89]. The patient has a mutation in the PTPN11 gene, which encodes the SHP2 phosphatase. A major disease phenotype in patients with the LEOPARD syndrome is hypertrophic cardiomyopathy. This study showed that cardiomyocytes derived from LEOPARD syndrome iPS cells are spontaneously hypertrophied in vitro and have a higher degree of sarcomeric organization compared with cardiomyocytes derived from human ES cells or wild-type iPS cells. They concluded that these features correlate with a potential hypertrophic state in patients. The other reported disease-specific iPS cell line mimics congenital long QT syndromes (LQTs) [90], which are heritable diseases associated with prolongation of the QT interval on an electrocardiogram and a high risk of sudden cardiac death due to ventricular tachyarrhythmia. The authors generated iPS cells from two patients with LQTs type 1 (LQTs 1), who have mutations in the KCNQ1 gene encoding the repolarizing potassium channel mediating the delayed rectifier I (Ks) current. Individual cardiomyocytes derived from LQTs 1 patients showed prolonged action potentials compared with cells from control subjects. Moreover, cardiomyocytes derived from patients with LQTs 1 had an increased susceptibility to catecholamine-induced tachyarrhythmia, and the phenotype was attenuated by beta-blockade, which is one of the most important clinical features of these syndromes. The study showed that LQTs 1 patient-specific iPS cell-derived cardiomyocytes totally reproduced the diseased phenotypes in a clinical setting.

These studies into patient-specific iPS cells indicate a tremendous potential for our increased understanding of pathogenesis. Such technologies will be the basis for novel industries in drug development and diagnostics.

7. Conclusions

Although iPS cells are artificial pluripotent stem cells, they can produce chimeric animals in mouse and rat, and a tetraploid complementation experiment demonstrated that mouse iPS cells have the ability to autonomously generate full-term mice. These results clarified the existence of pure pluripotent stem cells in iPS cells. Methods for the generation of iPS cells have now undergone tremendous and steady improvements. Although some problems remain such as genetic mutation during reprogramming, incomplete epigenetic reprogramming, and undesired gene expression, iPS cells could be applicable for regenerative medicine in the future after solving those problems. Studies of disease pathogenesis and drug discovery using this technology have already been undertaken and will shed light on the discovery of novel treatments for fatal cardiovascular diseases.

References

[1] S. Neubauer, "The failing heart—an engine out of fuel," *New England Journal of Medicine*, vol. 356, no. 11, pp. 1140–1151, 2007.

[2] D. Mancini and K. Lietz, "Selection of cardiac transplantation candidates in 2010," *Circulation*, vol. 122, no. 2, pp. 173–183, 2010.

[3] L. Bu, X. Jiang, S. Martin-Puig et al., "Human ISL1 heart progenitors generate diverse multipotent cardiovascular cell lineages," *Nature*, vol. 460, no. 7251, pp. 113–117, 2009.

[4] A. P. Beltrami, L. Barlucchi, D. Torella et al., "Adult cardiac stem cells are multipotent and support myocardial regeneration," *Cell*, vol. 114, no. 6, pp. 763–776, 2003.

[5] K. Takahashi and S. Yamanaka, "Induction of pluripotent stem cells from mouse embryonic and adult fibroblast cultures by defined factors," *Cell*, vol. 126, no. 4, pp. 663–676, 2006.

[6] K. Takahashi, K. Tanabe, M. Ohnuki et al., "Induction of pluripotent stem cells from adult human fibroblasts by defined factors," *Cell*, vol. 131, no. 5, pp. 861–872, 2007.

[7] J. Yu, M. A. Vodyanik, K. Smuga-Otto et al., "Induced pluripotent stem cell lines derived from human somatic cells," *Science*, vol. 318, no. 5858, pp. 1917–1920, 2007.

[8] J. Liao, C. Cui, S. Chen et al., "Generation of induced pluripotent stem cell lines from adult rat cells," *Cell Stem Cell*, vol. 4, no. 1, pp. 11–15, 2009.

[9] M. A. Esteban, J. Xu, J. Yang et al., "Generation of induced pluripotent stem cell lines from Tibetan miniature pig," *Journal of Biological Chemistry*, vol. 284, no. 26, pp. 17634–17640, 2009.

[10] I. Tomioka, T. Maeda, H. Shimada et al., "Generating induced pluripotent stem cells from common marmoset (Callithrix jacchus) fetal liver cells using defined factors, including Lin28," *Genes to Cells*, vol. 15, no. 9, pp. 959–969, 2010.

[11] S. Yuasa and K. Fukuda, "Cardiac regenerative medicine," *Circulation Journal*, vol. 72, pp. A49–A55, 2008.

[12] S. Yuasa and K. Fukuda, "Recent advances in cardiovascular regenerative medicine: the induced pluripotent stem cell era," *Expert Review of Cardiovascular Therapy*, vol. 6, no. 6, pp. 803–810, 2008.

[13] Y. Yoshida and S. Yamanaka, "IPS cells: a source of cardiac regeneration," *Journal of Molecular and Cellular Cardiology*, vol. 50, no. 2, pp. 327–332, 2011.

[14] M. J. Evans and M. H. Kaufman, "Establishment in culture of pluripotential cells from mouse embryos," *Nature*, vol. 292, no. 5819, pp. 154–156, 1981.

[15] J. A. Thomson, "Embryonic stem cell lines derived from human blastocysts," *Science*, vol. 282, no. 5391, pp. 1145–1147, 1998.

[16] T. Brambrink, R. Foreman, G. G. Welstead et al., "Sequential expression of pluripotency markers during direct reprogramming of mouse somatic cells," *Cell Stem Cell*, vol. 2, no. 2, pp. 151–159, 2008.

[17] K. Okita, T. Ichisaka, and S. Yamanaka, "Generation of germline-competent induced pluripotent stem cells," *Nature*, vol. 448, no. 7151, pp. 313–317, 2007.

[18] W. Zhou and C. R. Freed, "Adenoviral gene delivery can reprogram human fibroblasts to induced pluripotent stem cells," *Stem Cells*, vol. 27, no. 11, pp. 2667–2674, 2009.

[19] J. Yu, K. Hu, K. Smuga-Otto et al., "Human induced pluripotent stem cells free of vector and transgene sequences," *Science*, vol. 324, no. 5928, pp. 797–801, 2009.

[20] K. Kaji, K. Norrby, A. Paca, M. Mileikovsky, P. Mohseni, and K. Woltjen, "Virus-free induction of pluripotency and subsequent excision of reprogramming factors," *Nature*, vol. 458, no. 7239, pp. 771–775, 2009.

[21] K. Woltjen, I. P. Michael, P. Mohseni et al., "PiggyBac transposition reprograms fibroblasts to induced pluripotent stem cells," *Nature*, vol. 458, no. 7239, pp. 766–770, 2009.

[22] H. Zhou, S. Wu, J. Y. Joo et al., "Generation of induced pluripotent stem cells using recombinant proteins," *Cell Stem Cell*, vol. 4, no. 5, pp. 381–384, 2009.

[23] D. Kim, C. H. Kim, J. I. Moon et al., "Generation of human induced pluripotent stem cells by direct delivery of reprogramming proteins," *Cell Stem Cell*, vol. 4, no. 6, pp. 472–476, 2009.

[24] L. Warren, P. D. Manos, T. Ahfeldt et al., "Highly efficient reprogramming to pluripotency and directed differentiation of human cells with synthetic modified mRNA," *Cell Stem Cell*, vol. 7, no. 5, pp. 618–630, 2010.

[25] Y. Xu, Y. Shi, and S. Ding, "A chemical approach to stem-cell biology and regenerative medicine," *Nature*, vol. 453, no. 7193, pp. 338–344, 2008.

[26] D. Huangfu, R. Maehr, W. Guo et al., "Induction of pluripotent stem cells by defined factors is greatly improved by small-molecule compounds," *Nature Biotechnology*, vol. 26, no. 7, pp. 795–797, 2008.

[27] N. Urasawa, M. R. Wada, N. Machida et al., "Selective vacuolar degeneration in dystrophin-deficient canine Purkinje fibers despite preservation of dystrophin-associated proteins with overexpression of Dp71," *Circulation*, vol. 117, no. 19, pp. 2437–2448, 2008.

[28] H. J. Cho, C. S. Lee, Y. W. Kwon et al., "Induction of pluripotent stem cells from adult somatic cells by protein-based reprogramming without genetic manipulation," *Blood*, vol. 116, no. 3, pp. 386–395, 2010.

[29] A. Kunisato, M. Wakatsuki, H. Shinba, T. Ota, I. Ishida, and K. Nagao, "Direct generation of induced pluripotent stem cells from human nonmobilized blood," *Stem Cells and Development*, vol. 20, no. 1, pp. 159–168, 2011.

[30] J. Staerk, M. M. Dawlaty, Q. Gao et al., "Reprogramming of human peripheral blood cells to induced pluripotent stem cells," *Cell stem cell*, vol. 7, no. 1, pp. 20–24, 2010.

[31] Y. H. Loh, O. Hartung, H. Li et al., "Reprogramming of T cells from human peripheral blood," *Cell stem cell*, vol. 7, no. 1, pp. 15–19, 2010.

[32] M. E. Brown, E. Rondon, D. Rajesh et al., "Derivation of induced pluripotent stem cells from human peripheral blood T lymphocytes," *PLoS One*, vol. 5, no. 6, Article ID e11373, 2010.

[33] T. Seki, S. Yuasa, M. Oda et al., "Generation of induced pluripotent stem cells from human terminally differentiated circulating T cells," *Cell stem cell*, vol. 7, no. 1, pp. 11–14, 2010.

[34] S. Okano, Y. Yonemitsu, S. Nagata et al., "Recombinant Sendai virus vectors for activated T lymphocytes," *Gene Therapy*, vol. 10, no. 16, pp. 1381–1391, 2003.

[35] L. W. Van Laake, L. Qian, P. Cheng et al., "Reporter-based isolation of induced pluripotent stem cell-and embryonic stem cell-derived cardiac progenitors reveals limited gene expression variance," *Circulation Research*, vol. 107, no. 3, pp. 340–347, 2010.

[36] J. Xi, M. Khalil, N. Shishechian et al., "Comparison of contractile behavior of native murine ventricular tissue and cardiomyocytes derived from embryonic or induced pluripotent stem cells," *FASEB Journal*, vol. 24, no. 8, pp. 2739–2751, 2010.

[37] N. Yokoo, S. Baba, S. Kaichi et al., "The effects of cardioactive drugs on cardiomyocytes derived from human induced pluripotent stem cells," *Biochemical and Biophysical Research Communications*, vol. 387, no. 3, pp. 482–488, 2009.

[38] A. Kuzmenkin, H. Liang, G. Xu et al., "Functional characterization of cardiomyocytes derived from murine induced pluripotent stem cells in vitro," *FASEB Journal*, vol. 23, no. 12, pp. 4168–4180, 2009.

[39] S. Ueno, G. Weidinger, T. Osugi et al., "Biphasic role for Wnt/β-catenin signaling in cardiac specification in zebrafish and embryonic stem cells," *Proceedings of the National Academy of Sciences of the United States of America*, vol. 104, no. 23, pp. 9685–9690, 2007.

[40] A. T. Naito, I. Shiojima, H. Akazawa et al., "Developmental stage-specific biphasic roles of Wnt/β-catenin signaling in cardiomyogenesis and hematopoiesis," *Proceedings of the National Academy of Sciences of the United States of America*, vol. 103, no. 52, pp. 19812–19817, 2006.

[41] L. Lin, L. Cui, W. Zhou et al., "β-Catenin directly regulates Islet1 expression in cardiovascular progenitors and is required for multiple aspects of cardiogenesis," *Proceedings of the National Academy of Sciences of the United States of America*, vol. 104, no. 22, pp. 9313–9318, 2007.

[42] A. Klaus, Y. Saga, M. M. Taketo, E. Tzahor, and W. Birchmeier, "Distinct roles of Wnt/β-catenin and Bmp signaling during early cardiogenesis," *Proceedings of the National Academy of Sciences of the United States of America*, vol. 104, no. 47, pp. 18531–18536, 2007.

[43] L. Yang, M. H. Soonpaa, E. D. Adler et al., "Human cardiovascular progenitor cells develop from a KDR+ embryonic-stem-cell-derived population," *Nature*, vol. 453, no. 7194, pp. 524–528, 2008.

[44] S. Parisi, D. D'Andrea, C. T. Lago, E. D. Adamson, M. G. Persico, and G. Minchiotti, "Nodal-dependent Cripto signaling promotes cardiomyogenesis and redirects the neural fate of embryonic stem cells," *Journal of Cell Biology*, vol. 163, no. 2, pp. 303–314, 2003.

[45] P. Zhang, J. Li, Z. Tan et al., "Short-term BMP-4 treatment initiates mesoderm induction in human embryonic stem cells," *Blood*, vol. 111, no. 4, pp. 1933–1941, 2008.

[46] D. Kami, I. Shiojima, H. Makino et al., "Gremlin enhances the determined path to cardiomyogenesis," *PLoS One*, vol. 3, no. 6, Article ID e2407, 2008.

[47] S. Hong, J. K. Kang, C. J. Bae, E. S. Ryu, S. H. Lee, and J. H. Lee, "Dovelopment of efficient cardiac differentiation method of mouse embryonic stem cells," *Key Engineering Materials*, vol. 342-343, pp. 25–28, 2007.

[48] D. Kumar and B. Sun, "Transforming growth factor-β2 enhances differentiation of cardiac myocytes from embryonic stem cells," *Biochemical and Biophysical Research Communications*, vol. 332, no. 1, pp. 135–141, 2005.

[49] A. Behfar, L. V. Zingman, D. M. Hodgson et al., "Stem cell differentiation requires a paracrine pathway in the heart," *FASEB Journal*, vol. 16, no. 12, pp. 1558–1566, 2002.

[50] M. A. Laflamme, K. Y. Chen, A. V. Naumova et al., "Cardiomyocytes derived from human embryonic stem cells in pro-survival factors enhance function of infarcted rat hearts," *Nature Biotechnology*, vol. 25, no. 9, pp. 1015–1024, 2007.

[51] J. Hao, M. A. Daleo, C. K. Murphy et al., "Dorsomorphin, a selective small molecule inhibitor of BMP signaling, promotes cardiomyogenesis in embryonic stem cells," *PLoS One*, vol. 3, no. 8, Article ID e2904, 2008.

[52] T. Sumi, N. Tsuneyoshi, N. Nakatsuji, and H. Suemori, "Defining early lineage specification of human embryonic stem cells by the orchestrated balance canonical Wnt/β-catenin, activin/Nodal and BMP signaling," *Development*, vol. 135, no. 17, pp. 2969–2979, 2008.

[53] S. L. Paige, T. Osugi, O. K. Afanasiev, L. Pabon, H. Reinecke, and C. E. Murry, "Endogenous Wnt/β-catenin signaling is required for cardiac differentiation in human embryonic stem cells," *PloS One*, vol. 5, no. 6, Article ID e11134, 2010.

[54] S. Yuasa, Y. Itabashi, U. Koshimizu et al., "Transient inhibition of BMP signaling by Noggin induces cardiomyocyte differentiation of mouse embryonic stem cells," *Nature Biotechnology*, vol. 23, no. 5, pp. 607–611, 2005.

[55] S. Faure, P. De Santa Barbara, D. J. Roberts, and M. Whitman, "Endogenous patterns of BMP signaling during early chick development," *Developmental Biology*, vol. 244, no. 1, pp. 44–65, 2002.

[56] R. B. Fletcher, A. L. Watson, and R. M. Harland, "Expression of Xenopus tropicalis noggin1 and noggin2 in early development: two noggin genes in a tetrapod," *Gene Expression Patterns*, vol. 5, no. 2, pp. 225–230, 2004.

[57] S. Yuasa, T. Onizuka, K. Shimoji et al., "Zac1 is an essential transcription factor for cardiac morphogenesis," *Circulation Research*, vol. 106, no. 6, pp. 1083–1091, 2010.

[58] K. Shimoji, S. Yuasa, T. Onizuka et al., "G-CSF promotes the proliferation of developing cardiomyocytes in vivo and in derivation from ESCs and iPSCs," *Cell Stem Cell*, vol. 6, no. 3, pp. 227–237, 2010.

[59] B. Y. Hu, J. P. Weick, J. Yu et al., "Neural differentiation of human induced pluripotent stem cells follows developmental principles but with variable potency," *Proceedings of the National Academy of Sciences of the United States of America*, vol. 107, no. 9, pp. 4335–4340, 2010.

[60] J. M. Polo, S. Liu, M. E. Figueroa et al., "Cell type of origin influences the molecular and functional properties of mouse induced pluripotent stem cells," *Nature Biotechnology*, vol. 28, no. 8, pp. 848–855, 2010.

[61] K. Kim, A. Doi, B. Wen et al., "Epigenetic memory in induced pluripotent stem cells," *Nature*, vol. 467, no. 7313, pp. 285–290, 2010.

[62] Q. Hu, A. M. Friedrich, L. V. Johnson, and D. O. Clegg, "Memory in induced pluripotent stem cells: reprogrammed human retinal-pigmented epithelial cells show tendency for spontaneous redifferentiation," *Stem Cells*, vol. 28, no. 11, pp. 1981–1991, 2010.

[63] K. Fukuda and S. Yuasa, "Stem cells as a source of regenerative cardiomyocytes," *Circulation Research*, vol. 98, no. 8, pp. 1002–1013, 2006.

[64] Y.-H. Choi, T. Saric, B. Nasseri et al., "Cardiac cell therapies: the next generation," *Cardiovascular Therapeutics*, vol. 29, no. 1, pp. 2–16, 2011.

[65] I. Kehat, D. Kenyagin-Karsenti, M. Snir et al., "Human embryonic stem cells can differentiate into myocytes with structural and functional properties of cardiomyocytes," *Journal of Clinical Investigation*, vol. 108, no. 3, pp. 407–414, 2001.

[66] J. Zhang, G. F. Wilson, A. G. Soerens et al., "Functional cardiomyocytes derived from human induced pluripotent stem cells," *Circulation Research*, vol. 104, no. 4, pp. e30–e41, 2009.

[67] L. Zwi, O. Caspi, G. Arbel et al., "Cardiomyocyte differentiation of human induced pluripotent stem cells," *Circulation*, vol. 120, no. 15, pp. 1513–1523, 2009.

[68] T. Tanaka, S. Tohyama, M. Murata et al., "In vitro pharmacologic testing using human induced pluripotent stem cell-derived cardiomyocytes," *Biochemical and Biophysical Research Communications*, vol. 385, no. 4, pp. 497–502, 2009.

[69] L. W. Van Laake, R. Passier, P. A. Doevendans, and C. L. Mummery, "Human embryonic stem cell-derived cardiomyocytes and cardiac repair in rodents," *Circulation Research*, vol. 102, no. 9, pp. 1008–1010, 2008.

[70] O. Caspi, I. Huber, I. Kehat et al., "Transplantation of human embryonic stem cell-derived cardiomyocytes improves myocardial performance in infarcted rat hearts," *Journal of the American College of Cardiology*, vol. 50, no. 19, pp. 1884–1893, 2007.

[71] T. J. Nelson, A. Martinez-Fernandez, S. Yamada, C. Perez-Terzic, Y. Ikeda, and A. Terzic, "Repair of acute myocardial infarction with induced pluripotent stem cells induced by human stemness factors," *Circulation*, vol. 120, no. 5, pp. 408–416, 2009.

[72] K. Miura, Y. Okada, T. Aoi et al., "Variation in the safety of induced pluripotent stem cell lines," *Nature Biotechnology*, vol. 27, no. 8, pp. 743–745, 2009.
[73] S. J. Kinder, T. E. Tsang, G. A. Quinlan, A. K. Hadjantonakis, A. Nagy, and P. P. L. Tam, "The orderly allocation of mesodermal cells to the extraembryonic structures and the anteroposterior axis during gastrulation of the mouse embryo," *Development*, vol. 126, no. 21, pp. 4691–4701, 1999.
[74] S. J. Kattman, T. L. Huber, and G. Keller, "Multipotent Flk-1$^+$ cardiovascular progenitor cells give rise to the cardiomyocyte, endothelial, and vascular smooth muscle lineages," *Developmental Cell*, vol. 11, no. 5, pp. 723–732, 2006.
[75] F. Hattori, H. Chen, H. Yamashita et al., "Nongenetic method for purifying stem cell-derived cardiomyocytes," *Nature Methods*, vol. 7, no. 1, pp. 61–66, 2010.
[76] K. Hidaka, M. Shirai, J. K. Lee et al., "The cellular prion protein identifies bipotential cardiomyogenic progenitors," *Circulation Research*, vol. 106, no. 1, pp. 111–119, 2010.
[77] J. K. Yamashita, M. Takano, M. Hiraoka-Kanie et al., "Prospective identification of cardiac progenitors by a novel single cell-based cardiomyocyte induction," *FASEB Journal*, vol. 19, no. 11, pp. 1534–1536, 2005.
[78] T. J. Nelson, R. S. Faustino, A. Chiriac, R. Crespo-Diaz, A. Behfar, and A. Terzic, "CXCR4$^+$/FLK-1$^+$ biomarkers select a cardiopoietic lineage from embryonic stem cells," *Stem Cells*, vol. 26, no. 6, pp. 1464–1473, 2008.
[79] G. Blin, D. Nury, S. Stefanovic et al., "A purified population of multipotent cardiovascular progenitors derived from primate pluripotent stem cells engrafts in postmyocardial infarcted nonhuman primates," *Journal of Clinical Investigation*, vol. 120, no. 4, pp. 1125–1139, 2010.
[80] J. Leschik, S. Stefanovic, B. Brinon, and M. Pucéat, "Cardiac commitment of primate embryonic stem cells," *Nature Protocols*, vol. 3, no. 9, pp. 1381–1387, 2008.
[81] M. K. Gupta, D. J. Illich, A. Gaarz et al., "Global transcriptional profiles of beating clusters derived from human induced pluripotent stem cells and embryonic stem cells are highly similar," *BMC Developmental Biology*, vol. 10, article no. 98, 2010.
[82] A. Gore, Z. Li, H.-L. Fung et al., "Somatic coding mutations in human induced pluripotent stem cells," *Nature*, vol. 471, no. 7336, pp. 63–67, 2011.
[83] T. Zhao, Z.-N. Zhang, Z. Rong, and Y. Xu, "Immunogenicity of induced pluripotent stem cells," *Nature*, vol. 474, no. 7350, pp. 212–216, 2011.
[84] I. H. Park, N. Arora, H. Huo et al., "Disease-specific induced pluripotent stem cells," *Cell*, vol. 134, no. 5, pp. 877–886, 2008.
[85] F. Soldner, D. Hockemeyer, C. Beard et al., "Parkinson's disease patient-derived induced pluripotent stem cells free of viral reprogramming factors," *Cell*, vol. 136, no. 5, pp. 964–977, 2009.
[86] J. T. Dimos, K. T. Rodolfa, K. K. Niakan et al., "Induced pluripotent stem cells generated from patients with ALS can be differentiated into motor neurons," *Science*, vol. 321, no. 5893, pp. 1218–1221, 2008.
[87] A. D. Ebert, J. Yu, F. F. Rose et al., "Induced pluripotent stem cells from a spinal muscular atrophy patient," *Nature*, vol. 457, no. 7227, pp. 277–280, 2009.
[88] Á. Raya, I. Rodríguez-Piz, G. Guenechea et al., "Disease-corrected haematopoietic progenitors from Fanconi anaemia induced pluripotent stem cells," *Nature*, vol. 460, no. 7251, pp. 53–59, 2009.
[89] X. Carvajal-Vergara, A. Sevilla, S. L. Dsouza et al., "Patient-specific induced pluripotent stem-cell-derived models of LEOPARD syndrome," *Nature*, vol. 465, no. 7299, pp. 808–812, 2010.
[90] A. Moretti, M. Bellin, A. Welling et al., "Patient-specific induced pluripotent stem-cell models for long-QT syndrome," *New England Journal of Medicine*, vol. 363, no. 15, pp. 1397–1409, 2010.

Derivation of Induced Pluripotent Stem Cells from Human Peripheral Circulating T Cells

Tomohisa Seki,[1] Shinsuke Yuasa,[1,2] and Keiichi Fukuda[1]

[1]Department of Cardiology, Keio University School of Medicine, Tokyo, Japan
[2]Center for Integrated Medical Research, Keio University School of Medicine, Tokyo, Japan

ABSTRACT

This unit describes a protocol for the generation of induced pluripotent stem (iPS) cells from human peripheral circulating T cells. Initially, human dermal fibroblasts and retroviral vectors were used to generate human iPS cells. Invasive approaches, such as skin biopsy, and genomic insertion of transgenes into the host genome are not appropriate for routine clinical application. Peripheral circulating T cells are readily available from blood samples of patients and healthy volunteers. For the efficient generation of human iPS cells, efficient introduction of the transgene into host cells is necessary. Using a combination of activated T cell culture and Sendai virus allows for the easy and efficient introduction of transgenes into activated T cells and the generation of human iPS cells without genomic integration of extrinsic genes. The T cell-derived iPS (TiPS) cells exhibit monoclonal T cell receptor (TCR) rearrangement in their genome, a hallmark of mature terminally differentiated T cells. *Curr. Protoc. Stem Cell Biol.* 18:4A.3.1-4A.3.9.

Keywords: induced pluripotent stem cells • human • peripheral blood • T cells • Sendai virus

INTRODUCTION

This unit describes a protocol for the generation of induced pluripotent stem (iPS) cells from human peripheral circulating T cells. Although initially human dermal fibroblasts and retroviral vectors were used to derive human iPS cells (Takahashi et al., 2007; Yu et al., 2007), invasive approaches, such as skin biopsies, to obtain dermal fibroblasts are not appropriate for routine basic research and clinical application. Accumulating evidence from recent studies indicates that other human somatic stem cells can be used to generate human iPS cells (Aasen et al., 2008; Eminli et al., 2008; Kim et al., 2009; Sun et al., 2009; Ye et al., 2009; Aoki et al., 2010); however, it is difficult to obtain sufficient numbers of these cells. Thus, we focused on easily accessible tissues that can be obtained using less invasive procedures. Human peripheral circulating T cells are readily available in peripheral blood and can be induced to proliferate using simple cytokine stimulation (Sekine et al., 1993). Generating human iPS cells without genomic integration of extrinsic genes is also highly desirable to minimize unexpected and potentially harmful events. In an attempt to avoid transgene integration and achieve high infection efficiency during the generation of human iPS cells, we used a Sendai virus (SeV) vector, a minus-strand RNA virus that is not integrated into the host genome. Using SeV-mediated gene transfer, we succeeded in generating human iPS cells from human dermal fibroblasts (Fusaki et al., 2009). There is little transduction of SeV into non-activated human T cells, but the virus can be efficiently transduced into activated human T cells (Okano et al., 2003). Thus, we used a combination of SeV vectors encoding for human *OCT3/4, SOX2, KLF4*, and *c-MYC* and activated T cell culture (Seki et al., 2010). The temperature-sensitive mutated

Current Protocols in Stem Cell Biology 4A.3.1-4A.3.9
Published online September 2011 in Wiley Online Library (wileyonlinelibrary.com).
DOI: 10.1002/9780470151808.sc04a03s18

SeV vector we used in those studies cannot proliferate at standard culture temperatures and, after several passages, SeV-derived transgenes were not detected in the iPS cells generated. This combination method using the activated T cell culture of cells obtained from peripheral blood samples and SeV-mediated gene transfer generates human iPS cells with a very high efficiency.

NOTE: The following procedures are performed in a Class II biological hazard flow hood or a laminar-flow hood.

NOTE: All solutions and equipment coming into contact with live cells must be sterile, and proper aseptic technique should be used accordingly.

NOTE: All incubations are performed in a humidified 37°C, 5% CO_2 incubator unless noted otherwise.

BASIC PROTOCOL

GENERATION OF iPS CELLS FROM T CELLS

The first steps in generating iPS cells from T cells involve the collection of peripheral blood from patients and/or volunteers, the separation of mononuclear cells from whole blood samples, and then activation of T cells in a cell culture dish. The next step involves the introduction of four factors, namely human *OCT3/4*, *SOX2*, *KLF4*, and *c-MYC*, using SeV, into the activated T cells. The activation of T cells is necessary because the higher the proportion of activated T cells to whole mononuclear cells, the greater the efficiency of SeV infection. The final step is to pick up the initial human iPS cell colonies that emerge, to expand them, and then to store them prior to using them as human iPS cells.

In the first step, peripheral blood samples are obtained using heparin or other reagents to prevent clot formation. To eliminate the risk of bacterial contamination, a sterile technique is necessary. Routinely, we obtain 10-ml samples of whole blood from adults and 1-ml samples from children.

For gene transduction, we use an SeV vector, which is a negative-sense, single-stranded RNA virus that is not integrated into the host genome and is not pathogenic for humans (Li et al., 2000). SeV vectors carry human *OCT3/4*, *SOX2*, *KLF4*, and *c-MYC* independently. Thus, we use four kinds of SeV vectors. Frozen stock of the SeV vectors is thawed and simply added to the cell culture medium.

Before initial human iPS cell colonies emerge, we prepare mouse embryonic fibroblasts (MEFs) as feeder cells to maintain human iPS cells. After the emerging iPS cell colonies have been picked up, the subsequent procedures are same as those used to generate fibroblast-derived iPS cells.

CAUTION: All procedures involving SeV vectors should be performed in a safety cabinet while wearing gloves. All waste must be treated first with ethanol, and then with bleach (hypochlorous acid) before being finally autoclaved.

Materials

Heparinized whole blood from donors
Ficoll-Paque PREMIUM (GE Healthcare, cat. no. 17-5442-02; see manufacturer-provided protocol)
Purified NA/LE mouse anti-human CD3 (BD Pharmingen, cat. no. 555336)
D-PBS(–) (Wako, cat. no. 045-29795)
GT-T502 medium (KOHJIN BIO, cat. no. 16025020)
SeVs are available from DNAVEC Corporation (*http://www.dnavec.co.jp/*)
OCT3/4-SeV/TSΔF (DNAVEC)

SOX2-SeV/TSΔF (DNAVEC)
KLF4-SeV/TSΔF (DNAVEC)
c-MYC(HNL)-SeV/TS15ΔF (DNAVEC)
Irradiated MEF feeder cells (see Conner, 2000)
Human iPS cell medium (see recipe)
0.1% gelatin-coated 6-well plate (see recipe), preplated with MEFs (*UNIT 1A.2*)
Collagenase type IV (see recipe)
DMEM/F12 (Sigma, cat. no. D6421)
DAP213 solution (see recipe)
Liquid nitrogen

15-ml conical tubes
Centrifuge
100-mm tissue culture dish (Falcon, cat. no. 353003)
Dissection microscope
20- and 1000-μl pipets
0.2-ml cryovials
Liquid nitrogen tank

Prepare activated human T cells

1. Collect 1 to 10 ml heparinized whole blood from a donor.
2. Using a Ficoll-Paque PREMIUM gradient according to the manufacturer's instructions, separate mononuclear cells by centrifugation of the heparinized whole blood sample (Fig. 4A.3.1).
3. Prepare an anti-human CD3 antibody solution by dissolving anti-human CD3 antibody in D-PBS(–) to a concentration of 10 μg/ml. Add the anti-human CD3 antibody solution to the 6-well plates, soak the surface of each plate, and incubate the plates at 37°C in a 5% CO_2 incubator for at least 30 min. Before seeding the cells, remove the anti-human CD3 antibody solution and wash the dishes once with 2 ml D-PBS(–).

The plates can be coated overnight at 4°C for convenience.

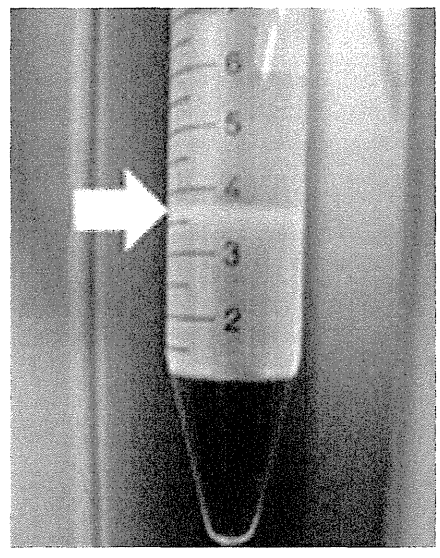

Figure 4A.3.1 Arrow indicates thin white layer, which contains mononuclear cells by a Ficoll gradient.

4. Seed mononuclear cells at a density of 3×10^4 cells/cm^2 on the anti-human CD3 antibody-coated 6-well plates into 2 ml GT-T502 medium per well. Incubate for 3 to 7 days until the T cells reach 80% to 90% confluency.

Infect human T cells with SeV vectors

5. After 3 to 7 days (Fig. 4A.3.2), collect the activated mononuclear cells by pipetting, transfer them into a 15-ml conical tube, and centrifuge 5 min at 200 × *g*, room temperature.

6. Remove the supernatant and add 1 ml fresh GT-T502 medium. Count the cells and plate 1.5×10^6 cells (in 2 ml fresh GT-T502 medium) in each well of a fresh anti-CD3 antibody-coated 6-well plate.

7. Incubate the plates for an additional 24 hr.

8. Add the solutions containing OCT3/4-SeV/TSΔF, SOX2-SeV/TSΔF, KLF4-SeV/TSΔF, and c-MYC (HNL)-SeV/TS15ΔF individually to the wells, each at an MOI of 5 to 10. Incubate for an additional 24 hr.

9. At a time point 24 hr after infection, collect the infected cells by pipetting, transfer them into a 15-ml conical tube, and centrifuge 5 min at 200 × *g*, room temperature.

10. Remove the supernatant, which contains the SeV vectors, and add 2 ml fresh GT-T502 medium. Replate the cells in each of the wells previously used for culture. Incubate for an additional 24 hr.

Reseed cells on MEF feeder cells

11. At a time point 48 hr after infection, collect the cells by pipetting, transfer them into a 15-ml conical tube, and centrifuge 5 min at 200 × *g*, room temperature.

12. Remove the supernatant and add 1 ml fresh GT-T502 medium. Count the number of cells and plate 5×10^4 and 5×10^5 cells (in 10 ml fresh GT-T502 medium) on a 100-mm dish with irradiated MEF feeder cells. Incubate the dish overnight.

13. The next day, and then every second day thereafter, change the medium to 10 ml human iPS cell (hiPSC) culture medium supplemented with 4 ng/ml bFGF.

14. Approximately 20 to 30 days after infection, colonies that closely resemble embryonic stem (ES) cells will appear (Fig. 4A.3.3). Using a dissection microscope and a 20-µl pipet, pick up colonies that resemble human ES cells and put each colony into

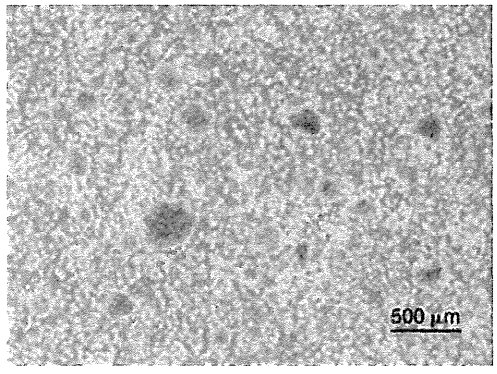

Figure 4A.3.2 Activated T cells, identified using CD-3 antibody and interleukin (IL)-2, on day 5 after blood sampling.

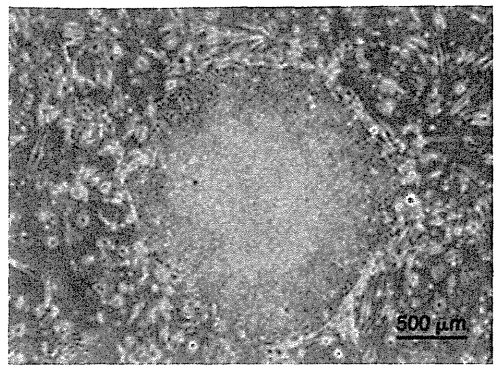

Figure 4A.3.3 The initial iPS cell colony.

one well of a 0.1% gelatin-coated 6-well plate that has been preplated (1 to 2 days before) with MEFs at a density of 1×10^4 cells/cm^2 (see *UNIT IA.2*).

Expand and maintain T cell-derived iPS cells

The T cell-derived iPS (TiPS) cells can be maintained and stored using the same techniques as for human iPS and human ES cells.

15. Change the hiPSC culture medium every 2 to 3 days.

16. Every 5 to 7 days, passage the cells using 1 mg/ml collagenase IV. When the colonies become confluent, aspirate the medium, add 1 mg/ml collagenase IV/DMEM/F12 (which is half the amount of the maintenance medium), and incubate the cells for 30 to 60 min at 37°C.

    If a 100-mm dish is used, the volume of human iPS culture medium is 10 ml per dish and the volume of 1 mg/ml collagenase IV/DMEM/F12 is 5 ml per dish.

17. When floating colonies appear, collect the supernatant into 15-ml tubes and centrifuge 2 min at 200 × *g*, room temperature.

18. Remove the supernatant. Add 1 ml fresh human iPS culture medium and break the colonies into small pieces with a 1000-µl pipet.

19. Place the broken colonies into a new gelatin-coated 100-mm dish or 6-well plate that has been preplated with irradiated MEFs and filled with fresh human iPS culture medium.

    The split ratio used for the cells depends on the cell lines and is usually in the range 1:4 to 1:10.

Store TiPS cells

When the TiPS cells have reached 80% to 90% confluency in the 100-mm dish, they can be frozen.

20. To freeze iPS cells, resuspend the colonies from step 16 in 3 ml fresh human iPS cell medium, divide the colonies across four 15-ml tubes, and centrifuge 2 min at 200 × *g*, room temperature.

21. Remove the supernatant from three of the 15-ml tubes and resuspend the pellets in 0.2 ml DAP213 solution by pipetting a few times using a 200-µl pipet. (Use the remaining pellet in the fourth 15-ml tube for further passaging, as described in step 17.)

22. Transfer 0.2 ml of the cell suspension into 2-ml cryovials.

23. Plunge the vials quickly into liquid nitrogen.

24. Store the vials in the liquid nitrogen tank.

REAGENTS AND SOLUTIONS

For culture recipes and steps, use sterile tissue culture–grade water. For other purposes, use deionized, distilled water or equivalent in recipes and protocol steps. For suppliers, see ***SUPPLIERS APPENDIX****.*

Collagenase IV

Dissolve collagenase IV (Invitrogen, cat. no. 17104-019) to a concentration of 1 mg/ml in DMEM/F12 (Invitrogen)
Filter using a 0.22-μm filter (Millipore, cat. no. SLGV033RS)
Divide into 10-ml aliquots
Store for up to 1 year at −20°C

DAP213 solution

To 5.37 ml human iPS culture medium (see recipe) add:
1.43 ml dimethyl sulfoxide (DMSO)
1 ml of 10 M acetamide
2.2 ml of propylene glycol
Store for up to 1 month at −80°C

Gelatin-coated 6-well plates, 0.1%

Dissolve 1 g of gelatin powder (Sigma, cat. no. G-1890) in 1000 ml of distilled water, autoclave, filter the solution with a 0.22-μm filter unit, and store at 4°C. To coat the culture dishes, add appropriate volume of 0.1% (1×) gelatin solution to cover the entire area of the dish bottom. For example, 1 ml of gelatin solution is used for a well of a 6-well plate. Incubate the plates for at least 30 min at 37°C in a sterile environment. Before using, aspirate off the excess gelatin solution.

Human iPS culture medium

DMEM/F12 medium (Sigma, cat. no. D6421) containing:
20% Knockout Serum Replacement (KOSR; Invitrogen, cat. no. 10828-028)
10 mM non-essential amino acids (Sigma, cat. no. M7145)
2 mM L-glutamine (GlutaMAX-I; Invitrogen, cat. no. 35050-061)
1× penicillin–streptomycin (Invitrogen, cat. no. 15140-122)
50 mM 2-mercaptoethanol
4 ng/ml bFGF (see recipe)
Store for up to 1 week at 4°C

Recombinant human bFGF

Resuspend lyophilized recombinant basic fibroblast growth factor, human (bFGF; Wako, cat. no. 064-04541) to a final concentration of 4 μg/ml in D-PBS(–) containing 0.1% bovine albumin fraction V solution (BSA; Invitrogen, cat. no. 15260-037)
Store for up to 1 month at −80°C

COMMENTARY

Background Information

The direct reprogramming of somatic cells to produce iPS cells is a significant recent advance in stem cell biology (Takahashi and Yamanaka, 2006). Initially, human dermal fibroblasts were used to derive human iPS cells