Pluripotent Stem Cell Therapy in Ischemic Cardiovascular Disease

Ting-Hsing Chao,[1,2] I-Chih Chen,[3] Shi-Ya Tseng[1,4] and Yi-Heng Li[1]

Stem cell therapy has been viewed as a promising therapeutic strategy in ischemic cardiovascular disease for almost a decade. Although many progenitor/stem cells obtained from patients have been investigated, and are alleged to be suitable for autologous transplantation, their therapeutic application has been limited by their inability to yield a sufficient number of stem cells, as well as impaired regeneration capacity from ageing and cardiovascular risk factors. Pluripotent stem cells, such as embryonic stem cells (ESCs) and induced pluripotent stem cells (iPSCs), have the capacity for functional multi-lineage differentiation and properties of self-renewal and immortality, and can generate clinically relevant amounts of stem cells. The regeneration capacity of these cells is not affected by ageing. Patient-specific pluripotent stem cells, iPSCs, can be established by epigenetically reprogramming somatic fibroblasts. iPSCs and iPSC-derived stem cells share similar phenotypes and gene expressions of ESCs and ESC-derived stem cells. Transplantation of pluripotent stem cell-derived endothelial cells, mural cells, cardiomyocytes, or cardiovascular progenitor cells contribute to neovascularization and cardiomyogenesis with better limb perfusion and recovery of myocardial contractility in the preclinical studies. Several strategies have been developed to enhance the efficacy of reprogramming and engrafting, and improve graft survival, proliferation, and electromechanical coupling by tissue engineering. However, the therapeutic application of ESCs and derivatives is limited by ethical concerns. Before wide clinical application of these cells in regeneration therapy occurs, substantial effort should be undertaken to discover the most promising cell type and derivatives, the best protocol regarding cell preparation, reprogramming and differentiation, and the most efficacious methods to avoid adverse effects.

Key Words: Embryonic stem cells • Induced pluripotent stem cells • Limb ischemia • Myocardial infarction

INTRODUCTION

Blood vessel growth is mediated by angiogenesis, which is defined as the formation of new blood vessel out of existing vessels. This process includes migration and proliferation of endothelial cells (ECs), extracellular matrix degradation, and capillary tube formation, as well as vasculogenesis, a *de novo* process that circulating progenitor cells contribute to adult neovascularization.[1,2] Since the time when endothelial progenitor cells (EPCs) were identified and isolated, many cell types have been reported to be able to participating in vasculo-angiogenesis, including bone marrow-derived mononuclear cells, circulating EPCs,[3] mesenchymal stem

Received: October 22, 2013 Accepted: January 20, 2014
[1]Division of Cardiology, Department of Internal Medicine, National Cheng Kung University College of Medicine and Hospital, Tainan; [2]Division of Cardiology, Department of Internal Medicine, National Cheng Kung University College of Medicine and Hospital, Dou-Liou Branch, Yun-Lin County; [3]Section of Cardiology, Department of Internal Medicine, Tainan Municipal Hospital, Tainan; [4]Department of Biological Science, National Sun Yat-Sen University, Kaohsiung, Taiwan.
Address correspondence and reprint requests to: Dr. Ting-Hsing Chao, Division of Cardiology, Department of Internal Medicine, National Cheng Kung University College of Medicine and Hospital, No. 138, Sheng-Li Road, North District, Tainan 704, Taiwan. Tel: 886-6-235-3535 ext. 2383; Fax: 886-6-275-3834; E-mail: chaotinghsing@yahoo.com.tw
I-Chih Chen shares equal contribution with the first author.

cells/adipose-derived stem cells, and skeletal myoblasts. The most significant advantage of these cells in translational therapy is that autologous transplantation is feasible through *ex vivo* expansion (or not). However, their therapeutic application is limited by the ability to yield stem cells numbers sufficient for transplantation,[4-6] a process that may cause some complications in patients with severe cardiovascular disease.[4] Furthermore, ageing[7] and underlying cardiovascular risk factors[8] affect the number and capacity of these cells, which further hampers the effectiveness of autologous cell therapy. Finally, most of the stem cell types implemented in clinical practice yield a modest improvement in cardiac function with a less than 5% increase in left ventricular ejection fraction.[9] Cardiomyogenic differentiation[9] or incorporation into neovascularization[10] from these stem cells is rare.

Pluripotent stem cells (Figure 1), such as embryonic stem cells (ESCs) and induced pluripotent stem cells (iPSCs), have functional multi-lineage differentiation capacity and can theoretically generate clinically relevant amounts of stem cells for regeneration therapy.[9,10] In the last 10 years, ESCs have been highlighted as a promising cell source for therapeutic angiogenesis[10-12] and cardiomyogenesis[13] because of their capacity to undergo unlimited expansion in an undifferentiated state, and their ability to undergo inducible differentiation into vascular ECs, mural cells (MCs) and cardiomyocytes (CMs) (Figure 1). However, concerns about ethics and immune system rejection largely limited ESCs in clinical application.[14] Recently, iPSCs have attracted increasing attention in the field of regeneration medicine regarding patient-specific cell therapy because they share common primitive programmed genes with ESCs and can be generated from reprogramming somatic fibroblasts[15-17] in adult patients and thus can be autologous (Figure 1).[9] Furthermore, they are easier to generate in clinically sufficient numbers of iPSC-derived CMs[18] and ECs,[19] as compared to adult stem cells with negligible immune rejection[20] and no concerns of an ethical nature. In addition, the functionality of generated iPSCs is not affected by ageing.[21] However, concerns regarding potentially uncontrolled cellular proliferation, oncogenesis or abnormal development from viral transduction during reprogramming should be answered and resolved before approval for human patients.[9] In addition to its application to regeneration therapy, pluripotent stem cell-derivatives, especially patient-specific iPSCs, can be used as a model of disease in exploring drug toxicity as well as disease pathogenesis.[22] This article reviews recent evidence relating to pluripotent stem cells in regeneration therapy of ischemic cardiovascular disease.

ESC-DERIVED ECs AND MCs IN LIMB ISCHEMIA

Deriving ECs, MCs, and vascular progenitor cells (VPCs) from mouse and human ESCs in monolayer culture

ESCs have an excellent capacity for self-renewal and pluripotency, and can be expanded without limit.[4,23] Theoretically, ESCs can be cultured to an immortal extent ex vivo, and can differentiate into virtually any cell type from all 3 germ layers in the adult body.[23,24] How-

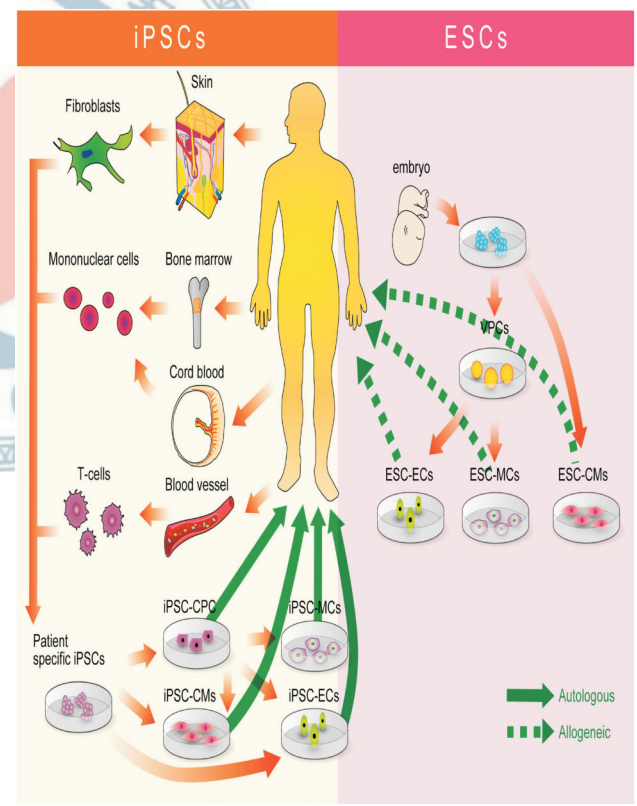

Figure 1. *Strategies for human embryonic stem cell (ESC)- and induced pluripotent stem cell (iPSC)-based therapy for ischemic cardiovascular disease. CMs, cardiomyocytes; CPCs, cardiovascular progenitor cells; ECs, endothelial cells; MCs, mural cells; VPCs, vascular progenitor cells.*

ever, to avoid tumorigenesis, minimize cellular misbehavior, and enhance the regeneration effect in transplantation with undifferentiated ESCs, committing progenitor lineage derived from undifferentiated ESCs for regeneration therapy is a promising and feasible strategy.[23] Vascular ECs and MCs can be differentiated from mouse ESCs by purifying and sorting fetal liver kinase 1 (Flk-1)+ cells, so-called VPCs (Figure 1), and recultured in type-IV collagen-coated dishes with serum and vascular endothelial growth factor (VEGF).[4,11] Then, $CD31^+$/vascular endothelial-cadherin$^+$ ECs, and α-smooth muscle actin-positive MCs are selectively induced (Figure 1).[4,11] VPCs or VPC-derived vascular cells can form mature vascular-like structures in vitro and contribute to neovascularization in vivo.[4,11] Human ESCs cultured on OP9 feeder, a mouse embryonic fibroblast feeder, also can be successfully differentiated into VPCs, Flk-1+/tumor rejection antigen 1-negaive/platelet-derived growth factor (PDGF)-positive cells, and then into ECs in the early differentiation stage induced by coincubation with VEGF and into MCs with PDGF-BB.[12] The combined transplantation of human VPC-derived ECs and MCs synergistically improves blood flow of ischemic hindlimbs remarkably, compared to single cell transplantations.[10] Transplanted VPC-derived vascular cells were effectively incorporated into host circulating vessels as ECs and MCs to maintain long-term vascular integrity, whereas transplanted EPCs were less incorporated into neovascularization sites.[10]

Deriving ECs, MCs, and VPCs from mouse and human ESCs in three-dimensional culture

In addition to being cultured in mouse embryonic fibroblasts as a feeder layer, human ESCs can be maintained in an undifferentiated state in suspension to aggregate and form embryoid bodies (EBs). Human ESC-VPCs can be also generated by spontaneous differentiation from EBs in three-dimensional (3D) culture.[23,25] EB-derived VPCs can be further differentiated into ECs in the early differentiation stage induced by coincubation with VEGF and into MCs with PDGF-BB.[23] However, the efficiency of endothelial differentiation of the 3D EB system is typically low, ranging from 1 to 3%.[23,25] Moreover, EBs are notoriously difficult to dissociate and subsequently challenging to get homogeneous cell types.[23,26] To circumvent this, multiple modifications have been tried to improve efficacy of EC differentiation from EBs.[23,26]

Preclinical application of human ESC-derived vascular cells in limb ischemia

Substantial preclinical studies report that transplantation of ESC-derived ECs, MCs, and VPCs is able to enhance neovascularization with improved limb perfusion and function in murine hindlimb ischemia.[10,12,23,25] Transplantation can be performed via intramuscular, intra-arterial or intravenous injection with similar effect.[27] Transplanted human ESC-derived vascular cells are capable of improving hindlimb ischemia mediated through engrafting into the ischemic microvasculature and enhancing angiogenesis and vasculogenesis by paracrine effect.[10] Of note, Yamahara et al. reported that transplanted human ESC-derived ECs and MCs were incorporated much more effectively into the neovascularization site than EPCs, with a similar beneficial effect in hindlimb ischemia, suggestive of more paracrine effect with EPC transplantation.[10]

Cell-enhancement strategy for human ESC-derived vascular cells in the ischemic hindlimb

Previous research on ESC-based therapy showed inadequate long-term engraftment and retention of transplanted ESC-derived vascular cells.[28] Several strategies have been developed to enhance cell therapy efficiency in hindlimb ischemia such as co-administration of vasculo-angiogenic factors,[29] modulation of the relevant signaling pathways to facilitate efficient differentiation of human ESCs into functional $CD34^+$ progenitor cells,[30] genetic engineering of human ESC-derived vascular cells using biodegradable nanoparticles before transplantation,[31] and modification of delivery strategy by scaffolds seeded with stem cells.[31]

ESC-DERIVED CMs AND VASCULAR CELLS IN MYOCARDIAL REGENERATION THERAPY

Methods deriving CMs from human ESCs

Despite a substantial number of methods reported, two essential principles for approaches have been developed to direct differentiation of human ESCs toward the cardiac lineage in vitro.[32] The first is to co-culture with mouse visceral endoderm-like cells or cells secret-

ing an endoderm-like signal;[32,33] the second principle is based on addition of the hormones and growth factors, such as transforming growth factor-superfamily including bone morphogenetic proteins (BMPs) and activin, known to be involved in heart development in vivo to human ESCs grown as EBs or in monolayer.[32,34] The combination of growth factors and co-culture with visceral endoderm-like cells provides efficient CM differentiation.[35] Serum-free condition[32] and insulin depletion[32] can further increase CM production. Recently, Yang et al.[36] reported that after induction with combinations of BMP4, activin A, basic fibroblast growth factor, VEGF and dickkopf homolog 1 in serum-free media, human ESC-derived EBs generate a Flk-1 (low)/c-kit(CD117) (negative) population that displays cardiac and vascular cells potential in vitro and, after transplantation, in vivo. When plated in monolayer cultures, these Flk-1 (low)/c-kit (negative) cells differentiate to generate populations, whereafter greater than 50% contracted cardiomyocytes.[36]

Preclinical application of human ESC-derived CMs and vascular cells in myocardial regeneration therapy

A lot of preclinical studies reveal that intramuscular injection with human ESC-derived CMs improves cardiac repair and myocardial performance in a rodent myocardial infarction model (Table 1).[37-40] In addition, the beneficial effects of cell therapy are also observed with transplantation of human ESC-derived VPCs or hemangioblasts or vascular cells in this model (Table 1).[41-43]

Table 1. Summary of human ESCs and iPSCs therapy in animal models of acute myocardial infarction

Cell type	Author/year of publication	Culture method/ surface markers	Animal species	Route/timing of delivery	Dose	Follow-up duration	Endpoints (vs. control)
ESCs							
ESC-CMs	Caspi et al.[37]/2007	EBs/-	Rat	IM/7days after MI	1.5×10^6	30-60 days	↑FS; ↓EDD
ESC-CMs	Ebelt et al.[38]/2007	EBs/-	Rat	IM/7 days after MI	3×10^5	4 weeks	↑FS; ↓EDD
ESC-CMs	van Laake et al.[39]/ 2007	EBs/-	Mouse	IM/-	-	4 weeks and 12 weeks	4 weeks: ↑EF; = EDV; = SV 12 weeks: = EF; = EDV; = SV
ESC-CMs	Laflamme et al.[45]/ 2007	Monolayer/-	Rat	IM/4 days after MI	1×10^7	4 weeks	↑FS; ↓EDD; ↑EF; ↑SWT
ESC-ECs	Li et al.[41]/2009	EBs+monolayer/ $CD31^+CD144^+$	Mouse	IM/immediately after MI	1×10^6	8 weeks	2 weeks: ↑FS; ↑viability 4 weeks: = FS; = viability 8 weeks: ↓EDV; = FS; = EF; = viability
ESC-VCs	Xiong et al.[42]/2011	Monolayer/ $CD34^+CD31^+$	Swine	IM+fibrin patch/ immediately after MI	1×10^6	4 weeks	↑FS; ↑EF
ESC-VCs	Xiong et al.[43]/2012	Monolayer/ $CD34^+CD31^+$	Mouse	IM+fibrin patch/ immediately after MI	1.25×10^5	4 weeks	↑EF
iPSCs							
iPSCs	Nelson et al.[55]/2009	EBs/-	Mouse	IM/immediately after MI	2×10^5	4 weeks	↑EF; ↑FS; ↑SWT
iPSC-CPCs	Mauritz et al.[53]/ 2011	Monolayer/ $Flk\text{-}1^+$	Mouse	IM/immediately after MI	5×10^5	2 weeks	↑EF; ↓infarct size; ↑SWT; ↓EDV
iPSC-CPCs	Dai et al.[57]/2011	EBs/ $NCX1^+CX43^+$	Mouse	Cell patch/7 days after MI	-	4 weeks	↑EF; ↑FS; ↑SWT; ↓EDD
iPSCs	Yan et al.[56]/2011	EBs/-	Mouse	IM/immediately after MI	5×10^4	2 weeks	↑EF
iPSC-CMs	Carpenter et al.[58]/ 2012	Monolayer/ $Troponin\ I^+$	Rat	IM/immediately after MI	2×10^6	10 weeks	↑EF; = infarct size; ↓ESV

↑ denotes significantly increased; ↓ denotes significantly decreased; = denotes not significantly affect, respectively.
CMs, cardiomyocytes; CPCs, cardiovascular progenitor cells; EBs, embryoid bodies; ECs, endothelial cells; EDD, end-diastolic diameter; EDV, end-diastolic volume; EF, ejection fraction; ESCs, embryonic stem cells; ESV, end-systolic volume; Flk-1, fetal liver kinase 1; FS, fractional shortening; IM, intramuscular; iPSCs, induced pluripotent stem cells; MI, myocardial infarction; SV, stroke volume; SWT, systolic wall thickness; VCs, vascular cells.

The mechanisms of therapeutic effects of ESC-derived CMs and vascular cells in myocardial regeneration therapy are presumed to be mediated through cell engraftment and proliferation, paracrine effects, and recruitment of intrinsic progenitor cells by releasing cardiac cytokines.[32,38]

Cell-enhancement strategy for human ESC-derived CMs and vascular cells in myocardium

Previous research on ESC-based therapy showed poor long-term engraftment of ESC-derived CMs.[28,38] Nevertheless, alternative transplantation protocols with the addition of matrix[44] or pro-survival factors[45] have been developed with promising results in an effort to prevent cell death after transplantation. Engineering of cardiac tissue employs the use of co-culture of stem cells with biodegradable scaffold microenvironment[46] or myocardial sheets[47] to improve graft survival, proliferation, and electromechanical coupling. In light of the pivotal role of vasculature formation in graft survival rather than repeated graft delivery,[40] a modified delivery strategy using prevascularized CMs grown in a triculture tissue construct (containing ESC-derived CMs, vascular ECs and embryonic fibroblasts) by tissue engineering has shown promising graft survival.[46] However, several technical obstacles and some issues remain to be resolved, such as graft survival, stable blood supply, occurrence of ventricular tachycardia, and adequate innervations before wider clinical applications can be further investigated and applied.[47]

The major challenges of ESC-based therapy in ischemic diseases

There are 4 major challenges of ESC-based therapy which remain to be resolved before clinical application in ischemic diseases: contamination of animal product (mouse feeder cells and bovine serum), tumorigenesis or teratoma formation, immune rejection, and ethical issues. Recently, a novel system comprising encapsulated multicellular aggregation of human ESC-derived ECs has been developed to utilize humoral factors secreted by human ESC derivatives that aid in the survivability and safety of transplanted cells without direct incorporation, thereby resulting in reduced tumorigenesis or unidentified side effects of injected cells in vivo.[48] In addition, a recent deviation of vascular ECs from human ESCs with feeder- and serum-free protocol under good manufacturing practice-compliant conditions has been developed, thereby leading the process even closer to clinical evaluation in ischemic diseases.[49] Although immune rejection has long been considered a potential problem in clinical application,[4,14,23,32] recent research suggests limited or negligible immunogenicity of transplanted cells differentiated from human ESCs.[20] However, the ethical dilemma involving the destruction of a human embryo is one factor that has limited the development of human ESC-based clinical therapy.[14] Therefore, patient-specific pluripotent stem cells may be the potential solution to this dilemma.

HUMAN IPSC-DERIVED ECS AND MCS IN LIMB ISCHEMIA

Development of patient-specific pluripotent stem cells

Patient-specific pluripotent stem cells, iPSCs (Figure 1), can be established by epigenetically reprogramming somatic fibroblasts by expressing exogenous transcription factors or gene expression regulators such as proteins and microRNAs.[9] iPSCs were first developed by Takahashi and Yamanaka[15] from reprogramming mouse fibroblasts in 2006 using retroviral expression of 4 pluripotency genes, including octamer-binding transcription factor 4 (OCT4), sex determining region Y (SOX2), c-MYC and Krüppel-like factor 4 (KLF4), to reset the cellular developmental program.[15] However, this study was limited by the low efficiency production of mouse iPSC lines and the fact that global gene expression profiles are dissimilar between iPSCs and ESCs, and failure to produce adult chimaeras.[14,15] Wernig et al.[16] reported a similar process but employed more stringent criteria (using Fbx15/Fbxo1, a downstream target of the OCT4, instead of OCT4) to establish iPSCs with more efficient production of stem cell lines and more similar gene expression profiles and DNA methylation patterns to those in ESCs with successful production of adult chimaeras.[14,16] Yamanaka and colleagues found that similar success could be achieved by selection for Nanog expression instead of c-MYC.[17] Further successful approach to establish human iPSCs by transducing adult human dermal fibroblasts with the four initially de-

scribed stemness factors was reported by Yamanaka team.[9] A similar result was also reported by Yu et al.[9] who transduced 3 different cell types with OCT4, SOX2, Nanog, and Lin28 to successfully produce human iPSCs.[9] The production of human iPSCs with near identical genetic and functional properties when compared to human ESCs has generated much excitement.[14] The technology to create iPSCs bypasses the production of an embryo and does not involve the collection of oocytes from female volunteers, by which patient-specific ESCs are established by somatic cell nuclear transfer.[14] Therefore, iPSCs can circumvent the ethical issue and can be used in autologous therapy.[9]

Pre-clinical application of human iPSC derivatives in the ischemic hindlimb

The differentiation of iPSCs in EBs or monolayer culture toward vascular lineage (ECs and MCs) or cardiovascular progenitor cells (CPCs) can be easily induced (Figure 1) by culture in the differentiation medium with or without relevant growth factors and purified based on CD31 or Flk-1 expression.[19,50] By using the ESC differentiation system, purified Flk-1$^+$ cells, that is CPCs, further differentiate to ECs and MCs by the addition of VEGF and serum, respectively (Figure 1). Diverse phenotypes of ECs such as arterial, venous, and lymphatic ECs can also be successfully induced. Self-beating CMs can be induced from CPCs by subculture on OP9 stroma cells (Figure 1).[50] The differentiation properties of iPSCs are almost completely identical to those of ESCs (Figure 1).[50] In addition, pericytes represent a unique subtype of microvessel-residing perivascular cells with diverse angiogenic functions and multilineage developmental features of mesenchymal stem cells.[51] Recently, iPSC-derived pericytes have been identified.[51] Transplantation of iPSC-derived vascular lineage cells restores limb perfusion and improves neovascularization in the rodent hindlimb ischemia model.[19,51]

HUMAN IPSC-DERIVED CMs AND CPCs IN MYOCARDIAL REGENERATION THERAPY

Pre-clinical application of human iPSC derivatives in myocardial regeneration therapy

Some studies have reported that functional CMs can be derived from differentiation of mouse or human iPSCs in EBs with similar cardiac gene expression patterns and electrophysiological characteristics, but with less efficient contracting EB formation than ESCs.[18] Besides, the functional properties related to excitation-contraction coupling of CMs clones differentiated from human foreskin fibroblast-derived iPSCs resemble in part those of adult CMs.[52] Partially committed progenitor-like cells, which are also named as CPCs with differentiation potential onto ECs, MCs and CMs, can also be derived from human iPSCs.[53,54] iPSC-derived CPCs have been characterized as progenitors with positive expression of Flk-1,[53] or expression of OCT4, stage-specific embryonic antigen 1 and mesoderm posterior 1.[54] Pre-clinical studies have shown that transplantation of human iPSC-derived CMs or CPCs resulted in improved cardiac function by 9-17% increase in left ventricular ejection fraction and reduced infarct size by 44-56% in rodent myocardial infarct models (Table 1).[9,53-58] However, transplantation of iPSC-derived CPCs not only repairs myocardium but also contributes to new vessel formation to increase blood supply. In addition, CPCs may exhibit superior survival in a hostile graft environment.[53] Accordingly, cell therapy with iPSC-derived CPCs may be more promising than iPSC-derived CMs in therapeutic myocardial regeneration. In addition to iPSCs from the somatic cells of healthy patients, recent derivation and CM differentiation of iPSCs from heart failure patients has also been established.[59] The authors found that in vivo transplantation studies in the rat heart revealed the ability of the iPSC-derived CMs to engraft, survive, and structurally integrate with host cardiomyocytes, a fact which indicates that cell therapy with iPSC derivatives is getting closer to clinical application.[59] The underlying mechanisms for translation therapy effects of iPSC derivatives in myocardial infarct include differentiation into the CM lineage commitment,[53-57] neovascularization[53,56] and paracrine effects.[60]

CELL-ENHANCEMENT STRATEGY FOR HUMAN IPSC DERIVATIVES IN ISCHEMIC DISEASES

Exploring efficient somatic cells for the generation of human iPSCs

Although skin fibroblasts are the most common cell

type used for the generation of iPSCs, fibroblasts reprogram with relatively low efficiency.[15] In order to determine an ideal cellular substrate, several sources of somatic cells have been used in the generation of iPSCs,[61-66] including T cells in peripheral blood,[61,62] hematopoietic stem cells in bone marrow,[63] and $CD34^+$ mononuclear cells from cord blood or in peripheral blood (Figure 1).[64-66] Hematopoietic stem cells can be reprogrammed with higher efficiency,[63,67] whereas peripheral T cells have a low capacity to expand in culture with low reprogramming efficiency.[61,62,67] Recently, human iPSCs have been derived from late outgrowth EPCs with higher reprogramming kinetics and efficiencies, which are around 10-fold more frequency, compared with dermal fibroblasts.[67]

Enhanced engraft and survival with bioengineering manipulation

Engraftment of iPSC derivatives can be enhanced by utilizing the iPSC cell sheet with tissue engineering technique.[68,69] Furthermore, the cell-sheet technique is one of the useful methods for transplanting large numbers of cells with corrected aligned CMs.[47,69] Recently, the feasibility and therapeutic efficacy of iPSC-CM sheets have been reported and the culture system used yields a large number of highly pure human iPSC-CMs, and human iPSC-CM sheets are able to improve cardiac function in a porcine ischemic cardiomyopathy model.[69] Besides, improved targeting, and enhanced retention of transplanted iPSCs can be also performed with the aid of the bioengineered, heterospecific, tetravalent antibodies, which have exquisite specificity and high affinity towards human iPSCs and the sarcomeres of the infarcted myocardium.[70]

The major challenges of iPSC-based therapy in ischemic diseases

Although reprogramming somatic cells to induced iPSCs may hold promise as a promising strategy for regeneration therapy, the well-known limitations of current reprogramming technologies include low efficiency, slow kinetics, transgene integration and residual expression, and genomic instability.[71] Virus-based delivery of reprogramming factors may cause permanent integration of transgene and/or virus sequences into the genome harboring a risk of tumorigenesis.[71] Residual expression of cMYC and KLF4, also known as oncogenes, may also result in tumorigenesis.[71] To avoid the tumorigenicity risk of transplanting iPSC derivatives, non-viral and on-integrating methods have been established such as the transient expression of reprogramming factors, including being delivered by minicircle vectors, episomal plasmid vectors, RNA and protein, without genomic integration.[9,71-75] However, a complete depletion of tumorigenicity is still difficult to achieve and the safety of iPSCs still has new challenges.[9]

Direct reprogramming of fibroblasts into CMs or ECs has been recently developed and emerged as a potential substitute for current iPSC-based cardiovascular regeneration therapy.[9] Transduction of some developmental transcriptional factors rapidly and efficiently reprograms adult cardiac fibroblasts directly into differentiated CM-like cells or ECs without first becoming iPSCs.[9,76-78] However, there are still some problems remaining such as oncogene delivery and formation of immature CM phenotypes in this approach.[9,77]

In spite of previous concerns about the occurrence and role of copy number variants with genomic instability in reprogramming somatic fibroblasts into iPSCs,[79] a recent report suggests that most of the line-manifested copy number variants reflect typical somatic mosaicism in the human skin.[80]

CONCLUSIONS AND PERSPECTIVES ON PLURIPOTENT STEM CELL THERAPY IN ISCHEMIC DISEASE

Pluripotent stem cells have multi-lineage differentiation capacity and can generate clinically relevant amounts of stem cells for regeneration therapy. Furthermore, the regeneration capacity of these cells is not affected by the age of the individual from whom the stem cells are derived, thus perhaps leading to the effectiveness of autologous cell therapy. Transplantation of pluripotent stem cell-derived ECs, MCs, CMs or CPCs contributes to neovascularization and cardiomyogenesis with resultant better limb perfusion and recovery of myocardial contractility mediated through paracrine effect, as well as direct incorporation from transplanted cells, according to preclinical studies. Several strategies have been developed to enhance the efficacy of repro-

gramming and engrafting, and improve graft survival, proliferation and electromechanical coupling by tissue engineering.

In the future, we expect that clinical trials can be approved and carried out to provide clinical benefits as seen in animal studies. Before wide clinical application of these cells in the regeneration therapy of ischemic disease can be implemented, substantial efforts should be made to ascertain the most promising cell type and pluripotent stem cell-derivatives, the best protocol for cell preparation, reprogramming, differentiation, and delivery, and finally the most attractive strategy to avoid tumorigenesis, contamination of animal product and viral vector, and immune rejection.

ACKNOWLEDGEMENT

This work was supported in part by the Multidisciplinary Center of Excellence for Clinical Trial and Research (grant number DOH 102-TD-B-111-002) from the Department of Health and the Ministry of Health (MOHW103-TDU-B-211-113002) of Taiwan. The authors would like to thank Mr. Shih-Hao Wang (from National Cheng Kung University College of Medicine and Hospital Dou-Liou Branch, Yun-Lin County, Taiwan) for assistance in figure preparation.

REFERENCES

1. Chao TH, Tseng SY, Li YH, et al. A novel vasculo-angiogenic effect of cilostazol mediated by cross-talk between multiple signalling pathways including the ERK/p38 MAPK signaling transduction cascade. *Clin Sci (Lond)* 2012;123:147-59.
2. Asahara T, Murohara T, Sullivan A, et al. Isolation of putative progenitor endothelial cells for angiogenesis. *Science* 1997;275: 964-7.
3. Chao TH, Tseng, SY, Chen IC, et al. Cilostazol enhances mobilization and proliferation of endothelial progenitor cells and collateral formation by modifying vasculo-angiogenic biomarkers in peripheral arterial disease. *Int J Cardiol* 2014;172:371-4.
4. Yamahara K, Itoh H. Potential use of endothelial progenitor cells for regeneration of the vasculature. *Ther Adv Cardiovasc Dis* 2009;3:17-27.
5. Sen S, McDonald SP, Coates PT, Bonder CS. Endothelial progenitor cells: novel biomarker and promising cell therapy for cardiovascular disease. *Clin Sci (Lond)* 2011;120:263-83.
6. Wang CH, Huang PH, Chen JW, et al. Clinical application of endothelial progenitor cell: are we ready? *Acta Cardiol Sin* 2013;29: 479-87.
7. Capogrossi MC. Cardiac stem cells fail with aging: a new mechanism for the age-dependent decline in cardiac function. *Circ Res* 2004;94:411-3.
8. Fadini GP, Sartore S, Albiero M, et al. Number and function of endothelial progenitor cells as a marker of severity for diabetic vasculopathy. *Arterioscler Thromb Vasc Biol* 2006;26:2140-6.
9. Wang WE, Chen X, Houser SR, Zeng C. Potential of cardiac stem/progenitor cells and induced pluripotent stem cells for cardiac repair in ischaemic heart disease. *Clin Sci (Lond)* 2013; 125:319-27.
10. Yamahara K, Sone M, Itoh H, et al. Augmentation of neovascularization [corrected] in hindlimb ischemia by combined transplantation of human embryonic stem cells-derived endothelial and mural cells. *PLoS One* 2008;3:e1666.
11. Yamashita J, Itoh H, Hirashima M, et al. Flk1-positive cells derived from embryonic stem cells serve as vascular progenitors. *Nature* 2000;408:92-6.
12. Sone M, Itoh H, Yamahara K, 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.
13. Kehat I, Kenyagin-Karsenti D, Snir M, et al. Human embryonic stem cells can differentiate into myocytes with structural and functional properties of cardiomyocytes. *J Clin Invest* 2001;108: 407-14.
14. Kastenberg ZJ, Odorico JS. Alternative sources of pluripotency: science, ethics, and stem cells. *Transplant Rev (Orlando)* 2008; 22:215-22.
15. Takahashi K, Yamanaka S. Induction of pluripotent stem cells from mouse embryonic and adult fibroblast cultures by defined factors. *Cell* 2006;126:663-76.
16. Wernig M, Meissner A, Foreman R, et al. In vitro reprogramming of fibroblasts into a pluripotent ES-cell-like state. *Nature* 2007; 448:318-24.
17. Okita K, Ichisaka T, Yamanaka S. Generation of germline-competent induced pluripotent stem cells. *Nature* 2007;448: 313-7.
18. Zhang J, Wilson GF, Soerens AG, et al. Functional cardiomyocytes derived from human induced pluripotent stem cells. *Circ Res* 2009;104:e30-41.
19. Rufaihah AJ, Huang NF, Jamé S, et al. Endothelial cells derived from human iPSCS increase capillary density and improve perfusion in a mouse model of peripheral arterial disease. *Arterioscler Thromb Vasc Biol* 2011;31:e72-9.
20. Araki R, Uda M, Hoki Y, et al. Negligible immunogenicity of terminally differentiated cells derived from induced pluripotent or embryonic stem cells. *Nature* 2013;494:100-4.
21. Suzuki H, Shibata R, Kito T, et al. Comparative angiogenic activities of induced pluripotent stem cells derived from young and old mice. *PLoS One* 2012;7:e39562.

22. Roden DM, Hong CC. Stem cell-derived cardiomyocytes as a tool for studying proarrhythmia: a better canary in the coal mine? *Circulation* 2013;127:1641-3.
23. Li Z, Han Z, Wu JC. Transplantation of human embryonic stem cell-derived endothelial cells for vascular diseases. *J Cell Biochem* 2009;106:194-9.
24. Thomson JA, Itskovitz-Eldor J, Shapiro SS, et al. Embryonic stem cell lines derived from human blastocysts. *Science* 1998;282: 1145-7.
25. Levenberg S, Golub JS, Amit M, et al. Endothelial cells derived from human embryonic stem cells. *Proc Natl Acad Sci U S A* 2002;99:4391-6.
26. Kane NM, Meloni M, Spencer HL, et al. Derivation of endothelial cells from human embryonic stem cells by directed differentiation: analysis of microRNA and angiogenesis in vitro and in vivo. *Arterioscler Thromb Vasc Biol* 2010;30:1389-97.
27. Huang NF, Niiyama H, Peter C, et al. Embryonic stem cell-derived endothelial cells engraft into the ischemic hindlimb and restore perfusion. *Arterioscler Thromb Vasc Biol* 2010;30:984-91.
28. Li Z, Suzuki Y, Huang M, et al. Comparison of reporter gene and iron particle labeling for tracking fate of human embryonic stem cells and differentiated endothelial cells in living subjects. *Stem Cells* 2008;26:864-73.
29. Kano MR, Morishita Y, Iwata C, et al. VEGF-A and FGF-2 synergistically promote neoangiogenesis through enhancement of endogenous PDGF-B-PDGFRbeta signaling. *J Cell Sci* 2005;118: 3759-68.
30. Park SW, Jun Koh Y, Jeon J, et al. Efficient differentiation of human pluripotent stem cells into functional CD34+ progenitor cells by combined modulation of the MEK/ERK and BMP4 signaling pathways. *Blood* 2010;116:5762-72.
31. Yang F, Cho SW, Son SM, et al. Genetic engineering of human stem cells for enhanced angiogenesis using biodegradable polymeric nanoparticles. *Proc Natl Acad Sci U S A* 2010;107:3317-22.
32. Freund C, Mummery CL. Prospects for pluripotent stem cell-derived cardiomyocytes in cardiac cell therapy and as disease models. *J Cell Biochem* 2009;107:592-9.
33. Mummery C, Ward-van Oostwaard D, Doevendans P, et al. Differentiation of human embryonic stem cells to cardiomyocytes: role of coculture with visceral endoderm-like cells. *Circulation* 2003;107:2733-40.
34. Fernandes S, Naumova AV, Zhu WZ, et al. Human embryonic stem cell-derived cardiomyocytes engraft but do not alter cardiac remodeling after chronic infarction in rats. *J Mol Cell Cardiol* 2010;49:941-9.
35. Bin Z, Sheng LG, Gang ZC, et al. Efficient cardiomyocyte differentiation of embryonic stem cells by bone morphogenetic protein-2 combined with visceral endoderm-like cells. *Cell Biol Int* 2006;30:769-76.
36. Yang L, Soonpaa MH, Adler ED, et al. Human cardiovascular progenitor cells develop from a KDR+ embryonic-stem-cell-derived population. *Nature* 2008;453:524-8.
37. Caspi O, Huber I, Kehat I, et al. Transplantation of human embryonic stem cell-derived cardiomyocytes improves myocardial performance in infarcted rat hearts. *J Am Coll Cardiol* 2007; 50:1884-93.
38. Ebelt H, Jungblut M, Zhang Y, et al. Cellular cardiomyoplasty: improvement of left ventricular function correlates with the release of cardioactive cytokines. *Stem Cells* 2007;25:236-44.
39. van Laake LW, Passier R, Monshouwer-Kloots J, 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.
40. van Laake LW, Passier R, den Ouden K, et al. Improvement of mouse cardiac function by hESC-derived cardiomyocytes correlates with vascularity but not graft size. *Stem Cell Res* 2009; 3:106-12.
41. Li Z, Wilson KD, Smith B, et al. Functional and transcriptional characterization of human embryonic stem cell-derived endothelial cells for treatment of myocardial infarction. *PLoS One* 2009;4:e8443.
42. Xiong Q, Hill KL, Li Q, et al. A fibrin patch-based enhanced delivery of human embryonic stem cell-derived vascular cell transplantation in a porcine model of postinfarction left ventricular remodeling. *Stem Cells* 2011;29:367-75.
43. Xiong Q, Ye L, Zhang P, et al. Bioenergetic and functional consequences of cellular therapy: activation of endogenous cardiovascular progenitor cells. *Circ Res* 2012;111:455-68.
44. Cao F, Sadrzadeh Rafie AH, Abilez OJ, et al. In vivo imaging and evaluation of different biomatrices for improvement of stem cell survival. *J Tissue Eng Regen Med* 2007;1:465-8.
45. Laflamme MA, Chen KY, Naumova AV, 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.
46. Lesman A, Gepstein L, Levenberg S. Vascularization shaping the heart. *Ann N Y Acad Sci* 2010;1188:46-51.
47. Fujita J, Itabashi Y, Seki T, et al. Myocardial cell sheet therapy and cardiac function. *Am J Physiol Heart Circ Physiol* 2012;303: H1169-82.
48. Moon SH, Kim JS, Park SJ, et al. A system for treating ischemic disease using human embryonic stem cell-derived endothelial cells without direct incorporation. *Biomaterials* 2011;32:6445-55.
49. Kaupisch A, Kennedy L, Stelmanis V, et al. Derivation of vascular endothelial cells from human embryonic stem cells under GMP-compliant conditions: towards clinical studies in ischaemic disease. *J Cardiovasc Transl Res* 2012;5:605-17.
50. Narazaki G, Uosaki H, Teranishi M, et al. Directed and systematic differentiation of cardiovascular cells from mouse induced pluripotent stem cells. *Circulation* 2008;118:498-506.
51. Dar A, Domev H, Ben-Yosef O, et al. Multipotent vasculogenic pericytes from human pluripotent stem cells promote recovery of murine ischemic limb. *Circulation* 2012;125:87-99.
52. Germanguz I, Sedan O, Zeevi-Levin N, et al. Molecular characterization and functional properties of cardiomyocytes derived

from human inducible pluripotent stem cells. *J Cell Mol Med* 2011;15:38-51.
53. Mauritz C, Martens A, Rojas SV, et al. Induced pluripotent stem cell (iPSC)-derived Flk-1 progenitor cells engraft, differentiate, and improve heart function in a mouse model of acute myocardial infarction. *Eur Heart J* 2011;32:2634-41.
54. Blin G, Nury D, Stefanovic S, et al. A purified population of multipotent cardiovascular progenitors derived from primate pluripotent stem cells engrafts in postmyocardial infarcted nonhuman primates. *J Clin Invest* 2010;120:1125-39.
55. Nelson TJ, Martinez-Fernandez A, Yamada S, et al. Repair of acute myocardial infarction by human stemness factors induced pluripotent stem cells. *Circulation* 2009;120:408-16.
56. Yan B, Abdelli LS, Singla DK. Transplanted induced pluripotent stem cells improve cardiac function and induce neovascularization in the infarcted hearts of db/db mice. *Mol Pharm* 2011; 8:1602-10.
57. Dai B, Huang W, Xu M, et al. Reduced collagen deposition in infarcted myocardium facilitates induced pluripotent stem cell engraftment and angiomyogenesis for improvement of left ventricular function. *J Am Coll Cardiol* 2011;58:2118-27.
58. Carpenter L, Carr C, Yang CT, et al. Efficient differentiation of human induced pluripotent stem cells generates cardiac cells that provide protection following myocardial infarction in the rat. *Stem Cells Dev* 2012;21:977-86.
59. Zwi-Dantsis L, Huber I, Habib M, et al. Derivation and cardiomyocyte differentiation of induced pluripotent stem cells from heart failure patients. *Eur Heart J* 2013;34:1575-86.
60. Gu M, Nguyen PK, Lee AS, et al. Microfluidic single-cell analysis shows that porcine induced pluripotent stem cell-derived endothelial cells improve myocardial function by paracrine activation. *Circ Res* 2012;111:882-93.
61. Loh YH, Agarwal S, Park IH, et al. Generation of induced pluripotent stem cells from human blood. *Blood* 2009;113:5476-9.
62. Seki T, Yuasa S, Oda M, et al. Generation of induced pluripotent stem cells from human terminally differentiated circulating T cells. *Cell Stem Cell* 2010;7:11-4.
63. Eminli S, Foudi A, Stadtfeld M, et al. Differentiation stage determines potential of hematopoietic cells for reprogramming into induced pluripotent stem cells. *Nat Genet* 2009;41:968-76.
64. Ho PJ, Yen ML, Lin JD, et al. Endogenous KLF4 expression in human fetal endothelial cells allows for reprogramming to pluripotency with just OCT3/4 and SOX2 -- brief report. *Arterioscler Thromb Vasc Biol* 2010;30:1905-7.
65. Koyanagi M, Iwasaki M, Rupp S, et al. Sox2 transduction enhances cardiovascular repair capacity of blood-derived mesoangioblasts. *Circ Res* 2010;106:1290-302.
66. Yin L, Ohanyan V, Pung YF, et al. Induction of vascular progenitor cells from endothelial cells stimulates coronary collateral growth. *Circ Res* 2012;110:241-52.
67. Geti I, Ormiston ML, Rouhani F, et al. A practical and efficient cellular substrate for the generation of induced pluripotent stem cells from adults: blood-derived endothelial progenitor cells. *Stem Cells Transl Med* 2012;1:855-65.
68. Kito T, Shibata R, Ishii M, et al. iPS cell sheets created by a novel magnetite tissue engineering method for reparative angiogenesis. *Sci Rep* 2013;3:1418.
69. Kawamura M, Miyagawa S, Miki K, et al. Feasibility, safety, and therapeutic efficacy of human induced pluripotent stem cell-derived cardiomyocyte sheets in a porcine ischemic cardiomyopathy model. *Circulation* 2012;126:S29-37.
70. Malecki M. Improved targeting and enhanced retention of the human, autologous, fibroblast-derived, induced, pluripotent stem cells to the sarcomeres of the infarcted myocardium with the aid of the bioengineered, heterospecific, tetravalent antibodies. *J Stem Cell Res Ther* 2013;3:138.
71. Hu K, Yu J, Suknuntha K, et al. Efficient generation of transgene-free induced pluripotent stem cells from normal and neoplastic bone marrow and cord blood mononuclear cells. *Blood* 2011; 117:e109-19.
72. Chou BK, Mali P, Huang X, et al. Efficient human iPS cell derivation by a non-integrating plasmid from blood cells with unique epigenetic and gene expression signatures. *Cell Res* 2011;21: 518-29.
73. Burridge PW, Thompson S, Millrod MA, et al. A universal system for highly efficient cardiac differentiation of human induced pluripotent stem cells that eliminates interline variability. *PLoS One* 2011;6:e18293.
74. Narsinh K, Narsinh KH, Wu JC. Derivation of human induced pluripotent stem cells for cardiovascular disease modeling. *Circ Res* 2011;108:1146-56.
75. Margariti A, Winkler B, Karamariti E, et al. Direct reprogramming of fibroblasts into endothelial cells capable of angiogenesis and reendothelialization in tissue-engineered vessels. *Proc Natl Acad Sci U S A* 2012;109:13793-8.
76. Ieda M, Fu JD, Delgado-Olguin P, et al. Direct reprogramming of fibroblasts into functional cardiomyocytes by defined factors. *Cell* 2010;142:375-86.
77. Qian L, Huang Y, Spencer CI, et al. In vivo reprogramming of murine cardiac fibroblasts into induced cardiomyocytes. *Nature* 2012;485:593-8.
78. Li J, Huang NF, Zou J, et al. Conversion of human fibroblasts to functional endothelial cells by defined factors. *Arterioscler Thromb Vasc Biol* 2013;33:1366-75.
79. Hussein SM, Batada NN, Vuoristo S, et al. Copy number variation and selection during reprogramming to pluripotency. *Nature* 2011;471:58-62.
80. Abyzov A, Mariani J, Palejev D, et al. Somatic copy number mosaicism in human skin revealed by induced pluripotent stem cells. *Nature* 2012;492:438-42.