Editorial

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Regenerative Medicine

Direct reprogramming into endothelial cells: a new source for vascular regeneration

"This approach has received notable attention and is regarded as the third-generation modality for cell therapy and regenerative medicine..."

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First draft submitted: 23 February 2017; Accepted for publication: 20 April 2017; Published online: 16 June 2017

Keywords: cell therapy • direct reprogramming • endothelial cells • ETV2 • pluripotency • stem cell • transcription factor • vascular regeneration • vector

Despite significant efforts over the last several decades, treating patients with severe conditions of myocardial ischemia and peripheral vascular disease remains challenging. For example, critical limb ischemia, a severe form of peripheral vascular disease, can lead to a 50% risk of amputation [1] with high incidences of second-leg loss and mortality within 2–5 years after the first amputation. Pathophysiologically, the main cause of these clinical entities is the loss or dysfunction of blood vessels, of which the major component is endothelial cells (ECs). Thus, therapeutic neovascularization has emerged as an attractive approach to re-establish functional vasculature, which can support proper blood perfusion and tissue repair [2].

Among several available modalities, cell-based therapy has garnered much attention as it can potentially supply functional ECs to form new blood vessels. Particularly, autologous ECs are in high demand, but their availability is limited. To resolve this problem, EC generation via adult stem or progenitor cells, such as endothelial progenitor cells, mesenchymal stem cells or bone marrow mononuclear cells, was attempted with limited success. It turned out that they work mainly through paracrine effects and even those effects were still modest [3–6]. More recently, human pluripotent stem cells (PSCs) including embryonic stem cells and induced pluripotent stem cells (iPSCs) were used for generating ECs. While PSC-derived ECs have shown potent vessel-forming effects, they have limitations for clinical use due to ethical issues (in case of embryonic stem cells), potential side effects such as tumorigenicity/aberrant tissue formation [7,8], lengthy and complex differentiation processes, low differentiation efficiency and difficulties in maintaining the phenotype [9,10].

Recently, a series of studies has reported a new method called direct reprogramming (or direct conversion or transdifferentiation) of somatic cells into specific lineage cells, such as ECs, neurons, cardiomyocytes or hepatocytes, using lineage or cell type-specific transcription factors or miRNAs without first dedifferentiating into a pluripotent state [11]. This approach has received notable attention and is regarded as the third-generation modality for cell therapy and regenerative medicine as it can reduce not only the time and cost of target cell generation, but also the potential side effects and inefficiency associated with the use of adult or PSCs mentioned above. Accordingly, this editorial will briefly review the progress of direct reprogramming of somatic cells into ECs.

Reprogramming into ECs via partial pluripotency

Margariti *et al.* reported generation of ECs by reprogramming human fibroblasts (HFs) using iPSC-inducing factors (OCT4, SOX2,

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KLF4 and c-MYC) and EC differentiation condition [12]. Short-term induction of iPSC-inducing factors altered the plasticity of HFs enough to generate partial-iPSCs, which were then differentiated into ECs under defined media and culture conditions. These ECs were referred to as partial-iPSCs–ECs. In line with these efforts, two other groups also reported reprogramming of HFs into ECs using iPSC-inducing factors [13,14]. By exposing HFs to these four iPSC-inducing factors for 8 days, followed by culture in mesodermal induction media, angioblast-like progenitor cells were obtained [13]. In this study, all four factors were necessary to convert HFs into the angioblast-like stage. Li *et al.* further optimized the process by using only two factors (OCT4 and KLF4) [14]. Although these methods may be considered as direct reprogramming, they are not direct reprogramming in a strict sense because they first need to undergo dedifferentiation processes to acquire partial pluripotency and then follow an endothelial differentiation pathway, which basically mimics the differentiation process from PSCs. While these methods are valuable to expedite the process of generating ECs, there remain concerns about tumorigenesis due to the use of iPSC-inducing factors. In addition, other concerns related to human PSC-derived ECs mentioned above still remain.

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Direct reprogramming of somatic cells into ECs

To achieve true 'direct reprogramming' of somatic cells into ECs by bypassing stem-cell-like stages, investigators attempted EC-lineage-specific transcription factors. Ginsberg *et al.* reported generation of ECs by direct reprogramming from human amniotic cells (ACs) using lentiviral ETS transcription factors (*ETV2, FLI1* and *ERG1*) along with a TGFβ inhibitor, and these expandable ECs were referred to as reprogrammed AC vascular endothelial cells (rAC-VECs) [10]. This combination of short-term TGFβ inhibition, transient *ETV2* expression and continuous expression of *FLI1* and *ERG1* were required for the transition from ACs to rAC-VECs. rAC-VECs were shown to have similar transcriptome to human umbilical vein endothelial cells. They also demonstrated that rAC-VECs formed tubes *in vitro* and *in vivo* with Matrigel, and were engrafted into the blood vessels in regenerating liver suggesting the capability of establishing functional vessels. The authors acknowledged that *ETV2* expression alone was insufficient to turn on all EC genes, and an attempt at using human postnatal cells as a source cells was unsuccessful. Additionally, due to the origin of source cells, it is not clear if the source cells were fully differentiated and if there is potential contamination with stem or progenitor cells. The authors suggested that this study did not aim to achieve autologous cell therapy, but allogenic cell therapy using banked ACs. Nonetheless, this report was the first study demonstrating feasibility of cellular reprogramming into ECs with EC-specific transcription factors. Two years later, Han *et al.* directly converted adult mouse skin fibroblasts into ECs, referred to as induced ECs (iECs), by using five transcription factors (*Foxo1, Etv2, Klf2, Tal1* and *Lmo2*) in lentiviral vector [15]. They observed that all five factors were required for efficient reprogramming into iECs (*Tie2*-GFP$^+$ 4%) and ETV2 alone was insufficient for successful reprogramming of mouse skin fibroblasts.

More recently, studies have shown successful reprogramming of postnatal HFs into ECs [16,17]. Morita *et al.* reported that the single-factor *ETV2*, which is transduced in doxycycline-inducible lentiviral vector, could directly convert HFs into ECs, referred to as ETV2-induced vascular endothelial cells (ETVECs) [16]. ETVECs are a proliferative CD31^{high} (PECAM1^{high}) cell population selectively sorted at 15 days (CD31$^+$ 3.5%) after *ETV2* overexpression and displayed endothelial phenotype *in vitro*. The authors demonstrated that ETVECs implanted with Matrigel promoted blood flow recovery in ischemic hindlimb of non-obsese diabetic/severe combined immunodeficiency (NOD SCID) mice, and were engrafted into blood vessels *in vivo*. However, during the culture period of more than 50 days, ETVECs maintained high expression of *ETV2*, which should be minimally expressed in any mammalian postnatal ECs. Therefore, it can be argued that ETVECs are not reprogrammed or iECs but rather selected cells displaying the ectopic expression of CD31 (PECAM1), which is one of the direct targets of ETV2 [18]. We recently reported that overexpression of *ETV2* alone via doxycycline-inducible lentiviral vector directly reprogramed HFs into ECs through at least two distinct stages [17]. At the early stage of reprogramming, KDR$^+$ cells sorted at day 7 after *ETV2* transduction displayed less mature but enriched endothelial characteristics. These cells were termed early reprogrammed ECs (rECs). Implantation of early rECs into ischemic hindlimbs demonstrated incorporation of rECs into the functional vessels, enhanced neovascularization and repaired tissue ischemia, indicating its functional and therapeutic potential. These early rECs were further cultured *in vitro* for another 2 months after transient reinduction of *ETV2* for 7 days. These rECs showed reduced *ETV2* expression, increased PECAM1 expression (as well as all other

EC proteins such as CDH5 [or VE-CADHERIN] and VWF), a transcriptome profile similar to human umbilical vein endothelial cells and NO production, indicating a mature EC phenotype and were thus referred to as late rECs. These late rECs also showed the capability of incorporation into vessels *in vivo*. This study clearly demonstrated that ETV2 alone can directly reprogram HFs into ECs, which have several phenotypes, and can be applied for cell therapy.

There is another approach modulating a signaling pathway of innate immunity to trigger cellular reprogramming. Sayed *et al.* reported a new method to directly convert HFs using polyinosinic:polycytidylic acid into ECs, which was referred to as iECs [19]. Polyinosinic:polycytidylic acid activated toll-like receptor 3, which consequently induced global changes in the expression and activity of epigenetic modifiers. Such enhanced epigenetic plasticity together with ECs transdifferentiation culture conditions induced the conversion of HFs into iECs. Flow cytometry revealed 2% of induced HFs expressed PECAM1 when 8-Br-cAMP was added for enhancing the transdifferentiation efficiency. In a mouse model of hindlimb ischemia, transplantation of iECs increased blood flow and capillary density without incorporation of iECs into the microvasculature, suggesting that therapeutic potential of iECs is mainly attributed to paracrine effects. Although this method has a clear advantage of avoiding genetic manipulation, concerns were raised against the failure to incorporate into the vasculature and the low reprogramming efficiency.

Conclusion & future perspective

Ground-breaking discovery has been made in the reprogramming of human somatic cells into ECs. Overexpression of several key genes including ETV2 and small molecules were found to induce changes in somatic cell identity into ECs. This novel approach cannot only be used for cell therapy but also direct application of these reagents for *in vivo* regenerative therapy. In addition, the reprogrammed cells can be applied for disease investigation, drug discovery and precision medicine. However, questions remain to be answered. Particularly, the use of lentiviral vectors and the low reprogramming efficiency limit clinical application of the reprogrammed or iECs. The use of lentiviral vectors raises safety issues as it may induce insertional mutagenesis, possible generation of replication competent lentiviruses and germline transfer. Thus, more clinically compatible materials should be developed such as adenoviral vectors, noncoding RNAs, exosomes and small molecules. Furthermore, the efficiency of direct reprogramming is low. When primary human somatic cells are used as source cells, the best efficiency is still less than 20% [13,16]. Particularly, to enhance the reprogramming efficiency, studies for exploring the reprogramming mechanisms should follow. Deep sequencing of RNAs and bioinformatics will help identify factors that can facilitate and augment reprogramming. Furthermore, the phenotypes of induced or rECs were not meticulously addressed. The stages of rECs compared with the developmental stages and their arterial, venous and lymphatic phenotypes need to be addressed in future studies.

Financial & competing interests disclosure

This work was supported by grants from the Korea Health Technology R&D Project through the Korea Health Industry Development Institute (KHIDI), funded by the Ministry of Health & Welfare, Republic of Korea (HI15C2782, HI16C2211), the Bio & Medical Technology Development Program of the National Research Foundation (NRF) funded by the Korean government (MSIP; No. 2015M3A9C6031514), NIDDK (DP3-DK108245) and NHLBI (R01HL127759, R01HL129511). The authors have no other relevant affiliations or financial involvement with any organization or entity with a financial interest in or financial conflict with the subject matter or materials discussed in the manuscript apart from those disclosed.

No writing assistance was utilized in the production of this manuscript.

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