Editorial

Stem Cell INVESTIGATION

Efficient CRISPR/Cas9-based gene correction in induced pluripotent stem cells established from fibroblasts of patients with sickle cell disease

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Since Takahashi and Yamanaka (1) first reported the successful establishment of induced pluripotent stem cells (iPSCs), these cells have been an important resource for regenerative medicine and gene therapy strategies (2). In particular, the use of iPSCs helps avoid the ethical concerns associated with the use of human embryonic stem cells (ESCs) established from donor oocytes, and mitigate the possibility of immune rejection by allowing the generation of "patient-specific" pluripotent stem cells from somatic cell types (3). Furthermore, the recent advent of site-specific genome-editing technologies, such as the CRISPR/Cas9 system, has enabled "gene correction" by inserting normal sequences or deleting mutated sequences from mutated sites in the genome (4,5). These techniques have opened the doors to curing genetic disorders caused by mutations in a specific gene. One promising approach is gene correction in iPSCs established from somatic cells of patients with genetic defects. The differentiated derivatives (e.g., neurons, hematopoietic cells, and cardiomyocytes) of successfully genome-edited iPSCs can be used for the replacement of damaged tissues through autologous transplantation. In fact, successful gene correction has been reported in iPSCs derived from patients with cystic fibrosis and β-thalassemia (6-10).

Generally, human ESCs derived from early embryos and iPSCs are in a primed state of pluripotency (hereafter referred to as "primed" cells) similar to mouse epiblast stem cells (11). These cells are distinct from the naïve pluripotent stem cells (hereafter referred to as "naïve" cells) of mouse ESCs and iPSCs in terms of colony morphology (flat or dome shape), single-cell passage ability [Rho-associated coiled-coil forming kinase (ROCK) inhibitor-independent or not], pluripotent gene expression profiles (similar to primed or naïve ESCs/iPSCs), signaling pathway [mitogen-activated protein kinase (MAPK) kinase (MEK)-extracellular signal-regulated kinase (ERK)-dependent or leukemia inhibitory factor (LIF)-signal transducer and activator of transcription 3 (STAT3)-dependent], and the ability to differentiate into various types of cells. The relatively low differentiation ability of primed ESCs/iPSCs is critical for their use in personalized medicine since it is often difficult to obtain the cell/tissue type that an investigator requires. Thus, several attempts have been made to convert the primed ESCs/iPSCs to naïve cells (12-15). For example, Theunissen *et al.* (14) incubated small colonies of iPSCs, which were transfected with vectors containing Yamanaka's factors to induce reprogramming, in N2B27 basal medium supplemented with inhibitors of MEK, glycogen synthase kinase 3β (GSK3β), ROCK, SRC, and B-Raf (BRAF) kinases, together with LIF and activin (the so-called "5i/L/FA" medium), for about 10 days. After the incubation period, the colony morphology changed to a dome-like shape and the cells exhibited pluripotent gene expression profiles similar to those of naïve ESCs/iPSCs. Furthermore, various types of differentiated cells were generated when these iPSCs were subcutaneously transplanted into immunocompromised mice, suggesting the acquisition of multipotency.

Yang *et al.* (16) successfully demonstrated that it is possible to obtain naïve iPSCs directly from fibroblasts isolated from patients with β-thalassemia. β-Thalassemia, which is also called sickle cell disease (SCD), is one of the most common genetic diseases worldwide. It is an inherited blood disorder that causes severe anemia and is characterized by reduction in or absence of synthesis of hemoglobin (HB) subunit β (HB β chain). The most common molecular defects are either point mutations or small fragment deletions in the *HBB* gene, which affect mRNA assembly or translation. Yang *et al.* (16) first transfected fibroblasts carrying the β-41/42 mutation with 3 plasmids carrying Yamanaka factors (obtained from Addgene) by electroporation. The transfectants were cultured on mitomycin C-inactivated mouse embryonic fibroblast (MEF) feeders in a conventional human ES medium for 6 days and gave rise to small iPSC colonies. Further culture of the cells for 14 to 20 days in human naïve medium (5i/L/LA medium) resulted in the generation of dome-shaped colonies. Analysis of these colonies revealed the expression of pluripotent marker genes such as *NANOG*, *KLF4*, *REX1* and *STELLA*—a global gene expression pattern similar to that of naïve ESCs/iPSCs, which have single-cell passage ability. More interestingly, inoculation of these colonies under the skin of immunocompromised mice resulted in the generation of solid tumors containing various types of differentiated cells. Next, they attempted to correct the β-41/42 mutation in the *HBB* gene in the resulting naïve iPSCs. For this, they used the knock-in (KI)-based CRISPR/Cas9 genome editing system. They constructed a KI donor vector in which the normal *HBB* sequence was flanked by ~250-bp long 5' and 3' homologous arms of the gene. The naïve iPSCs thus obtained were subjected to electroporation in the presence of a donor vector and the pX330 vector containing both the guide RNA and Cas9. Concomitantly, primed iPSCs derived from the same patient were transfected. Seven days after transfection, 40 colonies were picked up for genomic DNA analysis. Sequencing of the PCR-amplified fragments spanning the mutated site demonstrated that 57% of clones in the naïve iPSC group were successfully corrected at one allele of the *HBB* gene. In contrast, in the primed iPSC group, only 32% of the clones were corrected. This result implies that naïve iPSCs are more amenable to gene correction by the CRISPR/Cas9-based genome editing system than primed iPSCs are.

The most important aspect of this study would be how to efficiently differentiate genome-modified naïve iPSCs into hematopoietic progenitor cells. Yang *et al.* (16) cultured gene-corrected naïve iPSCs, parental naïve iPSCs, or primed iPSCs on the OP9-GFP stromal cells, a genetically modified murine stromal cell line expressing green fluorescent protein (GFP) for 8 days. Flow cytometry revealed that the proportion of $CD34^+$ cells among the corrected naïve iPSCs was approximately 4.3%, which was comparable to that of the parental naïve iPSCs (approximately 3.7%) and the primed iPSCs (approximately 4.0%). This might have been an unexpected result for the authors, because naïve iPSCs are expected to be more frequently differentiated into hematopoietic cells than primed iPSCs. In this context, a system that promotes more efficient differentiation of iPSCs into hematopoietic cells is needed.

When differentiated derivatives of iPSCs are used as therapeutic materials for treating damaged tissues, the risk of tumorigenicity arising from the residual iPSCs after autologous transplantation would be a major concern (17). Notably, Chandrakanthan *et al.* (18) recently demonstrated that adult human adipose cells isolated from fat tissues can be reprogrammed to tissue-regenerative multipotent stem cells (called iMS cells) by culturing them with 5-azacytidine (AZA) and platelet-derived growth factor-AB (PDGF-AB) for approximately 2 days, followed by treatment with the growth factor alone for a further 2–3 weeks. AZA is a demethylating nucleoside analog that is widely used in clinical practice. It is also known to induce cell plasticity, which is crucial for reprogramming cells. The resulting iMS cells exhibited long-term self-renewal, serial clonogenicity, and the potential to differentiate into cells of multiple germ layers. Importantly, unlike ESCs or iPSCs, iMS cells do not form teratomas. This new vector-free method of generating iMS cells from fat cells is a promising tool for safer cell-based therapy against genetic diseases.

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Footnote

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