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Non-Viral Co-Delivery of the Four Yamanaka Factors for Generation of Human Induced Pluripotent Stem Cells via Calcium Phosphate Nanocomposite Particles

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Generating of induced pluripotent stem cells (iPSCs) can be achieved by ectopic expression of defined transcription factor sets. However, most instances of iPSC induction have been achieved using viral vectors, which carry the risk of unpredictable genetic dysfunction. Here, for the first time, a non-viral vector based on calcium phosphate nanoparticles for the generation of virus-free iPSCs from human umbilical cord mesenchymal stem cells (HUMSCs) via co-delivery of the four plasmids (Oct4, Sox2, Klf4, and c-Myc) is reported. As a result, a total of 98 colonies from 200 000 cells have been obtained, with a reprogramming efficiency of 0.049%. The iPSCs shows positive expression of pluripotency markers, including OCT4, SSEA-3, SSEA-4, NANOG, and TRA-1-81. Moreover, the iPSCs are able to differentiate into all three germ layers in vitro. Subcutaneous injection of the iPSCs into immunocompromised mice results in the formation of teratomas containing a variety of tissues from all three germ layers. These findings indicate that co-delivery of the four Yamanaka factors via plasmid-encapsulated calcium phosphate nanoparticles can provide a simple, safe, and efficient method for the generation of virus-free iPSCs, which is crucial for their future clinical applications in the field of regenerative medicine.

1. Introduction

The reprogramming of differentiated cells into induced pluripotent stem cells (iPSCs) using exogenous transcription factors holds tremendous promise in both research and clinical applications to replace human embryonic stem cells (ESCs), which are hampered by ethical issues and obstacles associated with immune rejection after transplantation.[1,2] In 2006, Yamanaka and co-workers[3] first showed the generation of iPSCs from mouse embryonic fibroblasts by retroviral transduction of four reprogramming factors Oct4, Sox2, Klf4, and c-Myc.[4] Due to this pioneering work, which inspired many scientists into the area of iPSCs research, Yamanaka was awarded the 2012 Nobel Prize in Physiology and Medicine. Human iPSCs were also obtained by the introduction of the same four factors[2,5–7] or by Oct4, Sox2, Nanog, and Lin28.[8] The iPSCs are similar to ESCs in morphology, expression of pluripotency markers, epigenetic status, and ability to self-renew and differentiate into the three germ layers.[9] These findings have provided an invaluable source of patient-specific pluripotent cells for regenerative medicine, disease modeling, drug screening, and toxicology tests.[10,11] To date, most instances of direct reprogramming have been achieved by forced expression of defined factors using viral vectors[5,12–14] However, the permanent genome alteration caused by viral vector integration hinders their use in therapeutic applications because genome-integrating viruses usually cause unpredictable gene dysfunctions.[15,16] Although iPSCs can be obtained through the non-integrative adenoviral delivery of the factors Oct4, Sox2, Klf4, and c-Myc, the efficiency of reprogramming is extremely low, and a considerable percentage of clones are tetraploid.[17,18] Therefore, generating iPSCs with nonviral methods is critical for their clinical applications.

Recently, several virus-free methods for the generation of iPSCs have been developed, such as direct delivery of reprogramming proteins[18,19] or mRNAs,[20] plasmid transfection,[9,21] the piggyBac transposon,[22,23] non-integrating episomal vectors,[24] and microRNAs technologies.[25,26] A direct protein transduction system has provided a safe source of patient-specific iPSCs for regenerative medicine due to the elimination of the potential risks associated with virus, DNA transfection, and potentially harmful chemicals.[19] Nevertheless, all of the protein-based reprogramming reports suffered from extremely slow kinetics and low efficiencies (≈2 months, 0.001% in the case of human cells), in addition

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to the technical challenges of producing large quantities of pure and biochemically active proteins.[27] Thus far, the simplest approach for generating virus-free iPSCs is the serial transfection of plasmids, which can transiently express reprogramming factors.[18] Okita et al.[9] have successfully obtained mouse iPSCs with no sign of exogenous gene integration via serial transfection and the transient expression of two plasmids, one expressing c-Myc and the other expressing Oct4, Klf4, and Sox2 as a polycistronic unit. Similar work has been reported by Gonzalez and colleagues,[21] who generated virus-free iPSCs by nucleofection of a single plasmid construct expressing Oct4, Sox2, Klf4, and c-Myc. However, the reprogramming efficiencies described in these works were still much lower (≈0.001%) when compared with viral induction (≈0.1%).[13,15,28]

Currently, the bottleneck problem for the generation of iPSCs using nonviral strategies is the low reprogramming efficiency. Numerous nonviral vectors have been previously reported for gene delivery,[29–32] but only few of them have been used for iPSCs generation to improve reprogramming efficiency. Montserrat et al.[18] have obtained human iPSCs using poly-β-amino esters as the gene delivery system, resulting in enhanced reprogramming efficiency (≈0.01%). Nonetheless, this gene delivery system was cytotoxic, working against its high transfection efficiency. Another study reported the generation of iPSCs from mouse embryonic fibroblasts via magnetic nanoparticles-based transfection;[33] however, the aggregation of magnetic nanoparticles resulted in a relatively large particle size (400–900 nm), which led to a low reprogramming efficiency (0.001%–0.003%).

Here, for the first time, we were able to obtain virus-free iPSCs from human umbilical cord mesenchymal stem cells (HUMSCs) with enhanced reprogramming efficiency (0.049%) using calcium phosphate nanoparticles (CPNPs) for the co-delivery of four plasmids, with each transiently expressing one of the four Yamanaka factors: Oct4, Sox2, Klf4, and c-Myc (OSKM). Calcium phosphate has been widely used for DNA or drug delivery via calcium phosphate microparticles or nanoparticles.[34–38] Calcium ions are known to form ionic complexes with the helical phosphates of DNA through electrostatic interactions, and the resulting complexes are easily transported across the cell membrane via ion-channel mediated endocytosis.[35,39] CPNPs have been used as nonviral gene vectors for transfection in many somatic cell types,[36] due to their ability to facilitate DNA uptake[40] and endosomal escape.[41] Several reports have confirmed that calcium phosphate particles can encapsulate therapeutic nucleic acids and successfully deliver them into tumor cells,[42,43] fibroblasts,[44] osteoblasts,[45] and mesenchymal stem cells.[36] The widespread use of CPNPs as in vitro transfection reagents may be attributed to the following reasons. First, numerous studies have revealed that calcium phosphate has excellent biodegradability and biocompatibility, which account for the extremely low toxicity by virtue of its presence in the forms of amorphous calcium phosphate and crystalline hydroxyapatite throughout the body (e.g., bone, tendons, and tooth enamel).[35,46] Furthermore, calcium phosphate has been regarded as safe by the Food and Drug Administration (FDA)[47] and is bioresorbable with its byproducts, Ca^{2+} and PO_4^{3-}, with the concentrations of 1–5 mM in the bloodstream.[48] Second, CPNPs possess small particle size (<100 nm).[49] It is, in general, assumed that one of the mechanisms associated with the cellular uptake of nanosized particles is endocytosis which is limited to the size range of approximately 20–200 nm in diameter.[50] The size of CPNPs (<100 nm) falls in this range. Therefore, this can greatly increase the possibility of endocytosis-mediated cellular uptake, leading to relatively higher transfection efficiency.[48] Lastly, unlike the construction of polycistronic constructs, which is labor-intensive and time-consuming, the DNA-incorporated CPNPs can be simply prepared by forming ionic complexes via coprecipitation, due to the electrostatic interactions between the divalent cations Ca^{2+} and the negatively-charged helical phosphates of DNA.[34]

In this study, CPNPs were used for the first time as nonviral vectors for the co-delivery of the four Yamanaka factors (OSKM) into HUMSCs, successfully establishing virus-free human iPSCs. The findings of this study provide a valuable approach for the generation of virus-free and patient-specific iPSCs, which would make a great contribution for the development of regenerative medicine.

2. Results and Discussion

2.1. Synthesis and Characterization of the Plasmids (OSKM)-Encapsulated CPNPs (pOSKM-CPNPs)

The pOSKM-CPNPs were prepared using aqueous coprecipitation of calcium chloride and disodium hydrogen phosphate in the presence of the four plasmids (OSKM) within water-in-oil microemulsions as described in the Experimental Section. The plasmids (OSKM) encapsulation was accomplished through the designed addition of the four plasmids into the microemulsion during precipitation. The resulting particle suspension was were then isolated using the van der Waals chromatography laundering method as previously described.[36,49] The pOSKM-CPNPs products were observed with transmission electron microscopy (TEM). As shown in **Figure 1**A, the pOSKM-CPNPs displayed spherical shape, small size (20–50 nm), and a good monodispersity. As previously reported,[36,48] spherical shape can facilitate cellular uptake. Good monodispersity can ensure the stability of the nanoparticles solution and avoid particles aggregation,[33] thus promoting transfection. It has also been found that the pOSKM-CPNPs kept at 4 °C for two weeks showed no significant change in particle size and shape (Figure 1B). However, when placed for four weeks, some of the pOSKM-CPNPs displayed a slight enlargement in particle size (80–100 nm) (Figure 1C, shown using arrows).

To determine whether all four plasmids (OSKM) were encapsulated in the nanoparticles, agarose gel electrophoresis assays were performed. The pOSKM-CPNPs were treated with a sodium acetate solution (pH 3.0) to release the contents that were encapsulated in the nanoparticles. The resulted plasmid mixtures were concentrated, and the concentrations of total plasmids were then measured with an ultraviolet spectrophotometer. After these calculations, the plasmids-loading rate in

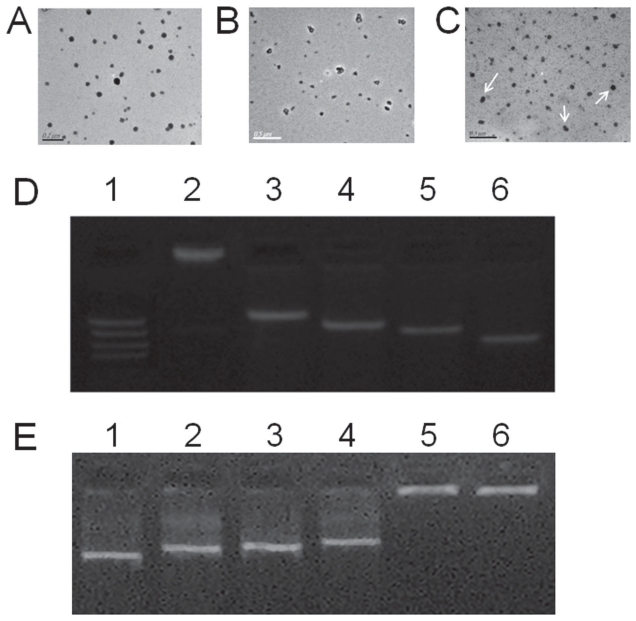

Figure 1. Characterization of plasmid Oct4, Sox2, Klf4, and c-Myc-calcium phosphate nanoparticles (pOSKM-CPNPs). A) A transmission electron microscopy (TEM) image of the pOSKM-CPNPs. B) The TEM image of the pOSKM-CPNPs kept for two weeks. C) The TEM image of the pOSKM-CPNPs for four weeks. D) Plasmid encapsulation evaluation and gel retardation assay using 1% agarose. Lanes from left to right: lane 1) pOSKM-CPNPs dissolved in a sodium acetate solution (pH 3.0); lane 2) pOSKM-CPNPs; lanes 3–6) free plasmids Klf4, c-Myc, Oct4, and Sox2, respectively. E) The detachment of the four factors from the pOSKM-CPNPs at different time points. Lanes from left to right: lanes 1–4) free plasmids Sox2, Oct4, c-Myc, and Klf4, respectively; lane 5) pOSKM-CPNPs kept for two weeks; lane 6) pOSKM-CPNPs for four weeks.

the pOSKM-CPNPs was (20.2 ± 0.1)% (w/w, $n = 3$). The plasmid mixtures were then subjected to agarose gel electrophoresis. If the plasmids were encapsulated within the nanoparticles, the corresponding electrophoretic bands would be observed in the gel. As shown in Figure 1D, the four plasmids (OSKM) were released from the pOSKM-CPNPs and produced four distinct ladder-like electrophoretic bands (Figure 1D1), with each indicating Klf4, c-Myc, Oct4, and Sox2 from top to bottom, respectively (Figure 1D3–6). This result demonstrates that all four plasmids were successfully encapsulated in the CPNPs. Furthermore, the nearly equal intensities of the four bands suggest that an equal amount of each plasmid was encapsulated in the CPNPs. A pOSKM-CPNPs sample was also tested via agarose gel electrophoresis to assess its gel retardation effect. As a result, the plasmid DNAs in the pOSKM-CPNPs was completely retained in the lane with no observed migration (Figure 1D2), indicating that the four plasmids can be combined with CPNPs to form physically stable pOSKM-CPNPs. Furthermore, the pOSKM-CPNPs that were kept for both two and four weeks displayed no plasmid migration across the gel (Figure 1E). Although some pOSKM-CPNPs showed slightly enlarged particle size when kept for four weeks (Figure 1C), there was still no plasmid DNA release from the pOSKM-CPNPs (Figure 1E).

2.2. Reprogramming of HUMSCs

Cell transfection efficiency is the key index for evaluating the properties of nonviral gene vectors. Although the efficient gene delivery of CPNPs has been demonstrated in many studies, this is the first study to use CPNPs as nonviral vectors to generate iPSCs. The pOSKM-CPNPs which encapsulated the four plasmids with each transiently expressing one of the four reprogramming factors, Oct4, Sox2, Klf4, and c-Myc (OSKM), were used to transfect HUMSCs. Primary HUMSCs (passage 0) were maintained in low glucose media (Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum (FBS) and 100 U mL^{-1} penicillin-streptomycin. Primary mouse embryonic fibroblasts (MEFs) were treated with mitomycin C (10 μg mL^{-1}), maintained in the fibroblast media (DMEM/F12 containing 10% FBS and 100 U mL^{-1} penicillin-streptomycin) and used as feeder cells for the following experiments.

The structure diagrams of the four plasmids are shown in **Figure 2**A. HUMSCs were transfected following the timeline depicted in Figure 2B. Due to the fact that the successful iPSCs generation requires sustained expression of OSKM for 8–12 days,[51,52] four consecutive transfection cycles (every other day; on day 0, day 2, day 4, and day 6) was employed in this study. Generating virus-free and transgene-free iPSCs for clinical application is very important.[9,53] This CPNPs-mediated strategy for the co-delivery of the four Yamanaka factors provides an effective solution for this goal. Further trials are being conducted to achieve sustained and high-efficient co-delivery of the four iPS genes (OSKM) using three-dimensional scaffolds for nonviral gene delivery. Notably, half of the cell samples were treated with valproic acid (VPA) from day 2 to day 6, while the other half did not receive VPA treatment. One day after the final transfection (day 7), cells were reseeded onto MEFs feeder layers. After approximately 23 days, ESC-like colonies appeared in both treatments. A total of 98 colonies from the initially seeded 200 000 cells were obtained with VPA treatment and a total of 20 colonies from 200 000 cells were generated without VPA treatment. VPA, a small-molecule histone deacetylase (HDAC) inhibitor, has been proven to increase the efficiency of reprogramming many differentiated cell types into a pluripotent state.[10] Our result confirms that VPA treatment increases the number of the ESC-like colonies by approximately five-fold in compared with non-VPA treatment. Importantly, the reprogramming efficiency with VPA treatment (0.049%) is significantly enhanced as compared with that of poly-β-amino esters-mediated polycistronic plasmid transfection (a total of 61 colonies from 400 000 cells, efficiency ≈0.015%), as reported by Montserrat and colleagues.[18] Although a lower reprogramming efficiency (0.01%) was obtained without VPA treatment, it is still higher than that of magnetic nanoparticle-based transfection, as previously reported.[33] These findings demonstrate that pOSKM-CPNPs are an efficient approach for the generation of virus-free iPSCs.

2.3. Characterization of Reprogrammed HUMSCs

The colonies were allowed to grow for eight days, after which they were mechanically selected based on human embryonic

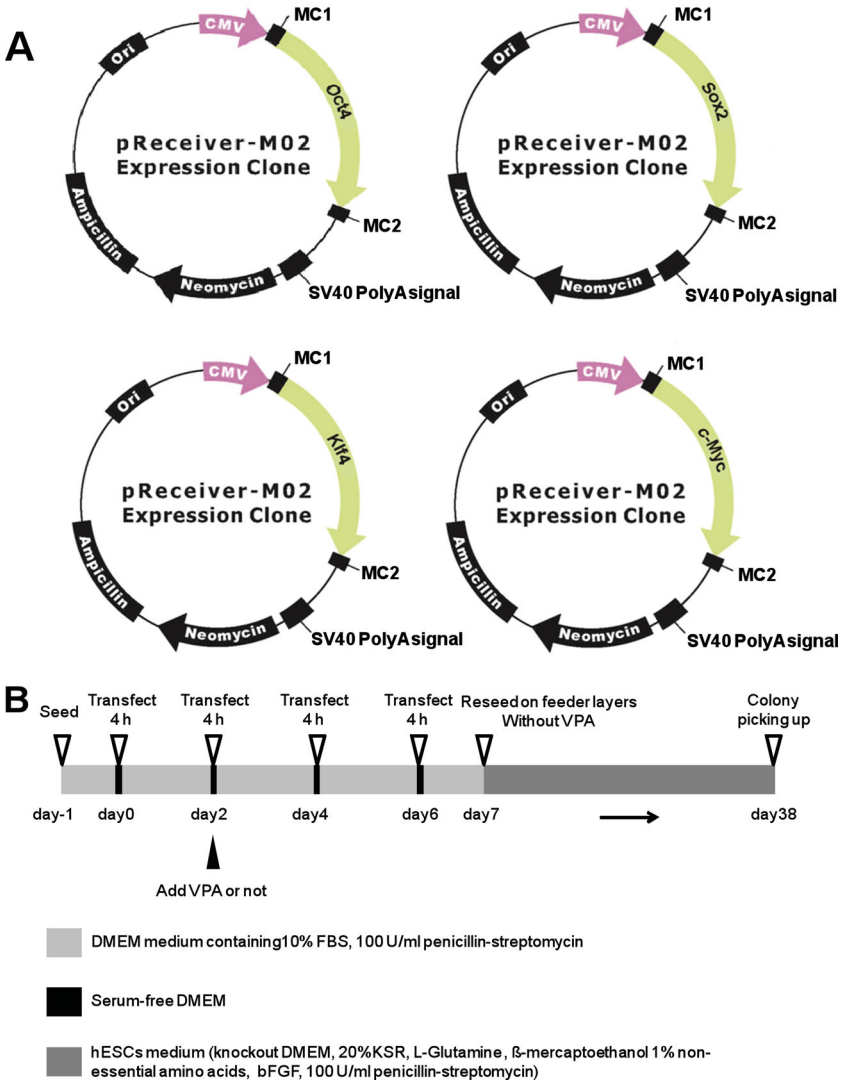

Figure 2. iPSC generation process via transfection of four plasmid-encapsulated calcium phosphate nanoparticles (i.e., pOSKM-CPNPs). A) Maps of the four plasmid constructs (Oct4, Sox2, Klf4, and c-Myc) used in this study. B) Timeline of iPSC production with four cycles of consecutive transfection.

stem cell (ESC)-like colony morphology (**Figure 3**A). The selected colonies were subsequently maintained on the MEFs feeder layers in human ESC media (knockout DMEM supplemented with 20% knockout serum replacement (KSR), 2 mM L-glutamine, 0.1 mM β-mercaptoethanol, 1% non-essential amino acids, 4 ng mL^{-1} basic fibroblast growth factor (bFGF), and 100 U mL^{-1} penicillin-streptomycin). As has been reported for reprogramming mouse cells,[54,55] human iPSCs colonies can be readily identified by ESC-like morphology, and most ESC-like colonies formed from transfected HUMSCs express pluripotency markers.[56,57] Therefore, we examined colonies (from cultures treated with VPA) that were chosen based on their ESC-like morphology. All of the colonies were then expanded and immunostained for alkaline phosphate (AP). As shown in Figure 3B, the iPSCs that are morphologically similar to hESCs stained positive (red) for alkaline phosphatase activity. To examine whether hESCs markers were expressed in these iPSCs colonies, primary antibodies for Oct-4, SSEA-3, SSEA-4, Tra-1-81, and NANOG, and the secondary antibody sheep antimouse IgG-Cy3 were employed. The Cy3-conjugated secondary antibody emits red fluorescence when excited by ultraviolet light, indicating the ESCs pluripotent markers. The nuclei were counterstained with 4′,6-diamidino-2-phenylindole (DAPI), a specific and simple fluorescent stain that can visualize nuclear DNA in both living and fixed cells. As expected, the results of immunofluorescence staining confirmed the obvious expression of pluripotency markers, including OCT4, SSEA-3, SSEA-4, NANOG, and TRA-1-81, in the iPSCs (Figure 3C).

To evaluate the expression levels of the four iPSC genes (Oct4, Sox2, Klf4, and c-Myc), enzyme-linked immunosorbent assay (ELISA) analyses were performed one day after the final transfection. The protein concentrations of the four factors (Oct4, Sox2, Klf4, and c-Myc) in the media are illustrated in **Figure 4**A. The pOSKM-CPNPs yielded significantly higher expression levels in both treatments (with or without VPA treatment) compared with the free plasmid control. This result demonstrates that pOSKM-CPNPs can successfully deliver the four Yamanaka factors into HUMSCs with a high transfection efficiency. Additionally, an approximately 30% increase in the expression of Sox2, Klf4, and c-Myc was observed in the pOSKM-CPNP groups treated with VPA. Even though there was no obvious increase in Oct4 expression in the VPA-treated pOSKM-CPNP group, an approximately 50% increase in the expression of each factor (Oct4, Sox2, Klf4, and c-Myc) was obtained after the naked plasmid group received VPA treatment (Figure 4A). This finding confirms the ability of VPA to facilitate iPSCs formation. More importantly, the expression level of each factor produced by the pOSKM-CPNPs (with or without VPA) met the requirement for the successful generation of human iPSCs. Unlike the use of polycistronic plasmids, which require a specific order for the arrangement of the iPSCs genes (Oct4, Sox2, Klf4, and c-Myc) to obtain an efficient derivation of iPSCs,[9] the co-delivery of the four factors via pOSKM-CPNPs are able to achieve iPSCs generation without the labor-intensive manipulations of constructing such polycistronic plasmids. These data indicate that pOSKM-CPNPs hold great potential in becoming efficient non-viral vectors for iPSCs generation.

The expression levels of the four Yamanaka factors (Oct4, Sox2, Klf4, and c-Myc) in the iPSC colonies were further examined with immunofluorescence staining. As shown in Figure 4B and indicated by the red fluorescence, the iPSC colonies showed robust expression of all four factors. Western

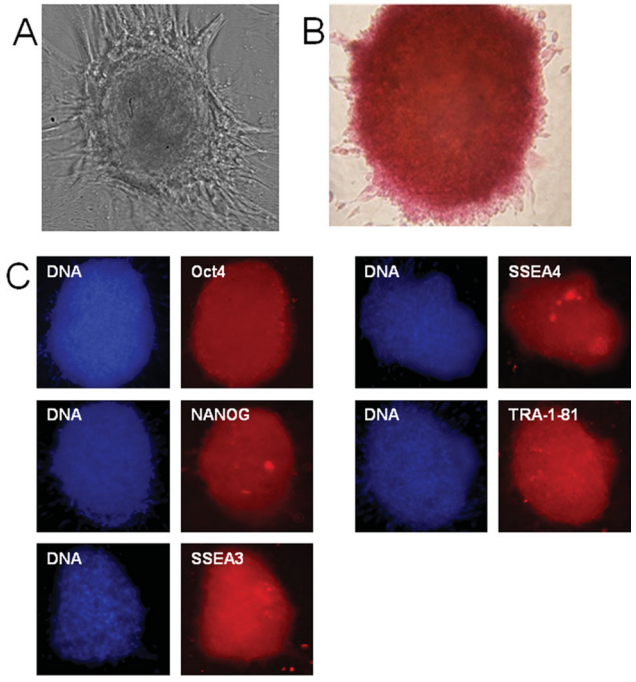

Figure 3. Characterization of the established iPSC colonies generated from human umbilical cord mesenchymal stem cells (HUMSCs) after pOSKM-CPNPs transfection. A) A bright field image depicting embryonic stem cell (ESC)-like morphology of the iPSCs colonies. B) Alkaline phosphatase staining (red) of iPSCs. C) Immunofluorescence staining assays for the expression of the pluripotency marker genes in iPSCs. Cellular expressions of pluripotency markers Oct4, NANOG, SSEA3, SSEA4, and TRA-1-81, all of which are human ESCs makers, were observed in the iPSCs. The nuclei were stained with DAPI.

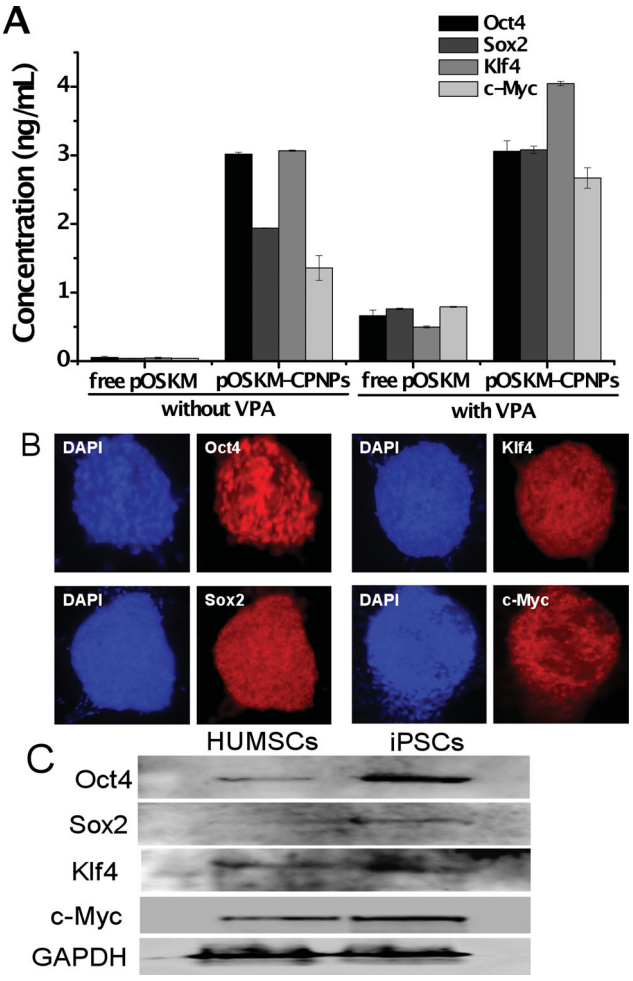

Figure 4. Expression of the four transcription factors (Oct4, Sox2, Klf4, and c-Myc; OSKM) in the iPSCs. A) The protein concentrations of the four factors expressed by the pOSKM-CPNPs (with or without VPA treatment) determined with ELISA assays; the free plasmid (OSKM)-transfected HUMSCs were used as the control group. B) Immunofluorescence staining assays for the expression of the four factors in iPSCs (blue, nuclei; red, the four factors). C) Western blot analyses for the expression levels of the four factors; HUMSCs were used as the control group. Human glyceraldehydes-phosphate dehydrogenase (GAPDH) was used as the internal standard.

blot analyses were performed to further determine the protein expression of each factor (Oct4, Sox2, Klf4, and c-Myc) in iPSCs. After separation by 10% SDS-PAGE, the samples were transferred onto nitrocellulose membranes and hybridized with primary antibodies against Oct4, Sox2, Klf4, and c-Myc. Goat anti-rabbit IgG-horseradish peroxidase (HRP) was used as the secondary antibody. Western blot images are shown in Figure 4C; each of the four factors achieved efficient protein expression. As a control, except for Sox2, which shows no band, the obvious weaker bands representing Oct4, Klf4, and c-Myc were observed in the HUMSCs group as compared with the iPSC group. These results indicate that small amounts of endogenous Oct4, Klf4, and c-Myc are expressed in HUMSCs. However, the endogenous expression levels of Oct4, Klf4, and c-Myc are not able to initiate the reprogramming of HUMSCs. These results suggest that HUMSCs can only be reprogrammed into a pluripotent state after the introduction of large amounts of exogenous Oct4, Sox2, Klf4, and c-Myc.

2.4. In vitro and in vivo Differentiation of iPSCs Generated from HUMSCs

The ability of ESCs to differentiate into all cell types is the basis for their potential in regenerative medicine. In this study, the differentiation capacity of iPSCs generated from HUMSCs was investigated. For in vitro differentiation, we used a suspension culture method that induced the formation of embryonic bodies in a suspended state. After three days of suspension culture at 37 °C in 5% CO_2, the embryoid bodies were transferred to gelatin-coated, 24-well plates in different media for neural, hepatocyte, and chondrocyte differentiation. The media was changed every three days. After four weeks, immunofluorescence staining was performed to assess whether the iPSCs differentiated into three germ layer lineages. To this end, primary antibodies against three germ layer markers, βIII-tubulin (Tuj1, a marker of ectoderm), α-fetoprotein (AFP, a marker of endoderm), and collagen II (a marker of mesoderm), were employed to specifically combine with the layer markers. Sheep

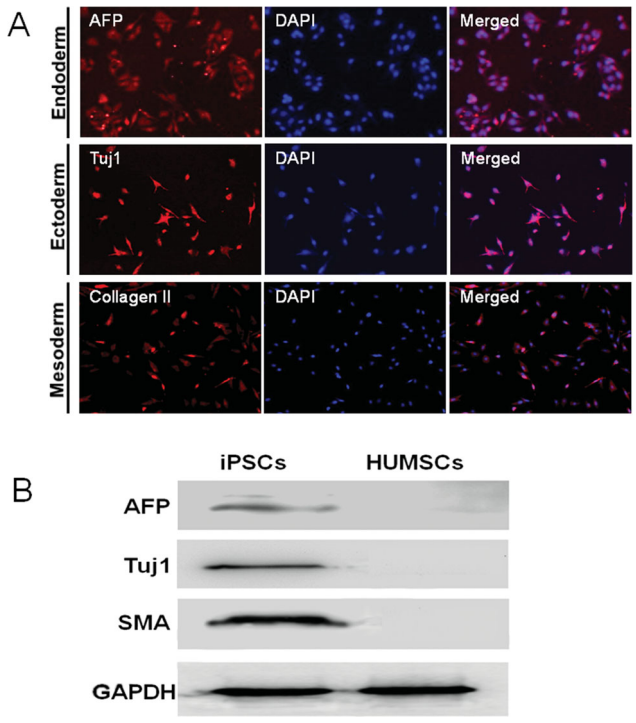

Figure 5. In vitro and in vivo differentiation potential of iPSCs. A) Immunofluorescence staining shows the in vitro differentiation of iPSCs into cells expressing markers that are characteristic of the three germ layers, including AFP (α-fetoprotein; endoderm marker), Tuj1 (βIII tubulin; ectoderm marker), and Collagen II (mesoderm marker). The nuclei were stained with DAPI. B) Western blots analyses demonstrating the in vivo differentiation potentials of iPSCs. Protein was isolated from teratomas and analyzed via Western blotting using antibodies against three germ layer markers: AFP, Tju1, and SMA (smooth muscle actin, mesoderm marker). Protein was isolated from HUMSCs and used as a control. Human glyceraldehydes-phosphate dehydrogenase (GAPDH) was used as the internal standard.

anti-mouse IgG-Cy3 was used as the secondary antibody. As shown in **Figure 5**A, the iPSC derivatives were positive (red) for all three markers (Tuj1, AFP, and collagen II), indicating that the iPSCs successfully differentiated into all three germ layers. These results demonstrate the induced differentiation of iPSCs in vitro.

A standard assay to test the pluripotency of human ESCs is to form teratomas when injected into immunocompromised mice.[58] To further estimate the differentiation potentials of iPSCs in vivo, we induced teratoma formation by subcutaneously injection of iPSCs into non-obese diabetic/severe combined immunodeficient (NOD-SCID) mice. The animal experimental protocol was approved by the University Ethics Committee for the use of experimental animals and conformed to the Guide for Care and Use of Laboratory Animals. Five to eight weeks after iPSC injection, the teratomas were collected for Western blot analysis, to examine the expression of the three embryonic germ layer marker genes. Primary antibodies against the three germ layer markers included Tuj1 (ectoderm), AFP (endoderm), and smooth muscle actin (SMA, mesoderm). The results demonstrated that the formed teratomas expressed all of the three germ layer markers (Tuj1, AFP, and SMA). However, the HUMSCs did not express any of the three markers (Figure 5B). These results indicate that the derived iPSCs had the ability to generate teratomas in NOD-SCID mice due to pOSKM-CPNP delivery, and the resulting teratomas harbored differentiated elements of all three primary embryonic germ layers. Taken together, these data suggest that even though HUMSCs can endogenously express small amounts of Oct4, Klf4, and c-Myc, they do not possess the ability to differentiate into the three germ layers either in vitro or in vivo without pOSKM-CPNP transfection.

3. Conclusions

In this study, we developed a simple, biocompatible, and efficient virus-free transfection system for iPSC generation using well-dispersed pOSKM-CPNPs with spherical shape and small particle size (20–50 nm). This system was used as a nonviral strategy for the co-delivery of the four plasmids that expressed the four Yamanaka factors (Oct4, Sox2, Klf4, and c-Myc) into HUMSCs for the generation of virus-free iPSCs. This strategy has two major advantages: 1) the transient expression of plasmids avoids viral integration and 2) the well-known efficient gene transfection of calcium phosphate nanoparticles enables the enhanced reprogramming efficiency for iPSC generation. As a result, a total of 20 ESC-like iPSC colonies from 200 000 cells were obtained without VPA treatment, and a total of 98 colonies from 200 000 cells were observed in the group treated with VPA, resulting in reprogramming efficiencies of 0.01% and 0.049%, respectively, which were greatly enhanced compared with previous reports.[18,33] The selected iPSC colonies based on the ESC-like morphology were positive for alkaline phosphatase activity and pluripotency markers, OCT4, SSEA-3, SSEA-4, NANOG, and TRA-1-81. ELISA analyses showed high expression of the four factors (Oct4, Sox2, Klf4, and c-Myc) when transfected with pOSKM-CPNPs. Furthermore, immunofluorescence staining and Western blot analyses confirmed the significant expression of the four factors in iPSCs. To investigate the differentiation potential both in vitro and in vivo, iPSCs underwent induced differentiation in different media and teratoma formation when injected into NOD-SCID mice, respectively. The result of in vitro induced differentiation demonstrated the positive expression of the three germ layer markers, including Tuj1 (a marker of ectoderm), AFP (a marker of endoderm), and collagen II (a marker of mesoderm). For in vivo differentiation, the teratomas formed in NOD-SCID mice and Western blot analyses using the total protein extracted from the teratomas demonstrated significant expression of Tuj1, AFP and SMA (a marker of mesoderm). In summary, the strategy to construct pOSKM-CPNPs as nonviral vectors achieved the generation of virus-free iPSCs with enhanced reprogramming efficiencies. The excellent biocompatibility and enhanced reprogramming efficiency, combined with the successful generation of virus-free human iPSCs, make pOSKM-CPNP delivery a competent strategy for regenerative medicine.

4. Experimental Section

Materials: Poly(oxyethylene)-nonylphenyl ether (Igepal CO-520) and cyclohexane were purchased from Sigma-Aldrich (St Louis, MO, USA); disodium hydrogen phosphate, glacial acetic acid, and absolute ethanol were obtained from Chemical Reagent Co., Ltd. of China National Pharmaceutical Group (Shanghai, China) and used without further purification. Silica spheres (50 μm in size, 60 Å in pore size) were purchased from Sepax Technologies (Newark, DE, USA). FBS, DMEM, DMEM/F12, knockout DMEM, KSR, bovine serum albumin, L-glutamine, penicillin, streptomycin, and trypsin were obtained from Gibco BRL(Invitrogen Co., Carlsbad, CA, USA). Mitomycin C, valproic acid, collagenase IV, β-mercaptoethanol, non-essential amino acids, and bFGF were purchased from Sigma-Aldrich (St. Louis, MO, USA). Human Oct4, Sox2, Klf4, and c-Myc ELISA kits were provided by Yantai Science and Biotechnology Co., Ltd., (Shandong, China). The animal experimental protocol was approved by the University Ethics Committee for the use of experimental animals and conformed to the Guide for Care and Use of Laboratory Animals.

Synthesis of Plasmids (OSKM)-Calcium Phosphate Nanoparticles (pOSKM-CPNPs): The preparation process of plasmids pOSKM-CPNPs followed one of the previously published reports with minor modification.[49] Briefly, the four different plasmids (1.25 μg for each plasmid) in $CaCl_2$ solution (1.36 mM, 70 μL) was added to Igepal CO-520/cyclohexane (25 mL, 29%, v/v) mixed solution, followed by continuous stirring at 4 °C for 2 min to form microemulsion A. Similarly, for microemulsion B, the same amount of the four plasmids (1.25 μg for each plasmid) in disodium hydrogen phosphate solution (0.35 mM, 70 μL) and tris-HCl buffer (0.2 M, 50 μL, pH 7.4) were added to the above-mentioned Igepal CO-520/cyclohexane mixed solution (25 mL). After 2 min of continuous stirring, microemulsion B was formed. The obtained microemulsions A and B were optically clear solutions. In the next step, the microemulsion B was added into microemulsion A dropwise with continuous magnetic stirring at 4 °C for 20 min for the generation of plasmids-encapsulated CPNPs emulsion. The resulting emulsion was diluted by pH-adjusted absolute ethanol (pH 7.0), from which the pOSKM-CPNPs were isolated with van der Waals chromatography laundering method as previously described.[36,49]

Morphology Observation of pOSKM-CPNPs: The morphology of pOSKM-CPNPs was observed with TEM under a JEM-2100 instrument (JEOL, Japan). Briefly, one drop of the freshly prepared nanoparticle suspension was placed onto a carbon film grid with a copper backing and was self-dried at room temperature (25 °C). The dried grid was then examined under the electron microscope.

Encapsulation Evaluation of pOSKM-CPNPs: To examine whether the four plasmids (OSKM) have been encapsulated in the CPNPs, the nanoparticles were centrifuged at 50 000 rpm for 15 min at 4 °C. One milligram of the pellet was then dissolved in a sodium acetate solution (pH 3.0). Thus, if the plasmids were encapsulated in the CPNPs, the four plasmids could be released from the pOSKM-CPNPs because the calcium phosphate was sensitive to the mildly acidic condition and dissolved in the solution to present itself in the forms of Ca^{2+} and PO_4^{3-}. The mixed plasmids solution was concentrated, and the concentration of total plasmids was then measured with an ultraviolet spectrophotometer (Shimadzu, Tokyo, Japan). After calculation, the plasmids encapsulation rate of pOSKM-CPNPs is 20%. Adjusting the concentration of mixed plasmids ensure that 400 ng of total plasmids were in 10 μL of solution. The mixed plasmids were subsequently subjected to the agarose gel electrophoresis. A plasmids solution (10 μL) was added to loading buffer (1 μL, 0.1% sodium dodecyl sulfate, 5% glycerol, and 0.005% bromophenol blue) and applied to a 1% agarose gel that contained ethidium bromide (EtBr, 0.1 mg mL^{-1}) in a trisborate-ethylenediaminetetraacetic acid buffer solution (TBE, pH 8.0). As controls, free plasmid solution (10 μL, containing 100 ng plasmid) for each one of Oct4, Sox2, Klf4, and c-Myc was subjected to the agarose gel, following the same procedure. In addition, the nanoparticle pOSKM-CPNPs solution (10 μL) was applied to the agarose gel electrophoresis as well, with the aim of evaluating the gel retardation effect. The electrophoretic evaluation was performed in TBE solution at 80 V for 90 min, after which the gel's image was taken with a UV transilluminator (Gel Doc 2000, BIO RAD laboratories, Hercules, CA).

Plasmid Preparation: The four plasmids with each expressing one of the four reprogramming factors Oct4 (catalog number EX-Z0092-M02), Sox2 (Catalog number EX-T2547-M02), Klf4 (catalog number EX-Z2845-M02), and c-Myc (catalog number EX-Z5703-M02) were purchased from GeneCopoeia, Inc. (Rockville, MD, USA), followed by amplification in *Escherichia coli* host strain DH5α. The plasmids DNA were extracted and purified by column chromatography with the PureYield Plasmid Maxiprep Start-Up Kit (Promega, Madison, Wisconsin, USA) according to the manufacturer's protocol. Ampicilin was used to select the OSKM-transformed *Escherichia coli* cells. The concentration of each plasmid DNA was quantified by measuring the UV absorbance at 260 nm with an ultraviolet spectrophotometer (Shimadzu, Tokyo, Japan). Using the four original plasmids as controls, the agarose electrophoresis was conducted to verify the plasmids extracted from *Escherichia coli* cells.

Cell Culture: Primary human umbilical cord mesenchymal stem cells (HUMSCs, passage 0) were a kind gift from Beike Jiangsu Stem Cell Bank (Taizhou, China). HUMSCs were maintained in the low glucose DMEM supplemented with 10% FBS andpenicillin-streptomycin (100 U mL^{-1}). Primary mouse embryonic fibroblasts (MEFs) which were used as the feeder cells were purchased from the Cell Bank at the Chinese Academy of Science (Shanghai, China). The MEFs were maintained in the fibroblast medium: DMEM/F12 containing 10% FBS and penicillin-streptomycin (100 U mL^{-1}).

The preparation of feeder cells was as described in the following procedure. Briefly, 3 mL of MEFs suspension were seeded on 6-cm gelatin-coated dishes at a density of 1×10^5 cells mL^{-1} and incubated at 37 °C in a humidified atmosphere of 5% CO_2 for 12–18 h to obtain a confluence of 80%. The supernate was then removed and replaced with fresh fibroblast medium (2 mL), followed by addition of mitomycin C (final concentration of 10 μg mL^{-1}). After 3 h of incubation at 37 °C, the medium was removed, and the cells were washed three times with phosphate-buffered saline (PBS, pH 7.4). Next, the cells were treated with 0.25% trypsin/EDTA at 37 °C for 3 min, followed by addition of a few drops of fibroblast medium. After centrifugation (1500 rpm for 5 min), the MEFs were re-seeded on another new 6-cm gelatin-coated dishes at the density of 1×10^5 cells mL^{-1} in fibroblast medium. The mitotically inactivated MEFs were incubated at 37 °C for 6–12 h before they could be used.

Induction of Pluripotent Stem Cells: 24 h before transfection, HUMSCs were seeded on a 24-well plate at the density of 2×10^5 cells per well in DMEM (containing 10% FBS and 100 U mL^{-1} penicillin-streptomycin), and then incubated at 37 °C in 5% CO_2 until the cells reached 80–90% confluence. The medium was then removed, and the cells were washed once with PBS. Four rounds of consecutive transfection with pOSKM-CPNPs were performed (of 4 h each) on days 0, 2, 4, and 6 in serum-free DMEM. Half of the cell-seeded wells were treated with 0.5 mM valproic acid (VPA) from day 2 to day 6. As control, the four naked plasmids (OSKM) were transfected using the same procedure. On day 7 (one day post-transfection), the transfected HUMSCs (with or without VPA treatment) were treated with type IV collagenase (1 mg mL^{-1}) at 37 °C for 30 min. After centrifugation (1500 rpm for 5 min), the cells were seeded on the MEFs feeder layers at the density of 1×10^3 cells mL^{-1} in human embryonic stem cell (hESC) medium. The medium consisted of knockout DMEM supplemented with KSR (20%), L-Glutamine (2 mM), β-mercaptoethanol (0.1 mM), non-essential amino acids (1%), bFGF (4 ng mL^{-1}), and penicillin-streptomycin (100 U mL^{-1}), which was refreshed daily with a new pre-warmed hESC medium. The iPSCs colonies were formed 23 days post-transfection (day 29) based on ESC-like colony morphology. To establish iPSCs lines, the colonies were then mechanically picked and maintained on MEFs feeder layers in hESC medium without VPA.

ELISA Assays: One day after the four-round transfection (day 7), the medium was removed and the cells were washed with PBS. The total protein of the cells was extracted using RIPA Lysis Kit (Beyotime Institute of Biotechnology, Haimen, China) according to the manufacturer's

protocol. After complete lysis, the reaction solution was centrifuged at 10 000 g for 5 min. The supernate was collected for enzyme-linked immunosorbent assay (ELISA) to determine ectopic expression level of each one of the four factors Oct4, Sox2, Klf4, and c-Myc.

Alkaline Phosphatase Staining: For alkaline phosphate (AP) staining, cells were washed twice with PBS, fixed in 4% paraformaldehyde (cold) for 2 min, and washed again with PBS. The alkaline phosphatase activity was examined using Alkaline Phosphatase Staining Assay Kit (Red) (8288, ScienCell Research Laboratory, San Diego, CA, USA) according to the instructions provided by the manufacturer. The images were acquired using microscopy (Olympus, Tokyo, USA).

Determination of Reprogramming Efficiency: The reprogramming efficiency was calculated as the number of iPSCs colonies formed per number of cells seeded for transfection. The iPSCs colonies were identified based on ESCs-like morphology and alkaline phosphatase staining was used to facilitate the identification of iPSCs colonies.

Western Blot: After removal of medium, cells were washed twice with PBS. The total protein was isolated with Cell & Tissue Protein Extraction Reagent (Beyotime Institute of Biotechnology, Haimen, China) according to the manufacturer's instruction. For western blotting assay, protein extracts (10 μg) were resolved on 10% SDS-PAGE, transferred onto nitrocellulose membranes(Schleicher & Schuell, Keene, NL), and blots were hybridized with antibodies against Oct4 (ab18976, Abcam, Cambridge, MA, USA), Sox2 (ab69893, Abcam, Cambridge, MA, USA), Klf4 (ab34814, Abcam, Cambridge, MA, USA), and c-Myc (ab9106, Abcam, Cambridge, MA, USA). The secondary antibody was goat anti-rabbit IgG-horseradish peroxidase (HRP) from Sigma (St. Louis, MO, USA).

Immunofluorescence Staining: For immunostaining, cells were fixed in 4% paraformaldehyde in PBS for 20 min. After washing twice with PBS, the cells were permeabilized with 0.1% Triton X-100 for 10 min. After another washing with PBS, cells were incubated in 4% bovine serum albumin for 1 h to block any non-specific binding. Cells were then incubated with the primary antibodies overnight at 4 °C, washed with PBS and incubated with secondary antibodies for 1 h. Nuclei were counterstained with DAPI (1:2000; Sigma, St. Louis, MO, USA). The primary antibodies included anti-Oct-4 (1:500), anti-SSEA-3(1:500), anti-SSEA-4(1:500), anti-Tra-1-81(1:500), and anti-NANOG (1:500), which were obtained from Abcam (Cambridge, MA, USA). The secondary antibodies included sheep anti-mouse IgG-Cy3 and goat anti-rabbit IgG-FITC from Sigma (St. Louis, MO, USA).

In Vitro Differentiation: For spontaneous differentiation through embryoid body formation, human iPSCs grown on the MEFs feeder layers were dissociated by collagenase IV treatment for 30 min at 37 °C and transferred to low attachment 6-well plates in hESC medium. After 3 days in suspension culture at 37 °C in 5% CO_2, embryoid bodies were transferred to gelatin-coated 24-well plates in different differentiation media. For neural differentiation, the embryoid bodies were cultured in a DMEM/F12 solution supplemented with sonic hedgehog (SHH, 10 ng mL^{-1}, Sigma), brain-derived neurotrophic factor (BDNF, 10 ng mL^{-1}, Sigma), and retinoic acid (RA, 1 μmol L^{-1}, Sigma) for 1 day. After that the solution was changed with 2% B27-supplemented neurobasal medium (Invitrogen, Carlsbad, CA, USA) with addition of SHH (10 ng mL^{-1}), BDNF (10 ng mL^{-1}), and RA (1 μmol L^{-1}). The medium was changed every 3 days. As control, cells were maintained in 2% B27-supplemented neurobasal medium without other supplements. For hepatocyte differentiation, the embryoid bodies were cultured in HepatoZYME-SFM (Invitrogen, Carlsbad, CA) medium containing hepatocyte growth factor (HGF, 20 ng mL^{-1}, Sigma), fibroblast growth factor4 (FGF4, 10 ng mL^{-1}, Sigma), 100 U mL^{-1} penicillin-streptomycin, 2% FBS, and dexamethasone (0.01 μmol L^{-1}, Sigma). The medium was changed every 3 days. For chondrocyte differentiation, the embryoid bodies were maintained in a DMEM medium supplemented with 10% FBS, transforming growth factor β1 (TGF-β1, 10 ng mL^{-1}, Sigma), bFGF (20 ng mL^{-1}, Sigma), and dexamethasone (0.01 μmol L^{-1}). Similarly, the medium was changed every 3 days. Four weeks later, the differentiated cells were processed for immunofluorescence staining, as described above. The primary antibodies were anti-βIII tubulin (Tuj1, ectoderm marker, ab18207, Abcam), anti-α-fetoprotein (AFP, endoderm marker, SAB3500533, Sigma), and anti-collagen II (mesoderm marker, SAB4500366, Sigma).

Teratoma Formation: For teratoma assay, human iPSCs were washed with PBS, treated with collagenase IV for 30 min at 37 °C, and the resulting cells were collected by centrifugation. The cells were resuspended in hESC medium at the density of 1×10^7 cells mL^{-1}. 100-μL of the cell suspension was subcutaneously injected into 4-week old immunocompromised non-obese diabetic-sever combined immunodeficient (NOD-SCID) mice (Comparative Medicine Center, Yangzhou University, Yangzhou, China). Five to eight weeks after cell injection, the teratomas were collected and processed for Western blotting. The antibodies used included AFP, Tuj1 and smooth muscle actin (SMA, a marker of mesoderm).

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[1] W. Deng, *EMBO Rep.* **2010**, *11*, 161.
[2] H. Liu, Z. Ye, Y. Kim, S. Sharkis, Y.-Y. Jang, *Hepatology* **2010**, *51*, 1810.
[3] K. Takahashi, S. Yamanaka, *Cell* **2006**, *126*, 663.
[4] N. Sun, N. J. Panetta, D. M. Gupta, K. D. Wilson, A. Lee, F. Jia, S. Hu, A. M. Cherry, R. C. Robbins, M. T. Longaker, *Proc. Natl. Acad. Sci. USA* **2009**, *106*, 15720.
[5] T. Aasen, A. Raya, M. J. Barrero, E. Garreta, A. Consiglio, F. Gonzalez, R. Vassena, J. Bilic, V. Pekarik, G. Tiscornia, M. Edel, S. Boue, J. C. I. Belmonte, *Nat. Biotechnol.* **2008**, *26*, 1276.
[6] W. Lowry, L. Richter, R. Yachechko, A. Pyle, J. Tchieu, R. Sridharan, A. Clark, K. Plath, *Proc. Natl. Acad. Sci. USA* **2008**, *105*, 2883.
[7] K. Takahashi, K. Tanabe, M. Ohnuki, M. Narita, T. Ichisaka, K. Tomoda, S. Yamanaka, *Cell* **2007**, *131*, 861.
[8] J. Yu, M. A. Vodyanik, K. Smuga-Otto, J. Antosiewicz-Bourget, J. L. Frane, S. Tian, J. Nie, G. A. Jonsdottir, V. Ruotti, R. Stewart, *Science* **2007**, *318*, 1917.
[9] K. Okita, M. Nakagawa, H. Hyenjong, T. Ichisaka, S. Yamanaka, *Science* **2008**, *322*, 949.
[10] D. Huangfu, K. Osafune, R. Maehr, W. Guo, A. Eijkelenboom, S. Chen, W. Muhlestein, D. A. Melton, *Nat. Biotechnol.* **2008**, *26*, 1269.
[11] J. O'Malley, K. Woltjen, K. Kaji, *Curr. Opin. Biotechnol.* **2009**, *20*, 516.
[12] G. L. Boulting, E. Kiskinis, G. F. Croft, M. W. Amoroso, D. H. Oakley, B. J. Wainger, D. J. Williams, D. J. Kahler, M. Yamaki, L. Davidow, C. T. Rodolfa, J. T. Dimos, S. Mikkilineni, A. B. MacDermott, C. J. Woolf, C. E. Henderson, H. Wichterle, K. Eggan, *Nat. Biotechnol.* **2011**, *29*, 279.
[13] C. Buecker, H. H. Chen, J. M. Polo, L. Daheron, L. Bu, T. S. Barakat, P. Okwieka, A. Porter, J. Gribnau, K. Hochedlinger, N. Geijsen, *Cell Stem Cell* **2010**, *6*, 535.

[14] M. Wernig, C. J. Lengner, J. Hanna, M. A. Lodato, E. Steine, R. Foreman, J. Staerk, S. Markoulaki, R. Jaenisch, *Nat. Biotechnol.* **2008**, *26*, 916.
[15] K. Okita, T. Ichisaka, S. Yamanaka, *Nature* **2007**, *448*, 313.
[16] S. Yamanaka, *Cell Stem Cell* **2007**, *1*, 39.
[17] M. Stadtfeld, M. Nagaya, J. Utikal, G. Weir, K. Hochedlinger, *Science* **2008**, *322*, 945.
[18] N. Montserrat, E. Garreta, F. González, J. Gutiérrez, C. Eguizábal, V. Ramos, S. Borrós, J. C. I. Belmonte, *J. Biol. Chem.* **2011**, *286*, 12417.
[19] D. Kim, C. H. Kim, J. I. Moon, Y. G. Chung, M. Y. Chang, B. S. Han, S. Ko, E. Yang, K. Y. Cha, R. Lanza, *Cell Stem Cell* **2009**, *4*, 472.
[20] L. Warren, P. D. Manos, T. Ahfeldt, Y. H. Loh, H. Li, F. Lau, W. Ebina, P. K. Mandal, Z. D. Smith, A. Meissner, *Cell Stem Cell* **2010**, *7*, 618.
[21] F. Gonzalez, M. Barragan Monasterio, G. Tiscornia, N. Montserrat Pulido, R. Vassena, L. Batlle Morera, I. Rodriguez Piza, J. C. I. Belmonte, *Proc. Natl. Acad. Sci. USA* **2009**, *106*, 8918.
[22] K. Woltjen, I. P. Michael, P. Mohseni, R. Desai, M. Mileikovsky, R. Hämäläinen, R. Cowling, W. Wang, P. Liu, M. Gertsenstein, *Nature* **2009**, *458*, 766.
[23] W. Wang, C. Lin, D. Lu, Z. Ning, T. Cox, D. Melvin, X. Wang, A. Bradley, P. Liu, *Proc. Natl. Acad. Sci. USA* **2008**, *105*, 9290.
[24] Q. Zhao, B. Lu, S. K. George, J. J. Yoo, A. Atala, *Biomaterials* **2012**, *33*, 7261.
[25] R. L. Judson, J. E. Babiarz, M. Venere, R. Blelloch, *Nat. Biotechnol.* **2009**, *27*, 459.
[26] N. Miyoshi, H. Ishii, H. Nagano, N. Haraguchi, D. L. Dewi, Y. Kano, S. Nishikawa, M. Tanemura, K. Mimori, F. Tanaka, *Cell Stem Cell* **2011**, *8*, 633.
[27] M. Li, J. C. I. Belmonte, *Am. J. Stem Cell* **2012**, *1*, 75.
[28] N. Maherali, R. Sridharan, W. Xie, J. Utikal, S. Eminli, K. Arnold, M. Stadtfeld, R. Yachechko, J. Tchieu, R. Jaenisch, *Cell Stem Cell* **2007**, *1*, 55.
[29] W.-W. Deng, X. Cao, M. Wang, Y. Yang, W.-Y. Su, Y.-W. Wei, Z. Ou-Yang, J.-N. Yu, X.-M. Xu, *Small* **2012**, *8*, 441.
[30] X. Xu, R. M. Capito, M. Spector, *J. Biomed. Mater. Res. A* **2008**, *84*, 73.
[31] X. Zhang, M. Oulad-Abdelghani, A. N. Zelkin, Y. Wang, Y. Haîkel, D. Mainard, J. C. Voegel, F. Caruso, N. Benkirane-Jessel, *Biomaterials* **2010**, *31*, 1699.
[32] F. M. Kievit, O. Veiseh, N. Bhattarai, C. Fang, J. W. Gunn, D. Lee, R. G. Ellenbogen, J. M. Olson, M. Zhang, *Adv. Funct. Mater.* **2009**, *19*, 2244.
[33] C. H. Lee, J. H. Kim, H. J. Lee, K. Jeon, H. J. Lim, E. R. Lee, S. H. Park, J. Y. Park, S. Hong, S. Kim, *Biomaterials* **2011**, *32*, 6683.
[34] I. Roy, S. Mitra, A. Maitra, S. Mozumdar, *Int. J. Pharm.* **2003**, *250*, 25.
[35] S. Bisht, G. Bhakta, S. Mitra, A. Maitra, *Int. J. Pharm.* **2005**, *288*, 157.
[36] X. Cao, W. Deng, Y. Wei, W. Su, Y. Yang, J. Yu, X. Xu, *Int. J. Nanomed.* **2011**, *6*, 3335.
[37] M. Kester, Y. Heakal, T. Fox, A. Sharma, G. P. Robertson, T. T. Morgan, E. I. Altınoğlu, A. Tabaković, M. R. Parette, S. M. Rouse, *Nano Lett.* **2008**, *8*, 4116.
[38] H. C. Wu, T. W. Wang, M. C. Bohn, F. H. Lin, M. Spector, *Adv. Funct. Mater.* **2009**, *20*, 67.
[39] V. L. Truong-Le, S. M. Walsh, E. Schweibert, H. Q. Mao, W. B. Guggino, J. T. August, K. W. Leong, *Arch. Biochem. Biophys.* **1999**, *361*, 47.
[40] V. V. Sokolova, I. Radtke, R. Heumann, M. Epple, *Biomaterials* **2006**, *27*, 3147.
[41] S. Neumann, A. Kovtun, I. D. Dietzel, M. Epple, R. Heumann, *Biomaterials* **2009**, *30*, 6794.
[42] T. Liu, A. Tang, G. Zhang, Y. Chen, J. Zhang, S. Peng, Z. Cai, *Cancer Biother. Radiopharm.* **2005**, *20*, 141.
[43] B. M. Barth, R. Sharma, E. I. Altınoğlu, T. T. Morgan, S. S. Shanmugavelandy, J. M. Kaiser, C. McGovern, G. L. Matters, J. P. Smith, M. Kester, *ACS Nano* **2010**, *4*, 1279.
[44] C. E. Pedraza, D. C. Bassett, M. D. McKee, V. Nelea, U. Gbureck, J. E. Barralet, *Biomaterials* **2008**, *29*, 3384.
[45] L. Chen, J. M. Mccrate, J. C. M. Lee, H. Li, *Nanotechnology* **2011**, *22*, 105708.
[46] A. Maitra, *Expert Rev. Mol. Diagn.* **2005**, *5*, 893.
[47] S. V. Dorozhkin, M. Epple, *Angew. Chem. Int. Ed.* **2002**, *41*, 3130.
[48] H. S. Muddana, T. T. Morgan, J. H. Adair, P. J. Butler, *Nano Lett.* **2009**, *9*, 1559.
[49] T. T. Morgan, H. S. Muddana, E. I. Altınoğlu, S. M. Rouse, A. Tabaković, T. Tabouillot, T. J. Russin, S. S. Shanmugavelandy, P. J. Butler, P. C. Eklund, *Nano Lett.* **2008**, *8*, 4108.
[50] S. D. Xiang, A. Scholzen, G. Minigo, C. David, V. Apostolopoulos, P. L. Mottram, M. Plebanski, *Methods* **2006**, *40*, 1.
[51] T. Brambrink, R. Foreman, G. G. Welstead, C. J. Lengner, M. Wernig, H. Suh, R. Jaenisch, *Cell Stem Cell* **2008**, *2*, 151.
[52] M. Stadtfeld, N. Maherali, D. T. Breault, K. Hochedlinger, *Cell Stem Cell* **2008**, *2*, 230.
[53] K. Kaji, K. Norrby, A. Paca, M. Mileikovsky, P. Mohseni, K. Woltjen, *Nature* **2009**, *458*, 771.
[54] A. Meissner, M. Wernig, R. Jaenisch, *Nat. Biotechnol.* **2007**, *25*, 1177.
[55] R. Blelloch, M. Venere, J. Yen, M. Ramalho-Santos, *Cell Stem Cell* **2007**, *1*, 245.
[56] M. Nakagawa, M. Koyanagi, K. Tanabe, K. Takahashi, T. Ichisaka, T. Aoi, K. Okita, Y. Mochiduki, N. Takizawa, S. Yamanaka, *Nat. Biotechnol.* **2008**, *26*, 101.
[57] I. H. Park, R. Zhao, J. A. West, A. Yabuuchi, H. Huo, T. A. Ince, P. H. Lerou, M. W. Lensch, G. Q. Daley, *Nature* **2007**, *451*, 141.
[58] J. A. Thomson, J. Itskovitz-Eldor, S. S. Shapiro, M. A. Waknitz, J. J. Swiergiel, V. S. Marshall, J. M. Jones, *Science* **1998**, *282*, 1145.