



Cellular reprogramming with multigene activation by the delivery of CRISPR/dCas9 ribonucleoproteins via magnetic peptide-imprinted chitosan nanoparticles



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ABSTRACT

Induced pluripotent stem cells are usually derived by reprogramming transcription factors (OSKM), such as octamer-binding transcription factor 4 (OCT4), (sex determining region Y)-box 2 (SOX2), Krüppel-like factor 4 (KLF4), and cellular proto-oncogene (c-Myc). However, the genomic integration of transcription factors risks the insertion of mutations into the genome of the target cells. Recently, the clustered regularly interspaced short palindromic repeat-associated protein 9 (CRISPR/Cas9) system has been used to edit genomes. In this work, dCas9-VPR (dCas9 with a gene activator, VP64-p65-Rta (VPR), fused to its c-terminus) and guide RNA (gRNA) combined to form ribonucleoproteins, which were immobilized on magnetic peptide-imprinted chitosan nanoparticles. These were then used to activate OSKM genes in human embryonic kidney (HEK) 293T cells. Four pairs of gRNAs were used for the binding site recognition to activate the OSKM genes. Transfected HEK293T cells were then prescreened for the high expression of OSKM proteins by immunohistochemistry images. The optimal gRNAs for OSKM expression were identified using quantitative real-time polymerase chain reaction and the staining of OSKM proteins. Finally, we found that the activated expression of one of the OSKM genes is up to three-fold higher than that of the other genes, enabling precise control of the cell differentiation.

1. Introduction

The activation and the repression of gene expression using the clustered regularly interspaced short palindromic repeat-associated protein 9 (CRISPR/Cas9) system (using a nuclease-deficient Cas9 [dCas9] protein that has lost its endonuclease activity but retains its ability to bind the guide RNA) is now widespread [1]. Transcription can be activated by binding transcription activators to the CRISPR complex [2], i.e. CRISPRa. The VP64-p65-Rta (VPR) activator was developed by modifying the conventional activator virion protein 64 (VP64) [3] by the addition of the transcription factors p65 and Rta to the C terminus of dCas9-Vp64 [4]. All three transcription factors are then targeted to the same gene, increasing its expression. When different genes are targeted by dCas9, they all exhibit significantly higher expression with dCas9-VPR than with dCas9-VP64 [5]. For example, dCas9-VPR has been used to increase the

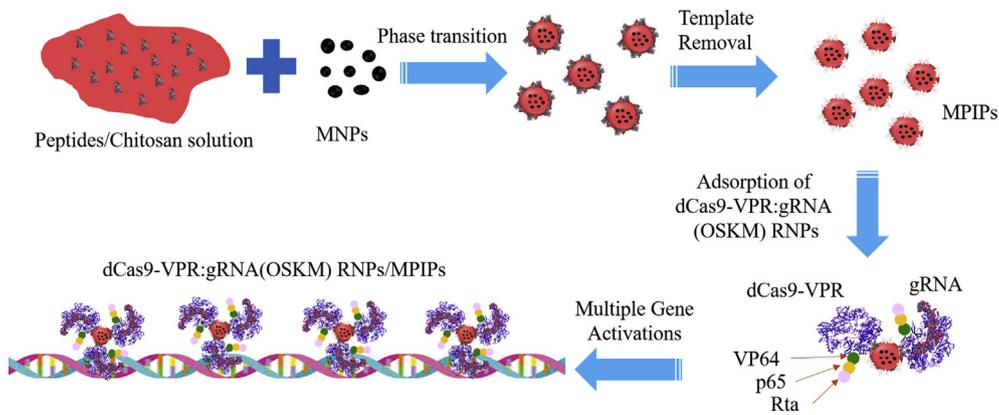
expression of multiple genes in the same cell by using multiple-guide RNAs to activate neurogenin2 and neurogenic differentiation-1 genes to differentiate induced pluripotent stem cells (iPSCs) into neurons [4]. Yang and Huang have reviewed applications of the CRISPR/Cas system for genome editing in stem cells [6]. Recently, CRISPRa-mediated octamer-binding transcription factor 4 (OCT4) activation was used to reprogram neuroepithelial stem cells into iPSCs using trimethoprim-stabilized SpdCas9VP192 that was fused with P65-HSF1 activator domain23 under a doxycycline-inducible promoter [7].

Three different protocols that have been used for the cellular delivery of the CRISPR/Cas9 system are (1) transfection with a DNA plasmid that encodes both the Cas9 protein and the guide RNA, (2) the use of mRNA for Cas9 translation alongside a separate single-guide RNA (sgRNA), and (3) the delivery of ribonucleoprotein (RNP) complex, comprised of Cas9 protein with a single attached guide sgRNA [3]. Several dCas9 activator

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Scheme 1. The synthesis of magnetic peptide-imprinted chitosan nanoparticles (MPIPs) and their adsorption of dCas9-VPR:gRNAs ribonucleoproteins (RNPs) for the activation of OSKM expression.

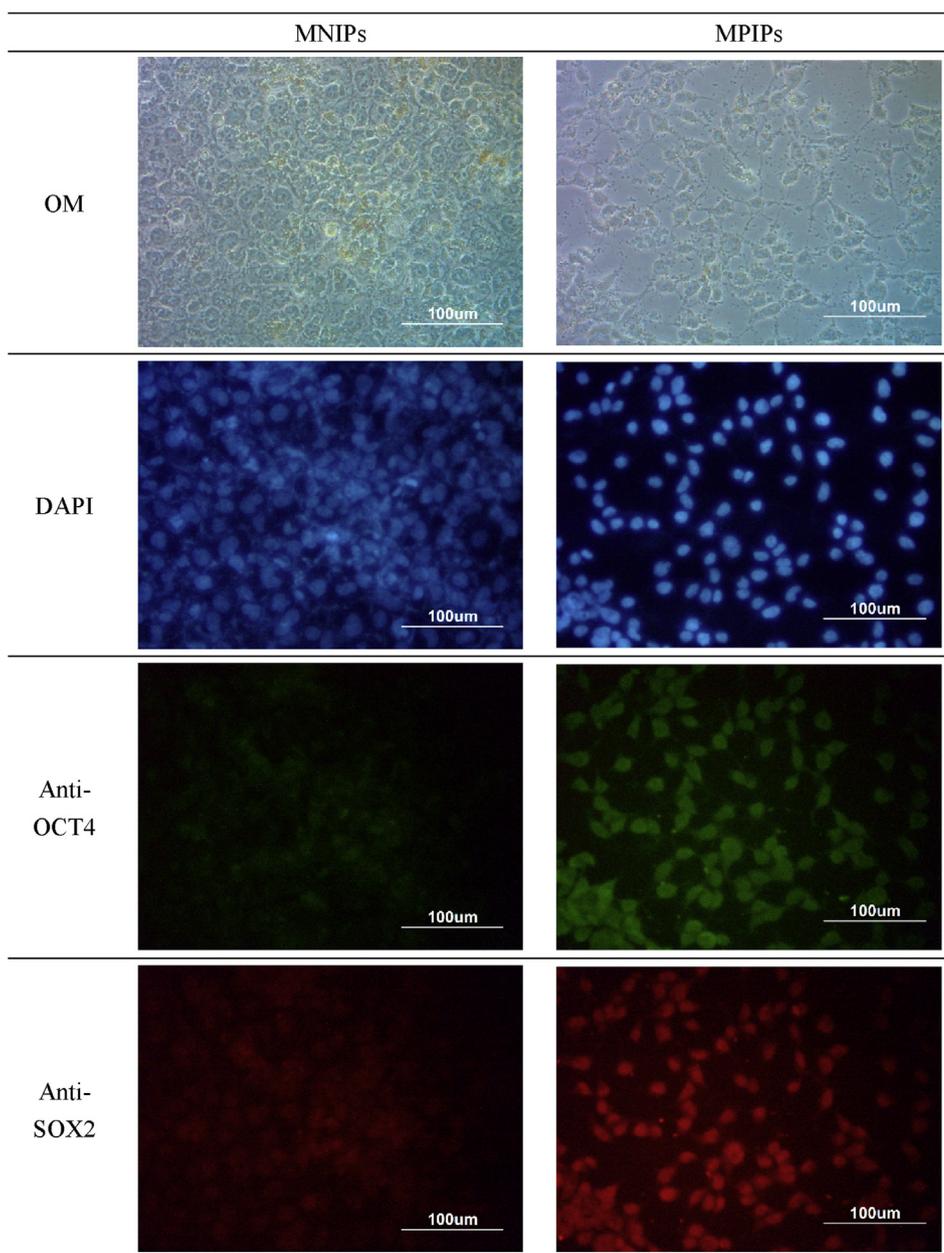


Fig. 1. Transfection of HEK-293T cells with magnetic peptide-imprinted nanoparticles/dCas9:gRNA and staining with DAPI, anti-OCT4, and anti-SOX2.

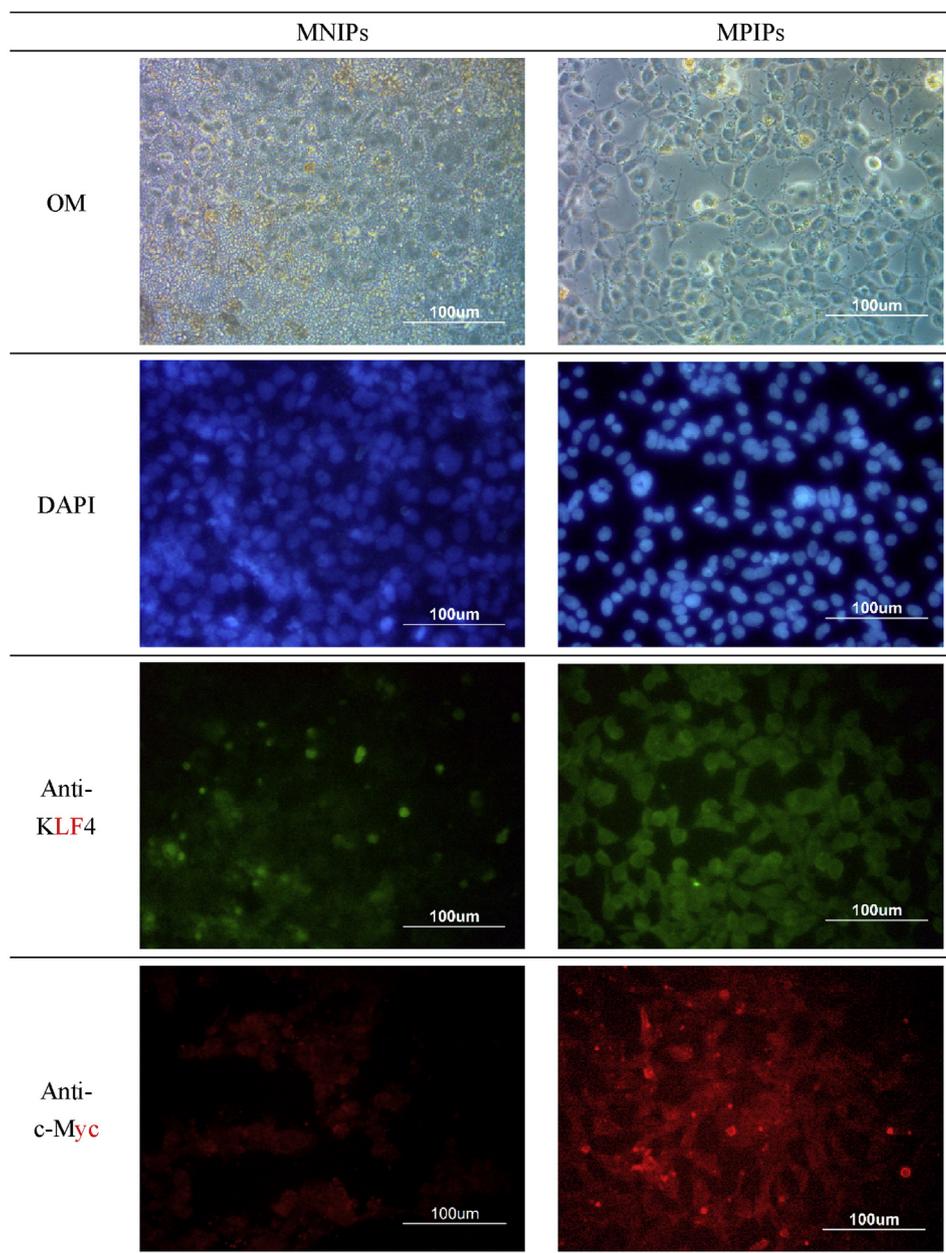


Fig. 2. Transfection of HEK-293T cells with magnetic peptide-imprinted nanoparticles/dCas9:gRNA and staining with DAPI, anti-KLF4, and anti-c-Myc.

systems have been investigated to quantify their ability to induce robust gene expression in several human, mouse, and fly cell lines [3]. Protocols for the CRISPR/Cas9 system [8] involve the delivery of RNP complex comprised of Cas9 protein with an attached sgRNA [9]. Compared with plasmid transfection approaches or the delivery of pDNA or mRNA, CRISPR/Cas9 RNP delivery has the advantages of immediate Cas9 onset expression, high editing efficiency, and low risk of off-target effects [8]. Highly efficient RNA-guided genome editing in human cells has been demonstrated using the delivery of purified Cas9 RNPs [10]. Diverse experimental and therapeutic genome engineering applications of Cas9 RNP technology have been confirmed in primary human T cells [11].

Magnetic molecularly imprinted polymers (MMIPs) are magnetic nanoparticles (MNPs) that are coated with tailor-made recognition polymers for biosensing [12], bioseparation [13,14] and delivery [13, 15,16]. In our previous work, seven peptides (13–18 amino acids) were synthesized and used as templates for the imprinting and recognition of Regenerating Protein 1 [14]. Recently, one peptide of programmed cell

death protein 1 was used as the template for molecular imprinting to form magnetic peptide-imprinted poly(ethylene-co-vinyl alcohol) composite nanoparticles [16]. The delivery of the CRISPR/Cas9 gene-editing system for *in vitro* genome editing [8,17], therapeutic applications [9], and challenges [18] have all been recently reviewed. However, precision medicine with the direct delivery of dCas9-VPR:gRNA RNPs for multiple gene activation cellular reprogramming is not well developed. In the present work, dCas9-VPR:gRNA RNPs with various gRNAs were immobilized on the magnetic peptide-imprinted nanoparticles (MPIPs) for the activation of OSKM genes in HEK 293T cells. The MPIPs were characterized by dynamic light scattering, high-performance liquid chromatography (HPLC), Brunauer–Emmett–Teller, the use of a superconducting quantum interference device for magnetometry, and atomic force microscopy (AFM). Four pairs of gRNAs were used to target the OSKM gene; cells were then prescreened for higher expression of OSKM proteins using immunohistochemical (IHC) images. The optimized gRNAs for OSKM

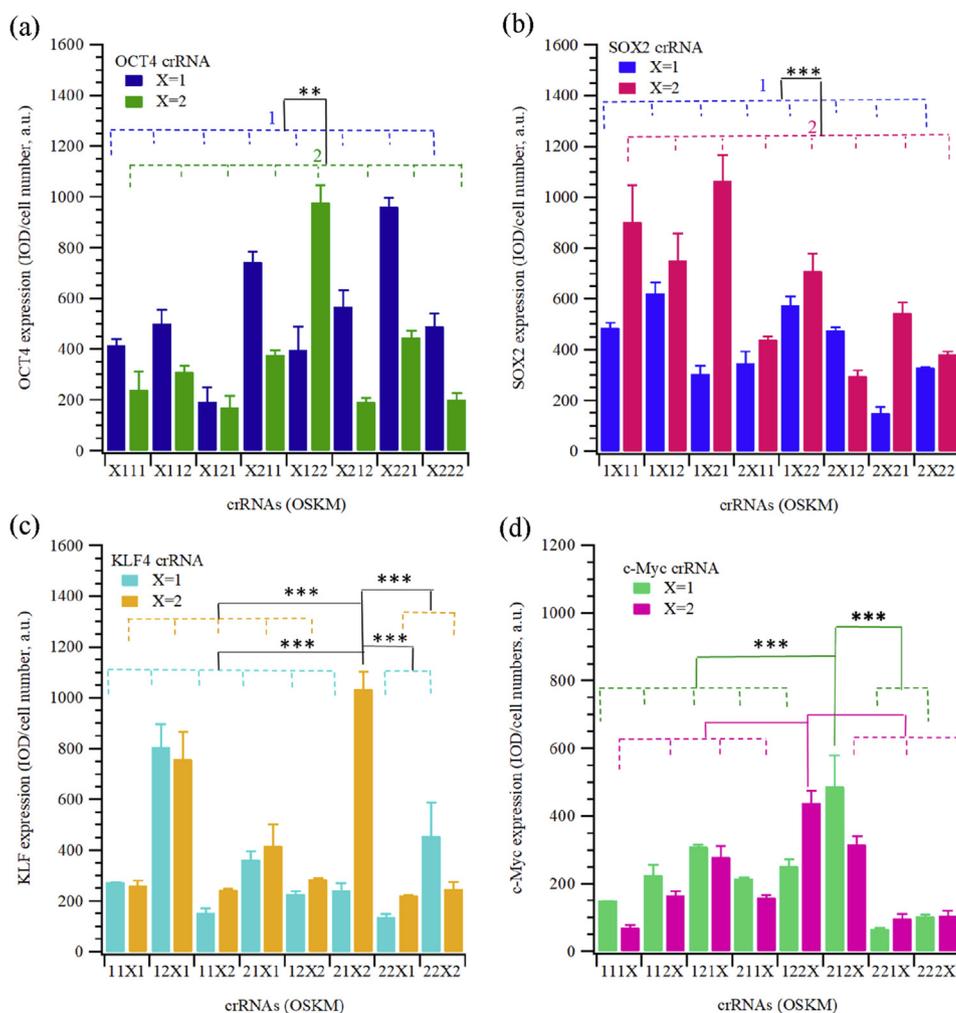


Fig. 3. Expression of (a) OCT4, (b) SOX2, (c) KLF4, and (d) c-Myc using immunohistochemistry images. HEK-293T cells were treated with dCas9 RNP/magnetic peptide-imprinted nanoparticles with four different crRNAs (targeting expression of each of the four proteins). For each protein, two crRNA sequences were studied (see Table S1), and all combinations of these (2^4) were examined. (Multicomparison statistical analyses of the expressions with X = 1 and X = 2 for paired *t*-test, and **: $p < 0.02$; ***: $p < 0.005$.)

expression were studied using quantitative real-time polymerase chain reaction (qRT-PCR) and the staining of OSKM proteins.

2. Experimental

2.1. Reagents and chemicals

Peptide of Cas9 protein in the sequence of QLFVEQHKHYLDE was purchased from Yao-Hong Biotechnology Inc. (HPLC grade, New Taipei City, Taiwan) for the template for molecular imprinting. Chitosan (C3646) and iron (III) chloride 6-hydrate (97%) were from Sigma-Aldrich Chemical Company (St. Louis, MO). Iron (II) sulfate 7-hydrate (99.0%) from Panreac (Barcelona, Spain), Acetic acid (ACS grade) from J. T. Baker (Phillipsburg, NJ) and sodium hydroxide from Mallinckrodt Chemical Inc. (St. Louis, MO). Human embryonic kidney cells (HEK 293T) were purchased from ATCC (American Type Culture Collection). The culture medium for HEK 293T cells contains high glucose Dulbecco's Modified Eagle's Medium (DMEM, including salts, L-glutamine, pyridoxine hydrochloride, and non-essential amino acids), and without sodium pyruvate and sodium bicarbonate, 10% v/v of fetal bovine serum (FBS, #12003C) and 1% v/v of antibiotics (penicillin and streptomycin) were from Life Technologies Co. (Grand Island, NY). Plasmid vector (dCas9-VPR-P2A) as shown in Fig. S1 was a precious gift from RNA technology platform and gene manipulation core, Academia Sinica, Taiwan. iPSC (A18945), essential 8 medium (A1517001), essential 8 supplement (A1517001), DMEM/F12+ and GlutaMAX (10565018), Geltrex (A1413301) were

purchased from Gibco (USA) and culture protocol can be found in (https://assets.thermofisher.com/TFS-Assets/LSG/manuals/episomal_hipsc_man.pdf).

2.2. Preparation and characterization of magnetic peptide-imprinted chitosan composite nanoparticles

The preparation of MPIPs is shown in Scheme 1. Briefly, MNPs were added to a chitosan solution (0.01 wt% chitosan and 0.01 wt % acetic acid in deionized [DI] water) to an MNP concentration of 0.2 mg/mL. The chitosan/MNPs solution was then mixed with 0 or 0.1 g/mL of peptide for the preparation of magnetic non-imprinted composite nanoparticles (MNIPs) and peptide imprinted MPIPs, respectively. In this solution, the acetic acid keeps the pH well below the pKa of chitosan (~6.5) and thus maintains chitosan solubility [19]. The solution was then dropwise diluted into 10 mL DI water at 4 °C, which causes chitosan to aggregate with MNPs and peptide [15]. The peptide template bound to the MPIPs was removed by washing with 10 mL DI water for 1 h and then separating the MPIPs using the field from a magnetic plate, both two times. All the nanoparticles, including MNPs and MPIPs before and after peptide removal, and after rebinding, were characterized by a particle sizer (90Plus, Brookhaven Instruments Co., New York). The magnetization of MNPs, MNIPs, and MPIPs after template removal was monitored with a magnetic property measurement MPMS XL-7 system (Quantum Design, San Diego, CA) at 298 K in $\pm 15,000$ Gauss.

The morphology of MPIPs after template removal was examined by AFM (Dimension Icon, Bruker Nano Surfaces Division, Santa Barbara,

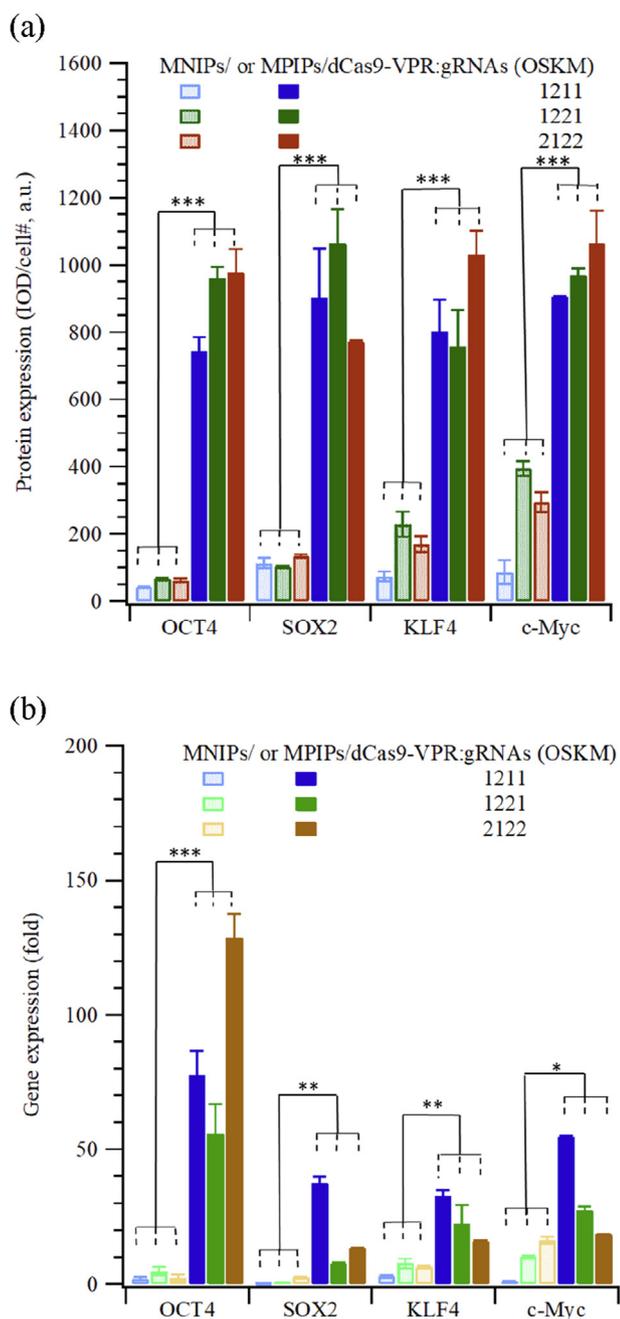


Fig. 4. HEK293T cells treated with magnetic peptide-imprinted nanoparticle/dCas9-VPR:gRNAs (OSKM), and their (a) OCT4, (b) SOX2, (c) KLF4, and (d) c-Myc expressions affected by various OCT4 gRNA concentrations. (Multi-comparison statistical analyses of the protein or gene expression with ribonucleoproteins on magnetic non-imprinted composite nanoparticles and magnetic molecularly imprinted polymers for paired *t*-test, and * $P < 0.05$; ** $p < 0.02$; *** $p < 0.005$.)

CA). Samples were sonicated and then dropwise added onto a cover glass and dried in an oven at 50 °C for 6 h. The sample was scanned in the tapping mode, using an ACTA probe (Applied NanoStructures, Inc., Mountain View, CA) at a scan rate of 0.8 Hz with a scan size of 5 μm , with resolution 512 \times 256 and integral gain 1.3. The adsorption of dCas9-VPR on MPIPs was quantified by a Cas9 ELISA Kit (PRB-5079) was from Cell Biolabs, Inc., (San Diego, CA). The Cas9 ELISA Kit is an enzyme immunoassay developed for the detection and quantitation of *Streptococcus pyogenes* Cas9 in cell or tissue lysate samples with a detection sensitivity limit of 1.5 ng/mL Cas9. The zeta potential of MNIPs and MPIPs were

measured with NanoPlus (Micromeritics Instrument Co., Norcross, GA).

The cytotoxicity experiments were carried out by seeding 7500 HEK293T cells per well in 96-well culture plates and incubating with different concentrations of nanoparticles for 24 h. 3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2H-tetrazolium bromide (MTT, Sigma-Aldrich) solution (10 μL) was added to each well and incubated for 3 h, followed by removal of media and addition of 100 μL DMSO to dissolve the formazan crystals. Emissions at 570 nm and 690 nm were measured with a plate reader (CLARIO star, BMG LABTECH, Germany). Cell viability (%) was then calculated as a percentage with respect to untreated cells.

2.3. Extraction of CRISPR/dCas9-VPR from transfected HEK293T cells with magnetic peptide-imprinted nanoparticles

Ten micrograms of dCas9-VPR-P2A plasmid (Fig. S1) was mixed with 500 μL jetPRIME buffer (jetPRIME, Polyplus Transfection, Illkirch, France). Then, 20 μL jetPRIME was added and vortexed for 10s and then spin down quickly. The transfection mixture was incubated for 10 min at room temperature and then added to the HEK 293T (2×10^6) cells for 24 h. These cells were washed with 10 mL PBS before adding 10 μL protease inhibitor cocktail (HY-K0010-1, MedChemExpress LLC, NJ, USA) and 1 mL RIPA lysis buffer (Biotechnology Grade, VWR International LLC, PA, USA) and then kept at 4 °C for 30 min. The mixture of cellular fluid and cells was sonicated for 10s and centrifuged at 10,000 rpm for 10 min. The MPIPs were then added into the supernatant for 30 min, and the MPIPs were then collected on the wall using a magnet. The CRISPR/dCas9-VPR was released from MPIPs by washing with DI water for 10 min.

2.4. Delivery of ribonucleoproteins with magnetic peptide-imprinted nanoparticles to HEK 293T cells

Both crRNAs and tracrRNA were from Dharmacon, Inc. (Lafayette, CO); the sequences of crRNA used to activate transcription of OCT4, SOX2, KLF4, and c-Myc (OSKM) are each listed in Table S1. For each protein two crRNAs were studied. Equal amounts of 5 μL crRNAs and tracrRNA at 5 nM were mixed at room temperature for 30 min to form gRNAs. Ten microliters of the extracted dCas9-VPR at 100 $\mu\text{g}/\text{mL}$ was loaded with four OSKM gRNAs (simultaneously) for 30 min at room temperature to form dCas9a OSKM RNPs. These dCas9a OSKM RNPs were then immobilized on 1.0 mg of MPIPs by the epitope recognition of Cas9 protein.

To deliver the dCas9-VPR OSKM RNPs to cells, cells were incubated with 100 $\mu\text{g}/\text{mL}$ MPIPs with bound OSKM RNPs for 2 days. Cells were then washed and grown in medium for 5 additional days (with two additional changes of media), and then cells were examined using immunohistochemistry (fluorescence) and PCR to study protein and mRNA expression.

crRNA was fluorescently labeled using LabelIT CX-Rhodamine (cat. no. MIR3100; Mirus), or Fluorescein (cat. no. MIR3200; Mirus) kits [20] according to the manufacturer's instructions. Labeling reagent and nucleic acid ratio were used at a ratio of 1:1 leading to 1 label per 20–60 bases [20] to track the binding of RNPs onto MPIPs.

2.5. Immunohistochemistry of OSKM proteins

HEK293T (2×10^4) cells were seeded in 24-well cell plates and kept at 37 °C in 5% CO_2 for 24 h. Cells were then washed with 400 μL PBS in each well and fixed in 3.7% formaldehyde in PBS (pH 7.4) for 10 min at room temperature. After washes with 350 μL PBS in each well, cells were permeabilized with 1% Triton X-100 for 5 min at room temperature, and then washed with 350 $\mu\text{L}/\text{well}$ PBS, followed by blocking of non-specific binding by washing in PBS supplemented with 5% BSA for 60 min. Finally, 350 μL PBS was added in each well, and cells were incubated overnight at 4 °C with 1:250 rabbit anti-OCT4 antibody (Sino Biological, #101282-T02) and mouse anti-SOX2 antibody (BioVision, 6772-100)/goat anti-KLF4 antibody (proteintech, 11880-1-AP) and mouse anti-c-

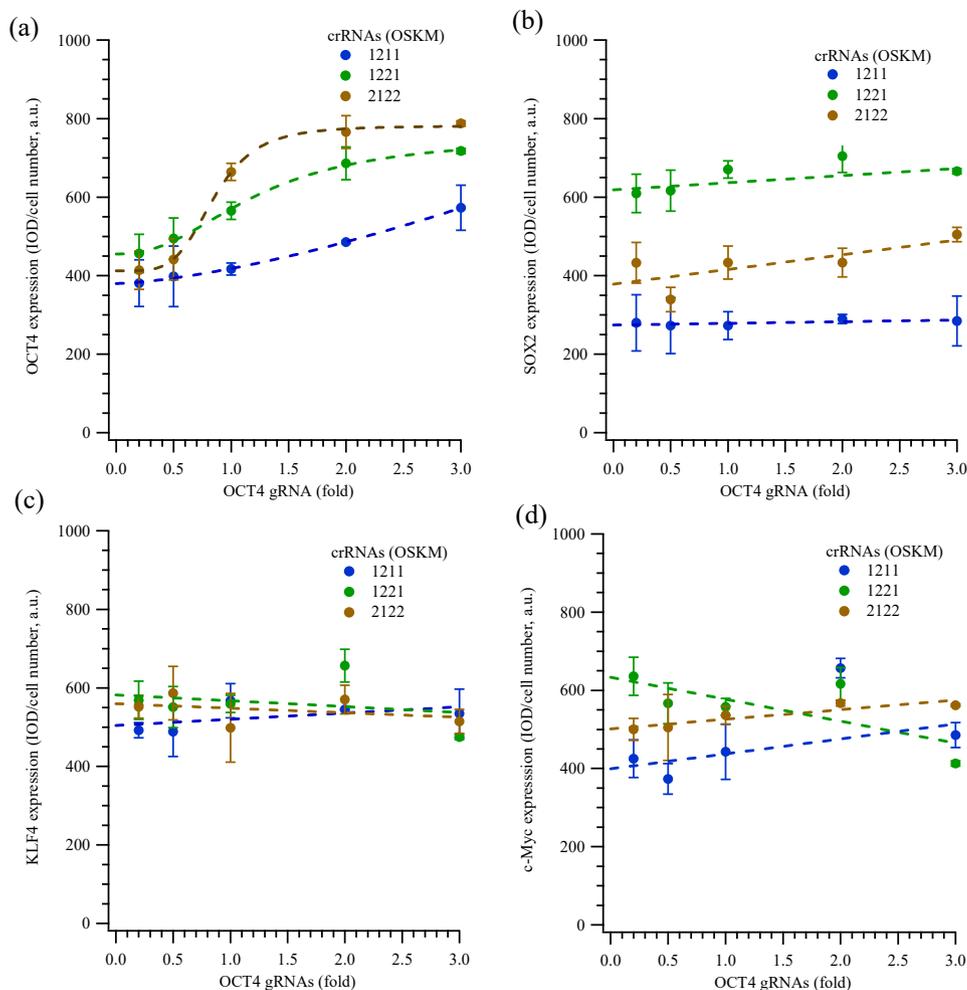


Fig. 5. Comparison of the expression of OCT4, SOX2, KLF4, and c-Myc with magnetic non-imprinted composite nanoparticles or magnetic peptide-imprinted nanoparticle/ribonucleoproteins (dCas9-VPR and the specific gRNAs (1211, 1221, and 2122) at the same concentrations) by (a) the relative immunohistochemistry images of and (b) the gene expression in HEK-293T cells.

Myc antibody (Biovision, A1450-100) to BSA. The cells were then washed three times in PBS for 5 min each and labeled with 300 μ L/well secondary antibody for 1 h at room temperature. After another wash in 250 μ L PBS at room temperature, the cells were co-stained with the nuclear dye DAPI for 15 min (Sigma). Finally, the cells were washed with PBS and examined with an inverted fluorescence microscope (CKX41, Olympus, Melville, NY).

Alkaline phosphatase (AP) live stain [10] of transfected HEK293T cells was performed using the AP staining kit (A14353, Thermo Fisher Scientific Inc.) according to the manufacturer's instructions. Briefly, the growth medium was removed, and the culture washed, and then 1X AP Live Stain solution was applied directly on to the adherent cell culture. The culture was incubated for 20–30 min, and the AP Live Stain was removed, followed by 2X wash prior to the visualization of fluorescent-labeled colonies using a standard FITC filter.

2.6. Gene expression of induced pluripotent stem cell and HEK293T cells treated with OSKM ribonucleoproteins on magnetic peptide-imprinted nanoparticles

The sequence (5'-3') of primers for GAPDH, OCT4, SOX2, KLF4, c-Myc, Akt1, β -catenin, STAT3, HRAS, KRAS, NRAS, ERK1, and ERK2 is listed in Table S2. The total RNA extraction from iPSCs and the HEK293T cells treated with various combinations of RNPs adsorbed on MIPs was performed using the Nucleospin RNA, Mini kit for RNA purification

(740955.50 Macherey-Nagel). Complementary DNA was obtained following a Magic RT Mastermix cDNA synthesis kit (BB-DBU-RT-100, Bio-genesis Technologies, Inc., Taiwan) protocol. The real-time PCR was then performed with IQ2 SYBR Green Fast qPCR System Master Mix (BB-DBU-006-5, Bio-genesis Technologies, Inc., Taiwan) in a StepOne Real-Time PCR System (LS4376357, Applied Biosystems, Waltham, MA). Relative gene expression was determined using a $\Delta\Delta$ Cq method [21] and normalized to a reference gene (GAPDH) and to control (HEK293T/iPSC).

2.7. Data analysis

All experiments were carried out in triplicate, and data are expressed as means \pm standard deviation. Images were analyzed using ImageJ (<http://imagej.nih.gov/ij/index.html>), measuring the area of blue or red pixels, keeping the saturation and brightness to a fixed level for all images. The gene expression data were analyzed with Student's *t*-test. Statistical significance was set at a *p*-value of less 0.05, highly significant as $p < 0.02$, and extremely significant as $p < 0.005$.

3. Results and discussion

Imprinted and non-imprinted nanoparticles were prepared and characterized as shown in Fig. S2 and discussed in the *Supplementary Information* [15]. We then used the imprinted nanoparticles for cellular

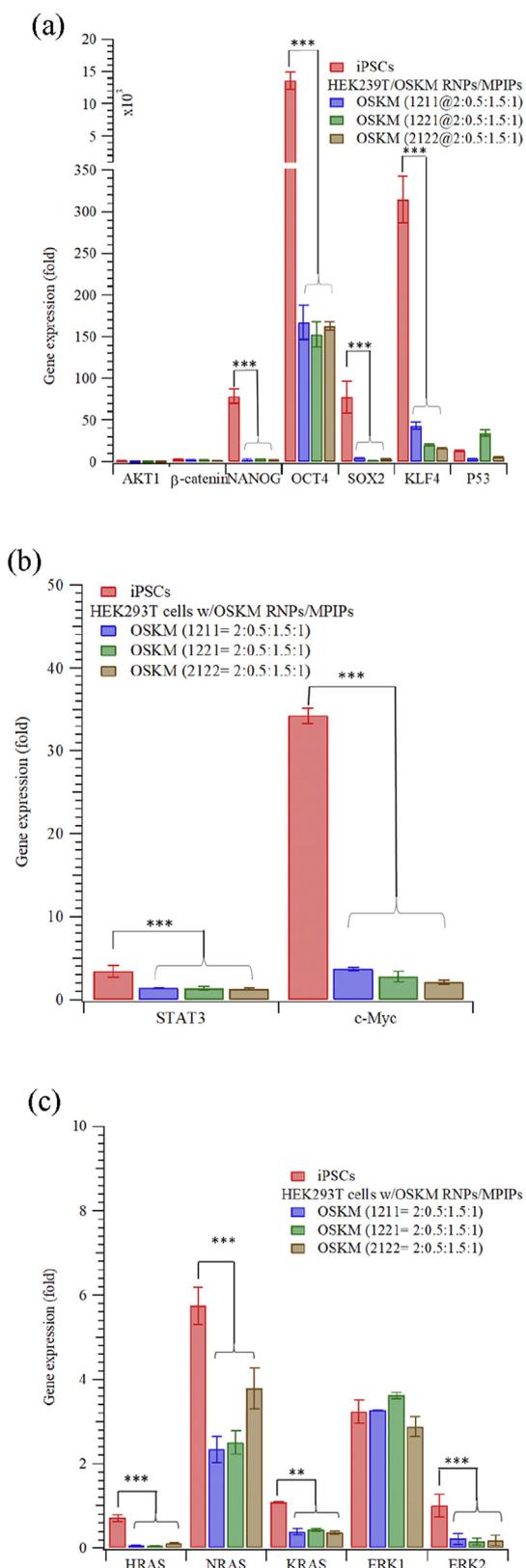


Fig. 6. Comparison of the expression of cellular self-renewal (a) reprogramming factors and (b) STATs/c-Myc and (c) cellular differentiation in iPSC and HEK293T cells. The HEK293T cells were treated with various combinations of magnetic peptide-imprinted nanoparticles (MPIPs)/ribonucleoproteins (RNPs) in the optimized gRNA ratio. (Multicomparison statistical analyses of the gene expression in iPSCs and HEK293T cells treated with various combinations of OSKM crRNAs in RNPs/MPIPs for paired *t*-test, and $***P < 0.005$.)

transfection using nanoparticle-bound dCas9a RNPs, to upregulate expression of four proteins (OCT4, SOX2, KLF4, and c-Myc) using each possible choice from two crRNAs (Table S1) that target each of the four proteins, i.e. 2^4 different experiments, each measuring the expression of four proteins. Thus, each experimental condition can be coded by a four-digit number consisting of ones or twos, according to whether the first or second choice of crRNA was made for each of the four protein targets. The dCas9a RNPs/MPIPs were introduced into cells by incubating with the cells for 2 days. Screening was done using fluorescence immunostaining (Figs. 1 and 2), followed by quantification of fluorescence levels via the integrated optical density (IOD) in each image. The expression of OCT4 protein is shown in Fig. 3(a). The highest IOD for OCT4 was obtained using the combination of crRNAs 1211 (see Figs. 1 and 2); the second highest IOD was obtained using 1112, 1212, 1221, or 1222, all of which gave similar expression levels. Most importantly, the expression level of one protein (OCT4) depends not only on which crRNA was used to target OCT4 but also which crRNAs were used in targeting the remaining three proteins.

Fig. 3(b) shows the expression of SOX2; the crRNA combinations 1211, 1212, 1221, and 1222 gave the highest SOX2 expression. Interestingly, the highest expression of KLF4 (Fig. 3(c)) was obtained using the crRNA combination 2122—i.e. using crRNA2 for targeting OCT4. Overall, the expression of c-Myc (Fig. 3(d)) was quite low, although three combinations (1222, 2121, and 2122) yielded higher expression than others. Based on this prescreening, from the 16 possible combinations of crRNAs, the combinations 1211 (highest OCT4), 1221 (second highest c-Myc but also relative good expression for the others) and 2122 (highest Klf4) were selected in the subsequent optimization of the reprogramming of HEK 293T cells.

Fig. 4(a) and (b) shows OSKM gene expressions, obtained by measuring IOD and by qRT-PCR. dCas9-VPR: gRNA (OSKM) was delivered with MNIPs or MPIPs. Since MPIPs are better able to bind to dCas9 proteins, the delivery of RNPs for the activation of all four genes was about seven- to eightfold higher (in IODs) for some combinations of OSKM crRNAs when using MPIPs compared with use of MNIPs; Fig. 4(a). However, the expression of mRNAs, as measured by qRT-PCR, was around only four- to sixfold higher when using MPIPs compared with MNIPs.

Figs. 5 and S4–S6 present the optimization of four OSKM gRNAs concentrations on MPIPs. A number of experiments were conducted in which the concentrations of the three gRNAs were held fixed (at 50 pM), while the fourth was varied. The three different combinations of crRNAs that were found to be most effective in prescreening (1211, 2122, and 1221) were studied. Fig. 5(a)–(d) plot the message expression levels of OSKM proteins when the gRNA concentration of one of the four administered was varied (For example, in Fig. 5(a), the OCT4 gRNA concentration was varied while the remaining three were fixed at 50 p.m.; the OCT4 ratio is the ratio of OCT4 gRNA concentration to that of the other gRNAs.). The expression of OCT4 increased with increasing OCT4 gRNA concentration, as expected; there is also a remarkable, almost step increase in OCT4 expression when using crRNA 2, which was not seen with crRNA 1. Interestingly, increasing the OCT4 gRNA concentration caused a small but significant increase in SOX2 expression, demonstrating synergy in gene activation. The other two proteins, KLF4 and c-Myc did not show clear trends as the concentration of OCT4 gRNA concentration was varied. Varying the SOX2 gRNA concentration gave very different results, Fig. S4. The expression of SOX2 did not increase monotonically with SOX2 gRNA concentration but peaked when the SOX2 gRNA concentration was cut in half relative to the other gRNAs (to 25 pM). OCT4 also peaked here. KLF4 and c-Myc also showed decreases with increasing SOX2 gRNA concentration, although in some cases a steady decline, rather than a peak, was observed.

Fig. S5 shows that KLF4 was mostly decoupled from the remaining three proteins, as changing the KLF4 gRNA concentration had a

significant effect only on the KLF4 expression. (There is one outlier point in the OCT4 expression.) Finally, in Fig. S6, the expression of all four OSKM proteins was maximal at c-Myc gRNA concentration ratios of from 0.5 to 2.0, thus showing very significant coupling. From these results, we anticipated that a concentration ratio of 2.0:0.5:1.5:1.0 for the OSKM gRNA would be highly effective as shown in Fig. S7.

Fig. 6 shows gene expression for differentiation and self-renewal pathways of both iPSC and HEK293T cells transfected with MPIPs/dCas9:gRNAs OSKM RNPs at the gRNA concentration ratio of 2.0:0.5:1.5:1.0. Fig. 6(a) and (b) show the expressions of AKT1, β -catenin, NANOG, OCT4, SOX2, KLF4, and p53/STAT3 and c-Myc for iPSC and reprogrammed HEK 293T cells, respectively. All markers in the reprogrammed HEK 293T cells varied similarly to the iPSCs expression; however, the expression of p53 in the reprogrammed HEK 293T cells with three combinations of gRNAs was greater than those of other markers in iPSCs. The expression of OCT4 was higher than NANOG (another reprogramming factor, Table S2) in both reprogrammed HEK 293T cells and iPSCs; however, the expression of OCT4 was about 26-fold that of NANOG in iPSCs and approximately eightfold that in reprogrammed HEK 293T cells. Finally, Fig. 6(c) compares the markers of cellular differentiation for iPSC and HEK 293T that had been treated with OSKM RNPs/MPIPs. The RAS and ERK expressions are similar in both iPSC and reprogrammed HEK 293T cells with three combinations of OSKM RNPs with their optimized ratios. These results suggest that reprogrammed HEK 293T cells are similar to iPSCs in their stemness [22].

To quantify the fraction of cells successfully reprogrammed, we stained transfected cells for AP, a marker for iPSCs, and with DAPI nuclear stain for cell counting. The reprogramming efficiency was found to be $75.4 \pm 5.3\%$, based on at least three images/sample and two individual samples. It is possible that not all four factors need be upregulated for AP expression, but, nonetheless, this is a very good reprogramming efficiency. For example, if each factor had an activation rate of one-third on successful cellular delivery of the dCas9 construct [8], the rate of fourfold activation (assuming independence) would be $1/3^4$ or merely 1.2%. While the mechanism for uptake of the chitosan nanoparticles and subsequent cellular delivery of dCas9 RNPs is unknown, it has been established that most synthetic non-viral vectors enter the targeted cells via endocytosis [23].

Finally, in this article, the nanoparticle carriers were ‘randomly’ loaded using a mixture of four OSKM RNPs. It is of interest to ask whether individual nanoparticles carried more than one kind of RNP. To check this, we used fluorescent labeling of crRNAs and examined nanoparticles using fluorescence microscopy. We confirmed that single nanoparticles contained (at least) two different RNPs, as shown in Fig. S9.

4. Conclusions

Precision medicine is currently being developed for personalized therapy to reduce the side-effects of mutation of gene-editing. The rapid development technique of gene editing (including the CRISPR/Cas9 system) has enabled the activation of genes by the fusion of activators on dCas9 proteins. dCas9a proteins that are immobilized on MMIPs can carry various crRNAs for biomedical applications, such as the reprogramming of HEK293T cells to iPSCs, which was demonstrated in this work. Moreover, the activation and optimization of multiple genes are adjustable for cellular cocktail therapies.

CRedit authorship contribution statement

Mei-Hwa Lee: Conceptualization, Methodology, Validation, Project

administration. **Cheng-Chih Lin:** Conceptualization, Investigation, Resources. **James L. Thomas:** Methodology, Formal analysis, Writing - review & editing. **Jin-An Li:** Investigation, Data curation. **Hung-Yin Lin:** Supervision, Writing - original draft, Visualization, Funding acquisition.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at <https://doi.org/10.1016/j.mtbio.2020.100091>.

References

- [1] D. Bikard, W. Jiang, P. Samai, A. Hochschild, F. Zhang, L.A. Marraffini, Programmable repression and activation of bacterial gene expression using an engineered CRISPR-Cas system, *Nucleic Acids Res.* 41 (15) (2013) 7429–7437.
- [2] X. Xiong, M. Chen, W.A. Lim, D. Zhao, L.S. Qi, CRISPR/Cas9 for human genome engineering and disease research, *Annu. Rev. Genom. Hum. Genet.* 17 (2016) 131–154.
- [3] A. Chavez, M. Tuttle, B.W. Pruitt, B. Ewen-Campen, R. Chari, D. Ter-Ovanesyan, S.J. Haque, R.J. Cecchi, E.J.K. Kowal, J. Buchthal, B.E. Housden, N. Perrimon, J.J. Collins, G. Church, Comparison of Cas9 activators in multiple species, *Nat. Methods* 13 (7) (2016) 563–567.
- [4] A. Chavez, J. Scheiman, S. Vora, B.W. Pruitt, M. Tuttle, E.P.R. Iyer, S. Lin, S. Kiani, C.D. Guzman, D.J. Wiegand, D. Ter-Ovanesyan, J.L. Braff, N. Davidsohn, B.E. Housden, N. Perrimon, R. Weiss, J. Aach, J.J. Collins, G.M. Church, Highly efficient Cas9-mediated transcriptional programming, *Nat. Methods* 12 (4) (2015) 326–328.
- [5] M. Pandelakis, E. Delgado, M.R. Ebrahimkhani, CRISPR-based synthetic transcription factors in vivo: the future of therapeutic cellular programming, *Cell Syst.* 10 (1) (2020) 1–14.
- [6] G. Yang, X. Huang, Methods and applications of CRISPR/Cas system for genome editing in stem cells, *Cell Regen.* 8 (2) (2019) 33–41.
- [7] J. Weltner, D. Balboa, S. Katayama, M. Bespalov, K. Krjutskov, E.-M. Jouhilahti, R. Trokovic, J. Kere, T. Otonkoski, Human pluripotent reprogramming with CRISPR activators, *Nat. Commun.* 9 (1) (2018) 2643.
- [8] A.K. Fajrial, Q.Q. He, N.I. Wirusanti, J.E. Slansky, X. Ding, A review of emerging physical transfection methods for CRISPR/Cas9-mediated gene editing, *Theranostics* 10 (12) (2020) 5532–5549.
- [9] C. Liu, L. Zhang, H. Liu, K. Cheng, Delivery strategies of the CRISPR-Cas9 gene-editing system for therapeutic applications, *J. Contr. Release* 266 (2017) 17–26.
- [10] S. Kim, D. Kim, S.W. Cho, J. Kim, J.-S. Kim, Highly efficient RNA-guided genome editing in human cells via delivery of purified Cas9 ribonucleoproteins, *Genome Res.* 24 (6) (2014) 1012–1019.
- [11] K. Schumann, S. Lin, E. Boyer, D.R. Simeonov, M. Subramaniam, R.E. Gate, G.E. Haliburton, C.J. Ye, J.A. Bluestone, J.A. Doudna, A. Marson, Generation of knock-in primary human T cells using Cas9 ribonucleoproteins, *Proc. Natl. Acad. Sci. U.S.A.* 112 (33) (2015) 10437–10442.
- [12] M.-H. Lee, J.L. Thomas, M.-H. Ho, C. Yuan, H.-Y. Lin, Synthesis of magnetic molecularly imprinted poly (ethylene-co-vinyl alcohol) nanoparticles and their uses in the extraction and sensing of target molecules in urine, *ACS Appl. Mater. Interfaces* 2 (6) (2010) 1729–1736.
- [13] M.-H. Lee, J.L. Thomas, H.-Y. Wang, C.-C. Chang, C.-C. Lin, H.-Y. Lin, Extraction of resveratrol from polygonum cuspidatum with magnetic orcinol-imprinted poly (ethylene-co-vinyl alcohol) composite particles and their in vitro suppression of human osteogenic sarcoma (HOS) cell line, *J. Mater. Chem.* 22 (47) (2012) 24644–24651.

- [14] M.-H. Lee, J.L. Thomas, C.-L. Liao, S. Jurcevic, T. Crnogorac-Jurcevic, H.-Y. Lin, Epitope recognition of peptide-imprinted polymers for Regenerating protein 1 (REG1), *Separ. Purif. Technol.* 192 (2018) 213–219.
- [15] M.-H. Lee, J.L. Thomas, J.-Z. Chen, J.-S. Jan, H.-Y. Lin, Activation of tumor suppressor p53 gene expression by magnetic thymine-imprinted chitosan nanoparticles, *Chem. Commun.* 52 (10) (2016) 2137–2140.
- [16] M.-H. Lee, K.-H. Liu, J.L. Thomas, J.-R. Chen, H.-Y. Lin, Immunotherapy of hepatocellular carcinoma with magnetic PD-1 peptide-imprinted polymer nanocomposite and natural killer cells, *Biomolecules* 9 (11) (2019) 651.
- [17] D. Wilbie, J. Walther, E. Mastrobattista, Delivery aspects of CRISPR/Cas for in vivo genome editing, *Acc. Chem. Res.* 52 (6) (2019) 1555–1564.
- [18] C.A. Lino, J.C. Harper, J.P. Carney, J.A. Timlin, Delivering CRISPR: a review of the challenges and approaches, *Drug Deliv.* 25 (1) (2018) 1234–1257.
- [19] M.-C. Chen, F.-L. Mi, Z.-X. Liao, C.-W. Hsiao, K. Sonaje, M.-F. Chung, L.-W. Hsu, H.-W. Sung, Recent advances in chitosan-based nanoparticles for oral delivery of macromolecules, *Adv. Drug Deliv. Rev.* 65 (6) (2013) 865–879.
- [20] M. Nasri, P. Mir, B. Dannenmann, D. Amend, T. Skroblyn, Y. Xu, K. Schulze-Osthoff, M. Klimiankou, K. Welte, J. Skokowa, Fluorescent labeling of CRISPR/Cas9 RNP for gene knockout in HSPCs and iPSCs reveals an essential role for GADD45b in stress response, *Blood Adv.* 3 (1) (2019) 63–71.
- [21] M.W. Pfaffl, A new mathematical model for relative quantification in real-time RT-PCR, *Nucleic Acids Res.* 29 (9) (2001) e45.
- [22] D. Melton, Chapter 2 – ‘stemness’: definitions, criteria, and standards, in: R. Lanza, A. Atala (Eds.), *Essentials of Stem Cell Biology*, third ed., Academic Press, Boston, 2014, pp. 7–17.
- [23] Y. Xu, R. Liu, Z. Dai, Key considerations in designing CRISPR/Cas9-carrying nanoparticles for therapeutic genome editing, *Nanoscale* 12 (41) (2020) 21001–21014.