Electronic Supplementary Materials:

Nanoparticle-Mediated CRISPR/dCas9a Activation of Multiple Transcription Factors to Engineer Insulin-Producing Cells

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S1. Experimental

S1.1 Reagents and chemicals.

Peptide of Cas9 protein in the sequence of QLFVEQHKHYLDE was from Yao-Hong Biotechnology Inc. (HPLC grade, New Taipei City, Taiwan). Chitosan (from shrimp shells, ≥75% (deacetylated), #C3646) and iron (III) chloride 6-hydrate (97%) were ordered from Sigma-Aldrich Chemical Company (St. Louis, MO). Iron (II) sulphate 7-hydrate (99.0%) was from Panreac (Barcelona, Spain). Acetic acid (ACS grade) and sodium hydroxide were purchased from J. T. Baker (Phillipsburg, NJ) and Mallinckrodt Chemical Inc. (St. Louis, MO), respectively.

S1.2 Preparation and characterization of magnetic peptide-imprinted nanoparticles (MQIPs).

The preparation of magnetic peptide Q- and non-imprinted chitosan composite nanoparticles (MQIPs and MNIPs, respectively,) was reported in our previous work.[20] Briefly, magnetic nanoparticles (MNPs) were added to the chitosan solution (chitosan/0.01 wt % acetic acid solution = 1.0 wt%) to a concentration of 0.1 mg/mL. The chitosan/MNP solution was then mixed with 0 or 1.0 μg/mL of peptide for MNIPs and MQIPs, respectively. The chitosan/peptide/MNPs suspension was dispersed in 10 mL deionized water at 4 ^{o}C followed

by removal of the peptide from MQIPs by washing with 10 mL deionized (DI) water for 1 min and separating on a magnetic plate two times. All composite nanoparticles were equilibrated with deionized water overnight before use.

The nanoparticles including MNPs and MQIPs with the adsorption of dCas9-VPR and *INS*, *NGN3*, *NKX6.1+MAFA* or *PDX1* RNPs were monitored by a dynamic light scattering (DLS) particle sizer (90Plus, Brookhaven Instruments Co., New York). Nitrogen adsorption measurements were performed with a NOVA 1000e, and Brunauer–Emmett–Teller (BET) analysis was performed with the Autosorb program (Quantachrome Instruments, Florida). The gradual release of the CRISPR/dCas9a from MQIPs were measured with Cas9 ELISA Kit (#PRB-5079, Cell Biolabs, Inc., San Diego, CA), and the protocol can be found from their website (https://www.cellbiolabs.com/cas9-crispr-elisa-kit).

S1.3 Delivery of RNP with MQIPs to HEK 293T cells

The sequences of tracrRNA and target sequence of crRNA (Dharmacon, Inc., Lafayette, CO, USA) are 5'AACAGCAUAGCAAGUUAAAAUAAGGCUAGUCCGUUAUCAACUU GAAAAAGUGG CACCGAGUCGGUGCUUUUUUU3' and listed in Table S1, respectively. Equal amounts of 1.0 μL tracrRNA and crRNAs at 10 μM were mixed at room temperature or 30 min to form gRNAs. Two microliters of the extracted dCas9-VPR proteins, which are

extracted from transfected HEK 293T cells with MQIPs,[20] at 100 ng/mL were loaded with above gRNAs for 30 min at room temperature to give dCas9a RNPs. These dCas9a RNPs were then immobilized with 100 μg of MQIPs and 496 μL DMEM medium by the epitope recognition of Cas9 proteins at room temperature (ca. 25°C) Unbound RNPs in medium were removed after 30 min, and 500 μL DMEM medium was added for cellular transfection. Different transfection treatment schedules for four-factor transfection are shown in Scheme 1.

S1.4 Measurement of glucose-stimulated insulin secretion (GSIS)

Culture medium was removed and cells were washed with PBS buffer, added DEME medium (without glucose) for incubation for an hour for the simulation before intake. The medium was replaced with medium containing 5 mg/mL glucose and incubated with cells for an hour at 37°C to simulate the intake, and the medium (500 μL) was then replaced with normal medium. These media were collected for measurement of the concentration of insulin with Human Insulin ELISA Kit (#KAQ1251, Thermo Fisher Scientific, Waltham, MA) The protocol can be found from their website. The same protocol was employed for the measurements of insulin concentrations on various day after transfection and stimulation.

S1.5 Immunocytochemistry of insulin, NGN3, NKX6.1, MAFA and PDX1 proteins

HEK 293T $2x10^4$ cells were seeded in a 24-well cell plate and kept at 37°C and 5% CO_2 for 24 hr. Each well was then washed with 400 μL PBS and the cells were fixed in 3.7 % formaldehyde in DI water for 10 min at room temperature. After further washes with 350 μL PBS in each well, cells were permeabilized with 1 % Triton X-100 for 10 min at room temperature, and then washed with 350 μL/well PBS, followed by blocking of nonspecific binding by washing in PBS supplemented with 5% BSA for 60 mins. Finally, 350 μL PBS was added in each well and cells were incubated overnight at 4 °C with (1) 1:800 rabbit anti-insulin antibody (Sino Biological, #101282-T02), or (2) 1:500 rabbit anti-PDx1 (Thermo Fisher) or anti-NKx6.1 (Thermo Fisher) and mouse anti-Ngn3 (Sigma-Aldrich) or anti-MaFA (Sigma-Aldrich) to BSA. The cells were then washed in PBS and labeled with 300 μL/well secondary antibody (CF488A goat anti-rabbit IgG (H+ green) and CF543 goat anti-mouse IgG (H+ red), both from Biotium) for 1 h at room temperature. After another wash in 250 μL PBS at room temperature, the cells were co-stained with the nuclear dye DAPI (Sigma) for 15 mins. Finally, the cells were washed with PBS and examined with an inverted fluorescence microscope (CKX41, Olympus, Melville, NY). Results of immunostaining are shown in the main manuscript in Fig. 2

S1.6 Gene expression of HEK293T cells treated with RNPs on MQIPs, before and after

glucose stimulation

Primers for activation of β cells and insulin release genes are listed in Tables S2 and S3, respectively. The total RNA extraction from (1) the HEK293T cells treated with various concentrations of RNPs adsorbed on MQIPs and (2) transfected cells before and after glucose stimulation was performed using the Nucleospin RNA, Mini kit for RNA purification (740955.50 Macherey-Nagel). The concentration of cellular RNA was quantified by determining the absorbance maximum at the wavelength of 260 and 280 nm to optimum OD about 1.5 in a UV/Vis spectrometer (Lambda 40, PerkinElmer, Wellesley MA). Complementary DNA was obtained following a Magic RT Mastermix cDNA synthesis kit (BB-DBU-RT-100, Bio-genesis Technologies, lnc., Taiwan) protocol. The real-time PCR was then performed with Fast SYBR™ Green Master Mix (#4385612, Thermo Fisher Scientific. Waltham, Massachusetts, US) in a StepOne™ Real-Time PCR System (LS4376357, Applied Biosystems, Waltham, MA). Relative gene expression was determined using a ΔΔCq method[26] and normalized to a reference gene (GAPDH) and to control (HEK293T).

S1.7 Data analysis

All experiments were carried out in triplicate, and data are expressed as means ± standard deviation. The gene expression data were analyzed with Student's t-test.

Table S1. The target sequence of crRNAs for Human *INS, NGN3, NKX6.1, MAFA, PDX1*.

crRNA	Target sequence
INS	GCGGCAGGGGTTGAGAGGTA
NGN3	CACAGCTGGATTCCGGACAA
NKX6.1	GCTGCCGCCTCCCGCGTGGA
MAFA	GCCCAGCTGTCAATCTCCTG
PDX1	CGGGCCGGCCGCCGCACCAT

Table S2. The sequence (5'- 3') of primers for *GAPDH, INS, NEUROG3, NKX6.1, MAFA, PDX1*.

mRNA	Forward/Reverse	Sequence (5'- 3')
GAPDH	forward	CTTTTGCGTCGCCAG
	reverse	TTGATGGCAACAATATCCAC
INS	forward	CCATCAAGCAGATCACTG
	reverse	CACTAGGTAGAGAGCTTCC
NGN3	forward	CCCCATTCTCTCTTCTTTTC
	reverse	AGGCGTCATCCTTTCTAC
NKX6.1	forward	CCTGTACCCCTCATCAAG
	reverse	GACCTGACTCTCTGACATC
MAFA	forward	ACATATCTGTAACTCCTGGG
	reverse	CCACAAACAAAACGAAACAC
PDX1	forward	AAAACGTATGTGATTGGAGG
	reverse	CCAGACCTTGAAAAGAAGAC

Table S3. The sequence (5'- 3') of primers for *GAPDH, PI3k, AKT1, PKA, Casp3, mTOR, FOXO, P53, cAMP, EzH2, ERK1, MEK1*.

mRNA	Forward/Reverse	Sequence (5'- 3')
GAPDH	forward	CTTTTGCGTCGCCAG
	reverse	TTGATGGCAACAATATCCAC
PI3k	forward	CAGATTCTACGAATCATGGAG
	reverse	TCCTATTTTGTCACCAGTTG
AKT1	forward	AAGTACTCTTTCCAGACCC
	reverse	TTCTCCAGCTTGAGGTC
PKA	forward	CTGAGATTATCCTGAGCAAAG
	reverse	GCCATTTCATAGATAAGAACCC
Casp3	forward	AAAGCACTGGAATGACATC
	reverse	CGCATCAATTCCACAATTTC
mTOR	forward	GGAGGAGAAATTTGATCAGG
	reverse	GGGCAACAAATTAACGATTG
FOXO	forward	GTCAAGACAACGACACATAG
	reverse	AAACTAAAAGGGAGTTGGTC
P53	forward	AGGATTCACAGTCGGATATG
	reverse	GGAGGAAGAAGTTTCCATAAG
EzH2	forward	AAGAAATCTGAGAAGGGACC
	reverse	CTCTTTACTTCATCAGCTCG
ERK1	forward	TTCGAACATCAGACCTACTG
	reverse	TAGACATCTCTCATGGCTTC
MEK1	forward	GATTACATAGTCAACGAGCC
	reverse	CTTCAAATCTGCTCTCTCTG

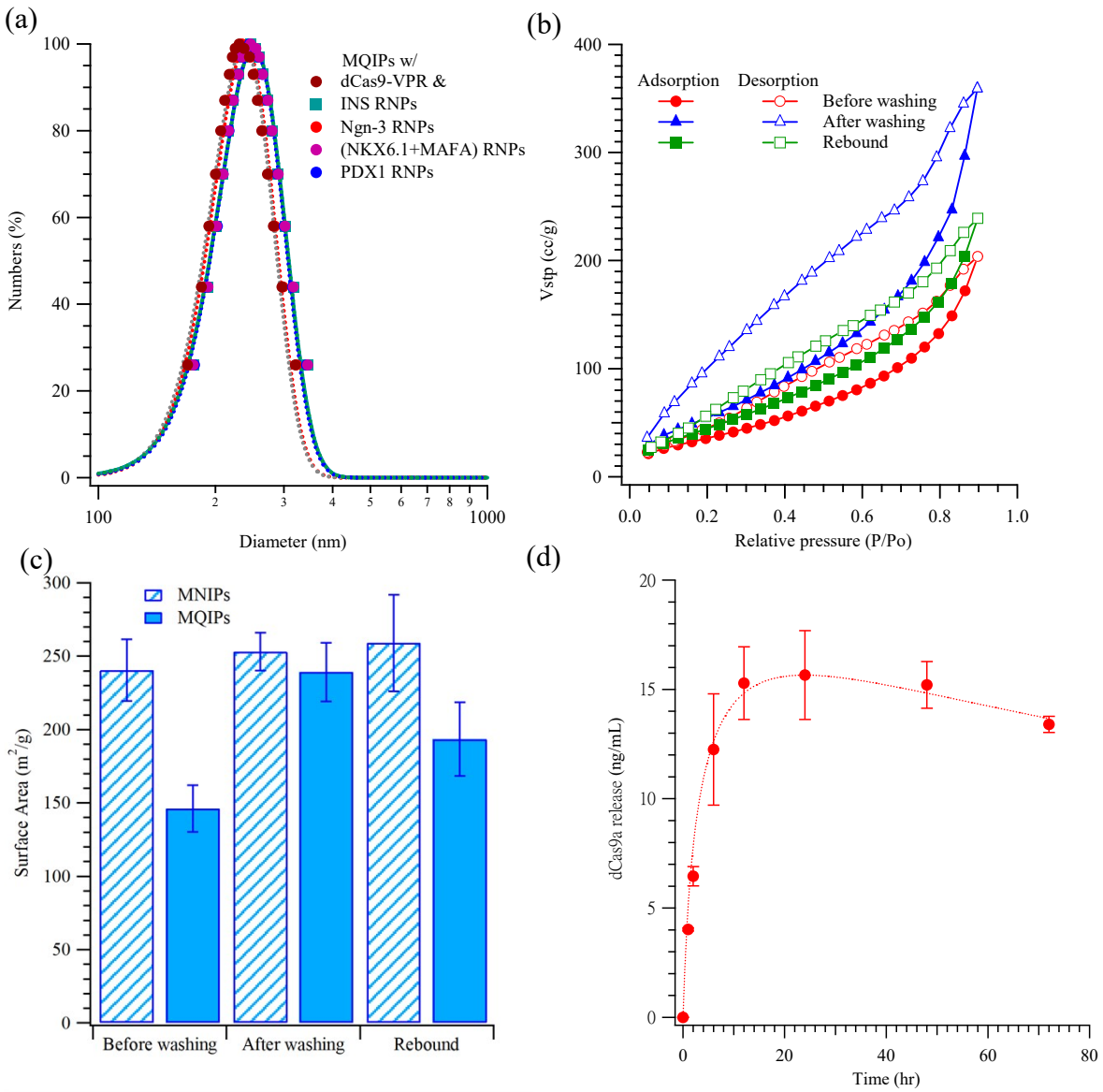

Figure S1. (a) The size distribution of the magnetic peptide-imprinted chitosan nanoparticles (MQIPs), bound dCas9-VPR and *INS*, *NGN3*, *NKX6.1+MAFA* or *PDX1* RNPs. (b) Nitrogen adsorption and desorption of MQIPs before and after washing, and rebound with peptide Q. (c) The specific surface area of MNIPs and MQIPs before and after washing, and rebound with peptide Q calculated by the BET analysis. (d) The gradual release of the CRISPR/dCas9a from MQIPs.

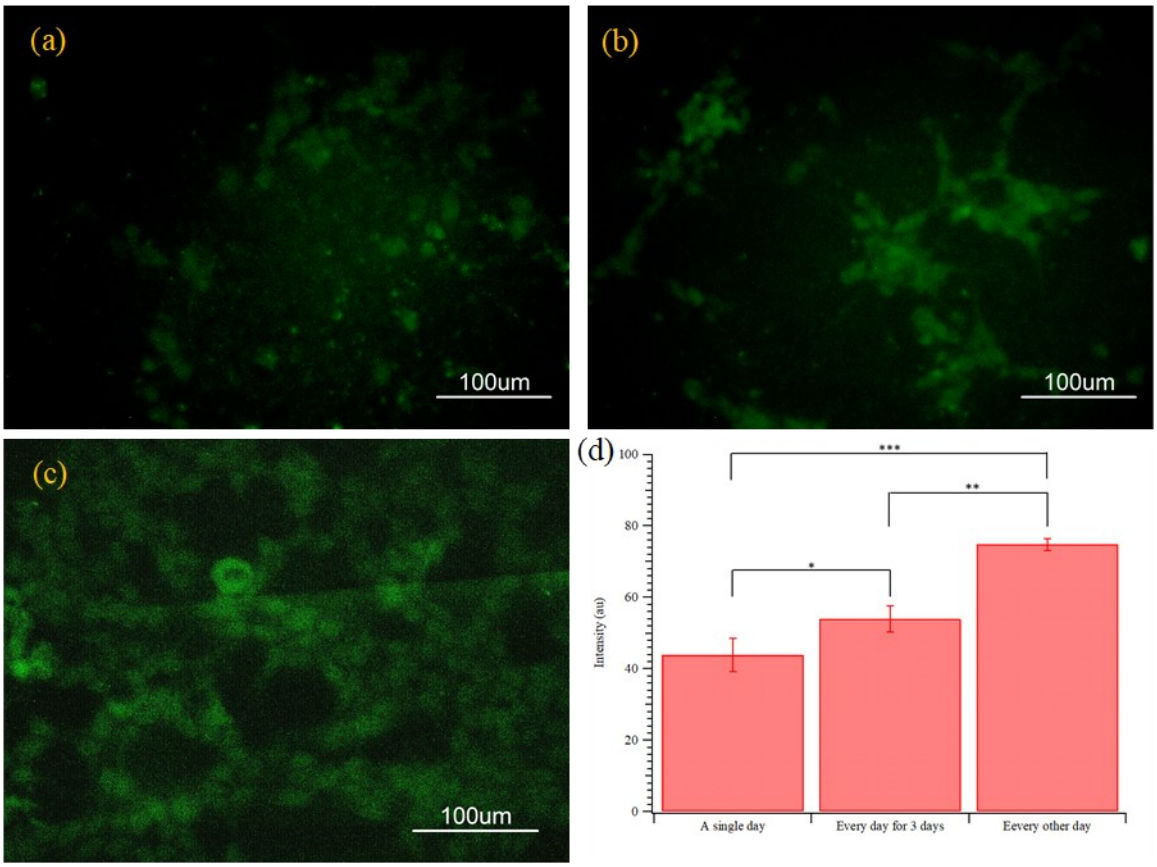

Figure S2. Immunocytochemistry images of anti-insulin staining in HEK-293T cells treated with MQIPs and four (NGN3, NKX6.1+MAFA, and PDX1) RNPs dCas9-VPR separately in (a) a single day, (b) every day for 3 days, or (c) every other day for five days. (d) Semi-quantitative measurements of fluorescence in previous images. Standard deviation is based on at least two images, and *: $p<0.05$, **: $p<0.005$, and ***: $p<0.0005$.

(a)	Before GSIS	After GSIS
PKA	1.91	3.62
MEK1	2.62	3.63
ERK1	2.40	3.35
Ezh2	2.23	2.58
PI3K	9.72	12.46
AKT	6.80	10.24
mTOR	4.30	9.27
P53	1.19	1.35
FOXO	1.24	3.55
Casp3	0.49	0.50

(b)

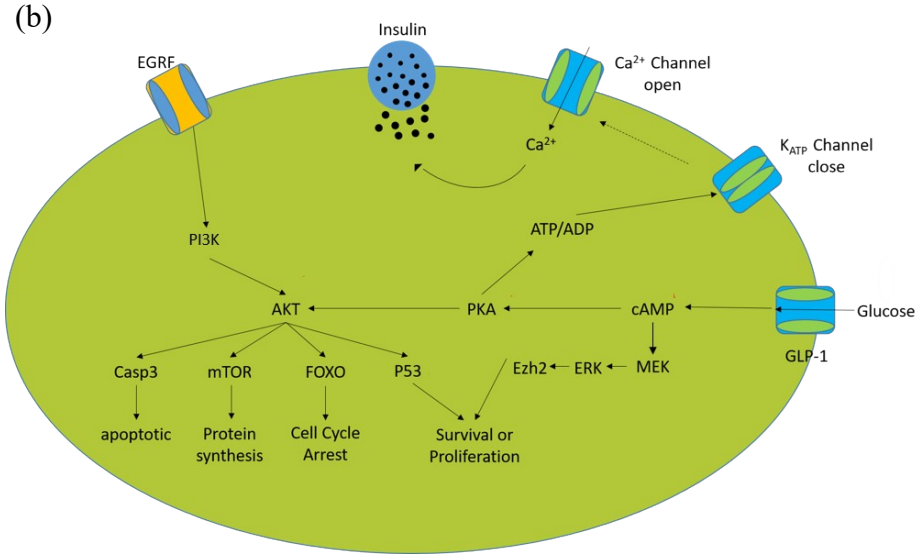

Figure S3. (a) Relative gene expression of proteins of insulin activated HEK-293T cells with MQIPs/RNPs in before and after glucose-stimulated insulin secretion (GSIS). Many genes responsible for cellular proliferation are concomitantly upregulated with insulin. (b) Schematic pathway of the glucose stimulation and gene expression.