Supramolecular Nanosubstrate-Mediated Delivery for Reprogramming and Transdifferentiation of Mammalian Cells

Shuang Hou, Jin-sil Choi, Kuan-Ju Chen, Yang Zhang, Jinliang Peng, Mitch A. Garcia, Jue-Hua Yu, Kaushali Thakore-Shah, Tracy Ro, Jie-Fu Chen, Parham Peyda, Guoping Fan,* April D. Pyle,* Hao Wang,* and Hsian-Rong Tseng*

Cellular machinery is governed by a complicated molecular circuitry consisting of a diverse range of genes that exhibit synergistic functions and interactions. Cell behaviors can therefore be modulated by introducing different genes that specifically perturb crucial effectors in the cellular circuitry.[1–8] Over the past few decades, many different approaches have been developed to deliver genes of interest into cells. For example, viruses have been the most commonly used carriers that can dependably deliver genes in vitro[9,10] and in vivo.[11,12] However, viral vectors raise safety concerns due to the risk associated with random insertions of viral DNA into the human genome.[13–15] To overcome these viral genomic integration issues, a broad collection of artificial nonviral vectors[7,8,16–22] has been engineered, yet the low delivery efficiency compromises their general utility in different cell types. Alternatively, physical delivery techniques,[23] such as microinjection,[24] electroporation,[25] continuous infusion,[26] sonication,[27] and gene gun,[28] were developed to directly introduce genes into cytoplasm. The problem arising from physical delivery techniques is the mechanical damage to the cells that may compromise their viability and interfere with cellular functions.[23] Overall, development of a highly efficient universal platform for gene delivery remains one of the major challenges in the field. In addition to the requirements of desired delivery performance, low toxicity, and minimal disruption to cell viability and functions, a promising technology must also be capable of multiple deliveries that sustains a steady supply of biomolecules over the duration of the desired biological process, e.g., direct conversion or reprogramming of somatic cells into different cell lineages.

Recently, we demonstrated a unique technology platform pioneering the concept of "supramolecular nanosubstrate-mediated delivery (SNSMD)" capable of highly efficient delivery of genes into a diverse array of mammalian cells.[29] SNSMD platform comprises of two functional components, including (i) Ad-SiNWS: adamantane-grafted silicon nanowire substrates and (ii) DNA⊂SNPs: supramolecular nanoparticle (SNP) vectors for encapsulation of DNA plasmid. The multivalent molecular recognition between the Ad motifs on Ad-SiNWS and the β-cyclodextrin (CD) motifs on DNA⊂SNPs leads to dynamic assembly and local enrichment of DNA⊂SNPs onto Ad-SiNWS. As cells settle onto the substrates, the intimate contact between the cell membrane and the nanowires on Ad-SiNWS facilitate the uptake of DNA⊂SNPs into the cells, resulting in highly efficient delivery of exogenous genes. In contrast to the existing nanosubstrate-mediated delivery approaches,[30,31] which must be regenerated after each single use, multiple deliveries of exogenous genes on the same batch of cells were realized with equivalent efficiency by sequential additions of the corresponding DNA⊂SNPs to the SNSMD-based gene delivery system. We attribute its unique performance to the combined use of nanostructured substrate, which enhances cell-surface

Dr. S. Hou, Prof. H. Wang
National Center for Nanoscience and Technology
Beijing, 100190, China
E-mail: wanghao@nanoctr.cn

Dr. S. Hou, Dr. J.-s. Choi, Dr. K.-J. Chen, Y. Zhang,
Dr. J. Peng, Dr. M. A. Garcia, T. Ro, Dr. J.-F. Chen,
P. Peyda, Prof. H.-R. Tseng
Department of Molecular and Medical Pharmacology
Crump Institute for Molecular Imaging (CIMI)
California NanoSystems Institute (CNSI)
University of California
Los Angeles, CA 90095-1770, USA
E-mail: hrtseng@mednet.ucla.edu

Dr. J. Peng
School of Biomedical Engineering
MED-X Research Institute
Shanghai Jiao Tong University
Shanghai 200030, China

Dr. J.-H. Yu, Prof. G. Fan
Department of Human Genetics
David Geffen School of Medicine
University of California Los Angeles
Los Angeles, CA 90095-7088
E-mail: gfan@mednet.ucla.edu

K. Thakore-Shah, Prof. A. D. Pyle
Molecular Biology Institute
Microbiology, Immunology, and Molecular Genetics
University of California Los Angeles
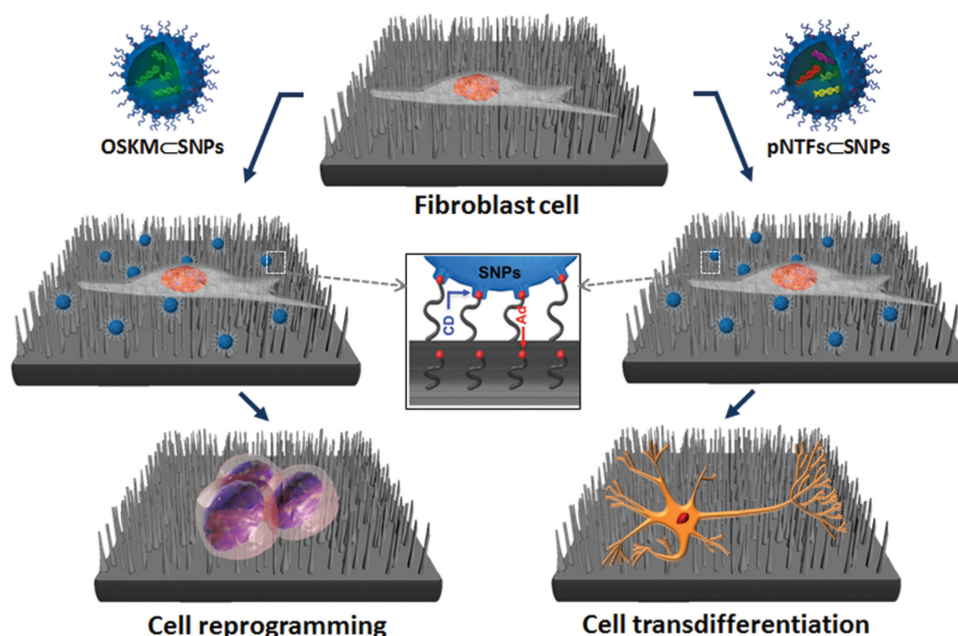Los Angeles, CA 90024, USA
E-mail: apyle@mednet.ucla.edu

Figure 1. Conceptual illustration of the operation mechanism of supramolecular nanosubstrate-medicated delivery (SNSMD) platform, and its applications for cell reprogramming and transdifferentiation.

interactions, and supramolecular chemistry based on Ad/CD recognition.[32,33]

The advent of reprogramming a somatic cell into human-induced pluripotent stem (iPS) cells has the potential to revolutionize regenerative medicine.[3,34] Standard reprogramming systems utilize viral expression of four crucial transcription factors (TFs): Oct4, Sox2, KLF4, and c-MYC (OSKM).[9,10,35] Equally important to the technology of induced pluripotency is the phenomenon of transdifferentiation, which coverts one cell lineage to the other without undergoing an intermediate pluripotent state or progenitor cell type. Research has demonstrated that fibroblast cells can be converted or transdifferentiated into induced neuronal like (iNl) cells via viral transfection, which introduces four neuron-specific transcription factors (NTFs, including Ascl1, Brn2, Myt1l, and NeuroD1).[36,37] Due to the use of viral vectors, the resulting iPS and iNl cells contain multiple viral integrations that raise safety concerns for cell-based therapy.[13–15,38] Further, the generation of both iPS and iNl cells require sequential and multiple delivery of the reprogramming genes in order to sustain a steady expression of these key factors over the duration of reprogramming and transdifferentiation. Given the advantages of SNSMD-based gene delivery system (i.e., no viral integration, high delivery efficiency, and sequential delivery), we demonstrate the feasibility of applying SNSMD platform for reprogramming and transdifferentiation of fibroblast cells (**Figure 1**).

We first demonstrated the feasibility of reprogramming human foreskin fibroblast (BJ) cells by performing multiple rounds of delivery of the OSKM plasmid with SNSMD platform. Similarly, this SNSMD platform is composed of two functional components: (i) Ad-SiNWS (prepared as previously described,[29] Figure S1, Supporting Information) and (ii) OSKM plasmid-encapsulated SNP vector (OSKM⊂SNPs) that is prepared by mixing OSKM plasmid with SNP building

blocks [i.e., cyclodextrin-grafted branched polyethylenimine (CD-PEI), adamantane-grafted polyamidoamine dendrimer (Ad-PAMAM), and Ad-grafted polyethylene glycol (Ad-PEG), as well as OSKM plasmid].[29,32] Cationic self-assembled hydrogel networks of CD-PEI and Ad-PAMAM, which are formed via Ad and CD recognition, can encapsulate DNA plasmids and form the cores of SNPs.[32] The capping/solvation reagent, Ad-PEG, constrains the growth of the OSKM-encapsulated hydrogel networks and simultaneously confers desired solubility and structural stability[33] to the resulting OSKM⊂SNPs with controllable sizes of ≈107 nm and surface charge of +16.7 meV (Figures S2–S4, Supporting Information). The multiround delivery protocol employed for BJ cell reprogramming is summarized in **Figure 2**a. BJ cells (5×10^5 cells mL^{-1}) were settled on Ad-SiNWS (1×2 cm), placed in each well of a two-well chamber slide (Lab-Tek). OSKM⊂SNPs (50 ng plasmid per well) in 1 mL of Dulbecco's modified Eagle's medium (DMEM) medium was added to each well on days 1, 3, 5, and 7. The treated cells were maintained in the same wells until termination of the studies on days 14 and 25. At day 14, the formation of cell clusters was observed. In contrast to the original BJ fibroblast cells that normally possess an elongated, stretched shape and grow separately (Figure 2b), cells in the clusters presented with round shape and gathered together (Figure 2c), suggesting their gaining of stem cell properties. Immunostaining was carried out on the clusters collected on day 25, and reprogramming of fibroblast cells was confirmed with the expression of embryonic markers. As shown in **Figure 3**, in addition to Oct4 and Sox2 which were delivered via SNSMD platform, the cells are also positive with Nanog,[39,40] a key regulator of embryonic stem cell self-renewal and pluripotency, and DNMT1 (DNA methyltransferase 1).[41] Nanog is known to be a downstream target of KLF4, Oct4, as well as Sox2[42] and DNMT1 expression is induced by Oct4 and
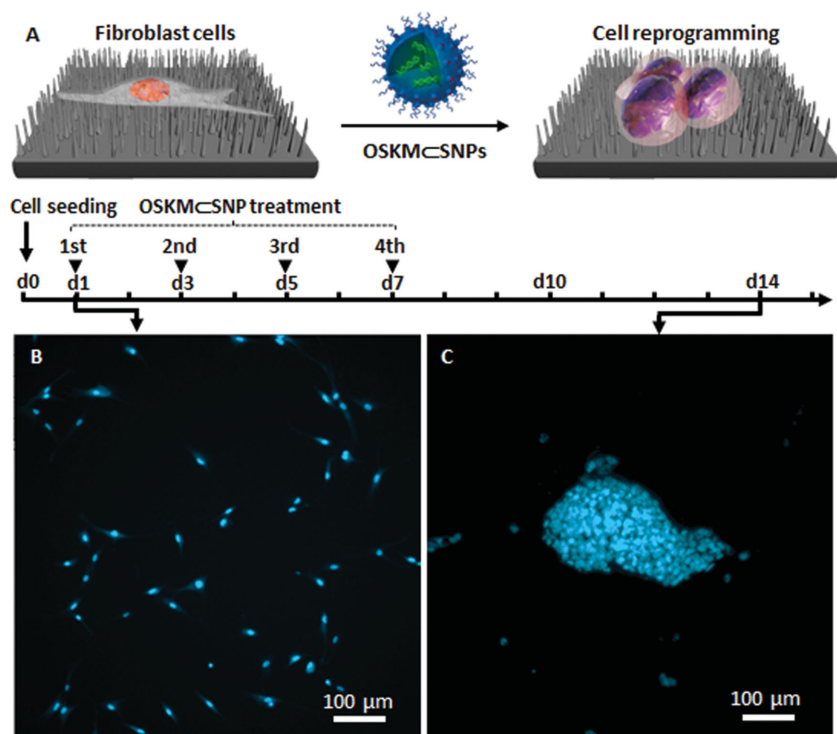
Figure 2. Cell reprogramming using SNSMD system. a) OSKM⊂SNP was treated four times to fibroblast cell (BJ) on SiNWS. Cells were visualized with DAPI staining (blue). Images of cells were taken at b) day 1 and c) day 14.

Nanog.[41] These findings indicated activation of stem cell-related pathways in the cluster cells and showed the feasibility of SNSMD platform's to reprogram the somatic cells. In addition, the biocompatibility of the SNSMD platform was examined. As shown in Figure S5, Supporting Information, the SNSMD-based gene delivery platform exhibited comparable biocompatibility compared to control groups. In the long term biocompatibility test with multiple treatments of DNA⊂SNPs (Figure S6, Supporting Information),

the SNSMD system showed decreased cell viability because of the slow proliferation rate on SiNWS[43] but still possessed a high live cell population.

On top of the fibroblast reprogramming study, we continued to exploit the utility of SNSMD platform for transdifferentiation of fibroblasts cells (human dermal fibroblasts, HDF) into iNl cells. In this case, four different NTF plasmids, including Ascl1, Brn2, Myt1l, and NeuroD1,[36,37] were encapsulated with SNP vector to make pNTFs⊂SNPs. The protocol employed for generating human iNl cells from human fibroblast cells is summarized in **Figure 4**a. After HDF cells settled on Ad-SiNWS in chamber slides for 24 h, pNTFs⊂SNPs (125 ng plasmid mL^{-1} for each NTF) in 1 mL of DMEM medium were added to individual chambers every 24 h for the first four days and every 48 h for the following six days in order to sustain a steady supply of the four NTFs over the conversion process. The cell fate conversion was assessed by immunofluorescence staining for neural stem cell- and neuron-specific proteins (i.e., Nestin: type VI intermediate filament (IF) protein, Tuj1: neuron-specific class III beta-tubulin, Map2: microtubule-associated protein 2, and NeuN: neuronal nuclei). We observed that around 30%–50% of Ad-SiNWS-immobilized cells expressed the neuron-specific markers, Tuj1 and Map2, at day 15 (Figure 4b, Figures S7 and S8, Supporting Information). Among the Tuj1- or Map2-positive cells, about 10% of the cell population expressed a neural stem cell marker, Nestin, and mature neuron markers, NeuN (Figure 4b, Figures S7 and S8, Supporting Information). In addition to the biochemical characterization of neuronal markers, dramatic morphological changes from elongated stretched fibroblasts to dendritic neurons were also observed. Furthermore, we carried out real-time PCR measurement to quantify the integration of exogenous plasmid DNA, one of the major drawbacks of viral vector, after the process of SNSMD-based gene delivery (Figure S9, Supporting Information). The results showed undetectable DNA integration among the transdifferentiated cells.

In order to evaluate whether the iNl cells could maintain the neuronal identity without additional pNTFs⊂SNPs, at day 13, the iNl cells were released from Ad-SiNWS and plated on PO/FN (poly-l-ornithine/fibronectin)-treated plates and cultured for additional four days (**Figure 5**). Until day 15, the replated iNl cells preserved their typical neuronal
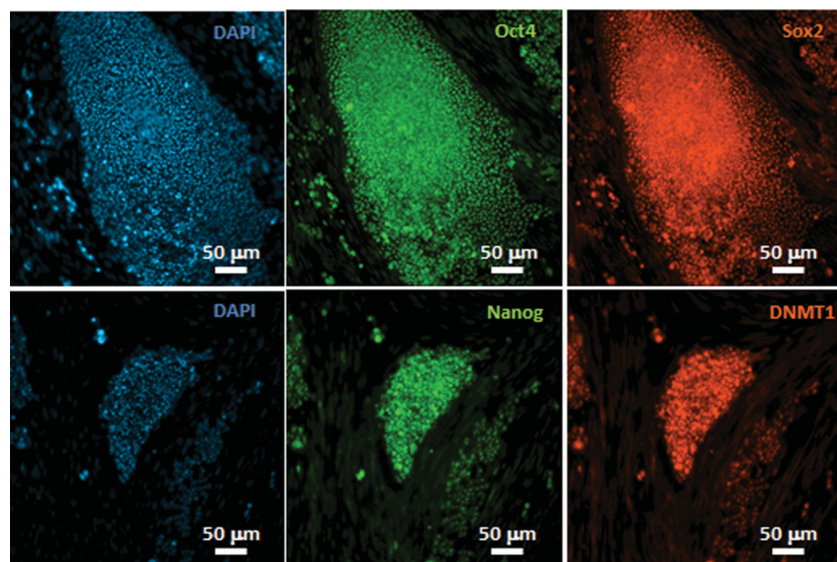

Figure 3. Immunostaining images of reprogrammed cells. The reprogrammed cells were stained with DAPI and stem cell-specific markers (i.e., Oct4, Sox2, Nanog, and DNMT) at day 25.
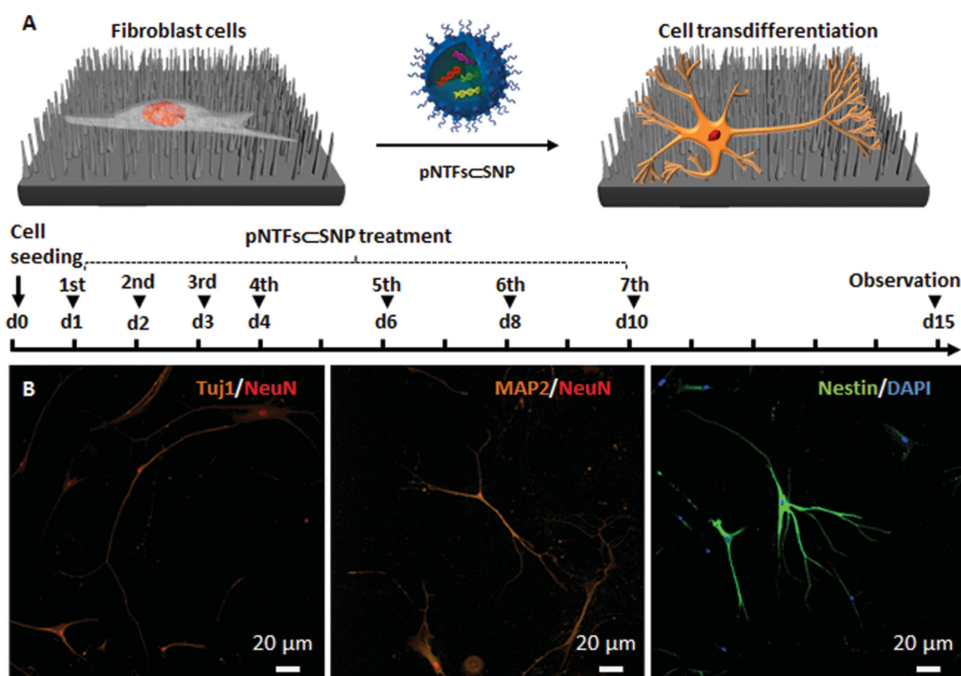
Figure 4. a) Graphic illustration and microscopic images depicting the transdifferentiation of fibroblast cells (HDF) on Ad-SiNWS in the presence of pNTFs⊂SNPs containing the four pNTFs (i.e., Ascl1, Brn2, Myt1l, and NeuroD1). A designated timeline summarizes the seven sequential treatments of pNTFs⊂SNPs, sustaining a steady supply of four NTFs over the transdifferentiation process of 15 days. b) Microscopic images of reprogrammed iNl cells. The iNl cells were immunostained for four neural-cell lineage markers (i.e., Tuj1, Map2, Nestin, and NeuN). Each merged image was processed with the pseudocolor based on images obtained from a monochrome CCD camera and the respective filter sets.
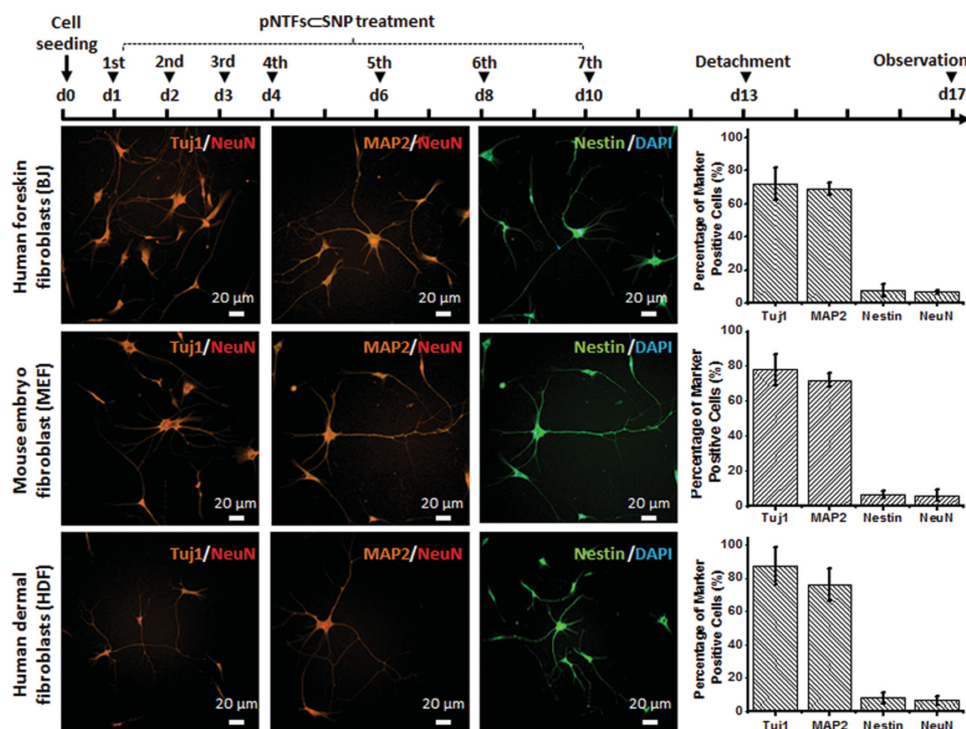
Figure 5. Cell transdifferentiation with various cell lines (i.e., HDF, BJ, and MEF) into iNl cells. After pNTFs⊂SNPs treatment, at day 13, iNl cells were released from Ad-SiNWS and plated on PO/FN treated plates for an additional four days. The iNl cells were immunostained for four neural-cell lineage markers (i.e., Tuj1, Map2, Nestin, and NeuN). The percentages of corresponding specific marker-positive cells were also presented to show the conversion efficiency. Each merged image was processed with the pseudocolor based on images obtained from a monochrome CCD camera and the respective filter sets.

morphology and the stable expression of neural stem cell-specific protein (Nestin) and neuron-specific proteins (i.e., Tuj1, Map2, and NeuN), similar to the iNl cells on substrates that continued to receive gene delivery (Figure 5). Additionally, other cell lines including BJ and mouse embryo fibroblast (MEF) also showed the characteristic neuronal morphology after being treated with pNTFs⊂SNPs and transferred to PO/FN treated plates. All types of cells expressed Tuj1 and Map2 in more than 70% of total cells. Nestin and NeuN were also expressed in around 5%–10% of total cells. Taken together, these results indicated SNSMD platform can effectively deliver NTFs to convert fibroblast into iNl cells.

In summary, we explored the use of SNSMD platform as an efficient transfection system to deliver various genes into target cells. Featuring the combination of Ad-SiNWS with specific DNA plasmid encapsulated by SNP vectors, SNSMD platform has high stability, minimal cellular toxicity, undetectable gene integration, and the capability of sequential delivery that sustains a steady supply of biomolecules over the duration of direct reprogramming of somatic cells into different cell lineages. By performing direct reprogramming of fibroblasts into a stem cell-like state and inducing transdifferentiation of fibroblasts into iNl cells, we demonstrated the feasibility and robustness of SNSMD platform. Our system is still limited in releasing reprogrammed cells from SiNWS after two weeks of culturing the reprogrammed cells on SiNWS. In a future study, we will explore cell reprogramming/transdifferentiation on thermoresponsive chips[44] which can change hydrophobicity depending on temperature, leading to an easy release of cells from chips. Ultimately, we expect this platform to provide a superior system for the applications in regenerative medicine and related researches.

Experimental Section

Synthesis of OSKM⊂SNPs: A self-assembly procedure was employed to prepare OSKM⊂SNPs. 2-μL DMSO solution containing Ad-PAMAM (3.96 μg) was added into a 600-μL PBS mixture with OSKM plasmid (500 ng), Ad-PEG (10.56 μg), and CD-PEI (9.0 μg). The resulting mixture was stirred vigorously to obtain OSKM⊂SNPs. The sizes (≈100 nm) of OSKM⊂SNPs were characterized by dynamic light scattering (DLS) and transmission electron microscope (TEM) (Figures S2–S4, Supporting Information).

Reprogramming of Fibroblast Cells: 5×10^5 fibroblast cells (BJ) were introduced into each well of a two-well chamber slide (Lab-Tek), in which a 1×2 cm^2 Ad-SiNWS was placed in the bottom of the chamber. Ad-SINWS was coated with MatriGel (0.1 mg mL^{-1}, BD Biosciences, USA) to increase its biocompatibility. After the cells fully attached onto Ad-SiNWS (for 24 h), the chambers were washed with PBS and refilled with fresh cell culture medium (DMEM). OSKM⊂SNPs (50 ng plasmid mL^{-1}) were introduced into individual chambers at days 1, 3, 5, and 7.

Synthesis of NTF Plasmid-Encapsulated Supramolecular Nanoparticles (pNTFs⊂SNPs): A self-assembly procedure was employed to prepare pNTFs⊂SNPs. 2-μL DMSO solution containing Ad-PAMAM (3.96 μg) was added into a 600-μL PBS mixture with NTF plasmid (125 ng for each NTF), Ad-PEG (10.56 μg),

and CD-PEI (9.0 μg). Four plasmids in a mixture were coencapsulated in the SNP vector. The resulting mixture was stirred vigorously to obtain pNTFs⊂SNPs. The sizes (≈100 nm) of pNTFs⊂SNPs were characterized by DLS and TEM (Figures S2–S4, Supporting Information).

Transdifferentiation of Fibroblast Cells into iNl Cells: 5×10^5 fibroblast cells were introduced into each well of a two-well chamber slide (Lab-Tek), in which a 1×2 cm^2 Ad-SiNWS was placed in the bottom of the chamber. Ad-SiNWS was coated with MatriGel (0.1 mg mL^{-1}, BD Biosciences, USA) to increase its biocompatibility. After 24 h, the chambers were washed with PBS and refilled with fresh cell culture medium (DMEM). pNTFs⊂SNPs (125 ng for each plasmid mL^{-1}) were introduced into individual chambers at days 1, 2, 3, 4, 6, 8, and 10.

Supporting Information

Supporting Information is available from the Wiley Online Library or from the author.

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