Generation of murine induced pluripotent stem cells by using high-density distributed electrodes network

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This study reports a robust method of gene transfection in a murine primary cell model by using a high-density electrodes network (HDEN). By demonstrating high cell viability after gene transfection and successful expression of transgenes including fluorescent proteins, the HDEN device shows great promise as a solution in which reprogramming efficiency using non-viral induction for generation of murine induced pluripotent stem cells (iPSCs) is optimized. High and steady transgene expression levels in host cells of iPSCs can be demonstrated using this method. Moreover, the HDEN device achieved successful gene transfection with a low voltage of less than 180 V while requiring relatively low cell numbers (less than 1.5×10^4 cells). The results are comparable to current conventional methods, demonstrating a reasonable fluorescent-plasmid transfection rate (42.4% in single transfection and 24.5% in triple transfection) and high cell viability of over 95%. The gene expression levels of each iPSC factor was measured to be over 10-fold higher than that reported in previous studies using a single mouse embryonic fibroblast cell. Our results demonstrate that the generation of iPSCs using HDEN transfection of plasmid DNA may be a feasible and safe alternative to using viral transfection methods in the near future. © 2015 AIP Publishing LLC. [<http://dx.doi.org/10.1063/1.4930866>]

I. INTRODUCTION

Pluripotent embryonic stem cells (ES cells), which have unique properties such as differentiation into a wide variety of cell types, self-renewal, and unlimited replication, were first derived from mouse embryos.^{1,2} Later on, human ES cells were also reported to possess the same functionality and properties.³ Although ES cells have been useful in human developmental study, drug discovery, and transplantation medicine, the applications of using ES cells for medical research are still controversial due to many ethical concerns, which requires the destruction of human embryos to obtain ES cells.^{4,5}

In 2006, induced pluripotent stem cells (iPSCs) was first derived from mouse somatic fibroblast cells with a defined set of transcription factors (OCT3/4, SOX2, KLF4, and c-MYC, also referred as OSKM) through a re-programming process.⁶ Later, human iPSCs were also generated from somatic cells with the same set of transcription factors⁷ and by other defined

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reprogramming factors (OCT4, SOX2, NANOG, and LIN28).⁸ These reprogramming factors led somatic cells to become ES-like cells in morphology and characteristics. In recent years, several studies have proven that the functionality of iPSC is very similar to ES cells such as the gene expression in epigenetic state,⁹ *in vitro* differentiation,^{10,11} and the ability to form teratomas in immune-deficient mice.¹²

These iPSCs have potential to differentiate into functional cells and may provide a useful model for therapeutic applications.^{13–15} However, as the iPSCs are formed by a process which involves either retroviral or lentiviral vectors being integrated into genome of host cells, it may alter genomic construction. Therefore, integrated viral-induced iPSCs, though with a high efficiency, are not suitable for therapeutic applications. While considering the safety issues to iPSCs, non-viral generation of iPSCs is a relatively safer approach to avoid genomic insertion of viral vectors.¹⁶

Use of traditional electroporation devices usually results in relatively low transfection rate and poor cell viability for mammalian cell lines¹⁷ and primary cells.¹⁸ Furthermore, hundreds of volts of electricity is commonly used in the traditional gene transfection process, which raises safety concerns to manipulators. Recent study also suggested that the buffer composition and local pH change, creating strongly acidic or basic environment around the cells during electroporation,¹⁹ might result in a destructive effect on cells near the electrodes, and led to low cell survival rate after gene transfection. Therefore, there is a great need to develop a safe and high-efficiency approach for generation of iPSCs.

Recently, a high-density distributed electrode network (HDEN) device based on tri-phase electric stimulation, which can control the local pH change, has been demonstrated for gene transfection with promising viability and transfection rate.²⁰ Compared with the single-phase electroporation mode, the tri-phase stimulation mode generates a relatively uniform electric field covering the entire electroporation region. The HDEN achieves relatively low biomaterial consumption for a wide range of sample volumes ranging from 20 μ l to 300 μ l with a 96-well plate, and has flexibility for bio-molecular delivery in different cell types including mammalian cell lines and primary cells. In this study, we showed a high cell survival rate and stable gene expression with a relatively low sample ($<1.5 \times 10^4$ cells) or reagent consumption (only 2 μ g DNA) for the generation of murine iPSCs. With the non-viral induction and the HDEN gene transfection approach, we demonstrated a promising way to obtain safe (non-viral alternation) iPSCs as therapeutic materials in a relatively high efficient approach.

II. MATERIALS AND METHODS

A. Cell culture

Mouse embryonic fibroblasts (MEFs) were selected as the model for induction of iPSCs and were cultured in Dulbecco's modified Eagle medium (DMEM, 500 ml, 11995-065, Life Technologies Co., USA), supplemented with 10% (50 ml) fetal bovine serum (10091-148, Life Technologies Co., USA), 0.1 mM (5 ml) Minimum Essential Media (MEM) non-essential amino acids solution (11140-050, Life Technologies Co., USA), 2 mM (5 ml) L-glutamine (25030-081, Life Technologies Co., USA), 0.1 mM (1.3 μ l) 2-mercaptoethanol (M3148, Sigma-Aldrich, Inc., USA), and 0.06 mg/0.1 mg (5 ml) penicillin/streptomycin (L0010, Biowest, Inc., France). For collection of cells, cell culture medium was first removed, and MEFs were washed twice with 5 ml $1\times$ phosphate buffer saline (PBS), detached by using 0.025% (w/vol) trypsin-ethylenediaminetetraacetic acid (EDTA) (X0930, Biowest, Inc., France) at 37 °C for 1 min, and re-suspended in the cell culture medium to stop the action of trypsin. Cell pellets were then collected by centrifugation at 1000 rpm for 5 min, and re-suspended in an electroporation buffer composed of 25 mM KCl, 0.3 mM KH₂PO₄, 0.85 mM K₂HPO₄, 36 mM myo-inositol (Sigma-Aldrich, Inc., USA, pH 7.2, conductivity 3.5 mS/cm at 25 °C).

B. HDEN-based transfection

Three plasmids, including EGFP (enhanced green fluorescent protein), DsRED (*Discosoma* sp. red fluorescent protein), and ECFP (enhanced cyan fluorescent protein) (Clontech Co., USA),

were used for quantification of transfection efficiency, as schematically shown in Figure 1(a), which was determined by counting the number of cells expressing fluorescence under a fluorescent microscope (BX43F, Olympus Co., Japan). Murine *oct3/4*, *klf4*, and *sox2* cDNAs (complementary DNAs) were encoded and connected in the same order and inserted into a pCX plasmid (pCX-OKS-2A, 19771, Cambridge, MA, USA) such that they could be transfected and expressed simultaneously. Note that murine *c-myc* cDNA was also inserted into the pCX plasmid (pCX-*cmyc*, 19772, Cambridge, MA, USA), as shown in Figure 1(b).

Detailed information about the HDEN device (Etta Biotech Co. Ltd., China) and its optimal design could be found in our previous work.²⁰ Briefly, the microelectrodes were arranged as a hexagonal cellular array. They consisted of a printed circuit board (PCB) and bio-compatible stainless-steel acupuncture needles, as shown in Figures 2(a) and 2(b). Note that the upper part of the electrode was measured to be 1.9 cm. 1.4 cm of the needles was immersed in cell suspension for applying the required electric field. Totally 57 of needles were used in a PCB with dimensions of 2 cm × 2 cm. In order to provide the optimal (maximum) distribution of the electric field in a round area (in the 96-well plate) during the electroporation, a hexagonal arrangement (57 electrodes) of the electrodes was adopted in the previous work such that they may fill most of the electroporation area. Note that a higher density of the electrodes could cover more electroporation area. However, the density of the electrodes cannot be too high such that the short circuit may occur. It is also limited by the size of PCB. The electrodes were connected to a power supply with three polarities through patterned wires on the PCB, which were compatible with a single well of the traditional 96-well plate. Tri-phase electric stimulation mode was adopted in this work and the control of the power supply was through wireless communication. The HDEN device was operated at voltages ranging from 90 to 270 V with tri-phase stimulation for 6 pulses. The duration and interval of the operating electroporation pulse was 100 μ s, and 2 s, respectively. The procedure of the HDEN gene transfection is illustrated in Figure 2(c). After MEF pellets were collected by centrifugation at 1000 rpm for 5 min, and re-suspended in the electroporation buffer, the cell concentration was adjusted to be 6×10^5 cells/ml for MEFs experiments. Note that each plasmid was mixed with the cell suspension (100 ng/ μ l) prior to HDEN-based transfection. Twenty microliters of cell suspension mixed with DNA was then added into a 96-well plate, and used for gene transfection by using the HDEN device.

C. Cell viability

The viability assay was carried out on MEFs after gene transfection. Calcein acetoxymethyl ester (CaAM, C3099, Life Technologies Co., USA) and propidium iodide (PI, P3566, Life

FIG. 1. Schematic illustration of plasmids used in HDEN-based electroporation with MEFs. (a) Three different fluorescent protein DNA encoded plasmids were used to determine the transfection efficiency. (b) The reprogramming factor genes (*oct4*, *sox2*, *klf4*, and *c-myc*) in two separated plasmids were used to verify the transfection efficiency and stability after transfection.

FIG. 2. (a) Cross-sectional view and (b) top view of the HDEN gene transfection device; (c) a schematic illustration of procedures to transfect OSKM plasmids into MEFs.

Technologies Co., USA) were used to stain cells such that one may count alive and dead cells, respectively. Total MEFs were collected after HDEN-based transfection and mixed with CaAM (10^5 -fold dilution from 1 mg/ml stock, 400 μ l) and PI (400-fold dilution from 1 mg/ml stock, 400 μ l) at room temperature for 10 min. Cell viability and cell counting were then analyzed by using flow cytometry (Accuri C6, BD Biosciences, USA).

D. Transfection efficiency and expression of iPS factor genes

MEFs transfected with the above-mentioned three kinds of plasmids encoding fluorescence genes (EGFP, DsRED, and ECFP) were cultured on sterile glass slides (154534, Thermo Fisher Scientific, Inc., USA) with cell culture medium. On the second day, the glass slides were sealed and observed under an optical microscope. The transfection efficiency was then quantified in a whole field (100 $\times$ magnification) as follows:

$$\frac{\text{total fluorescence} - \text{expressing cells in dark field}}{\text{total cells in bright field}} \times 100\%. \quad (1)$$

Similarly, total RNA of MEFs transfected with four factor genes, including pCX-OKS-2A and pCX-cmyc (OSKM) plasmid DNAs, was collected from 24 h to 96 h by performing RNA extraction following the procedures of TRIzol RNA Isolation Reagents (15596-018, Life Technologies Co., USA), and by using the SuperScript III First-Strand Synthesis SuperMix kit (18080-400, Life Technologies Co., USA). Note that cDNA was synthesized from total RNA and used as index for gene expression. Detection of OSKM was accomplished by using cDNA and the StepOnePlus Real-Time PCR Systems (4376600, Life Technologies Co., USA). The detail information for evaluating the efficiency of gene expression could be found in previous works^{21,22} and then calculated by using following equations:

$$\Delta CT = CT_{\text{transfected gene}} - CT_{\text{internal control gene}}, \quad (2)$$

$$\frac{2^{-\Delta CT}, \text{ Fold of gene expression}}{\text{Total cell number}} = \text{Average gene expression/Single cell}, \quad (3)$$

$$\frac{\text{Average gene expression in experiment group}}{\text{Average gene expression in control group}} = \text{Efficiency of gene transfection}, \quad (4)$$

where CT (threshold cycle) was the calculated fractional cycle number at which the PCR product crossed a threshold value of detection. ΔCT indicated the change in RNA transcription

caused by OSKM transfection, and negative ΔCT indicated an upregulation of the gene's transcription following OSKM transfection, as shown in Equation (2). Note that the transfected genes were OSKM, and the internal control gene was a house-keeping gene. Herein, we chose murine beta actin as the internal control. Gene expression could be then quantified by ΔCT , as shown in Equation (3). Finally, efficiency of gene transfection in this study could be obtained by comparing average gene expression in the experimental group (OSKM transfected MEFs) to average gene expression in the control group (nil MEFs), as shown in Equation (4).

III. RESULTS AND DISCUSSION

A. Morphology and viability of MEFs after HDEN transfection

In order to optimize the operating conditions for MEFs in a healthy status with a high transfection efficiency, a series of applied voltages ranging from 90 V to 270 V were tested. Nil MEFs, which was a control group, were cultured on the glass slide to compare with experimental groups, as shown in Figure 3(a). MEFs maintained normal cell morphology when transfection was carried out at low voltages (Figures 3(b)–3(d)), while morphology deformation occurred at 180 V (Figure 3(e)). It was also observed that some cells detached from the glass slide surface at applied voltages ranging from 210 to 270 V, as shown from Figures 3(f)–3(h). It indicated that the HDEN device successfully achieved low-voltage transfection (<180 V) for MEFs, which maintained normal cell morphology.

Viability of MEFs after gene transfection was further determined by flow cytometry with CaAM and PI double staining. Figure 4(a) shows that the MEFs were selected for analysis. CaAM dye was used for alive MEFs staining and a threshold value was defined by nil MEFs, as shown in Figure 4(b-1). The fluorescent CaAM signal could be linked to alive MEFs specifically, as dead cells were not stained by CaAM dye, as shown in Figures 4(b-2) and 4(b-3), respectively. Similarly, PI dye was used to stain the dead MEFs while nil MEFs were selected as a threshold setting, as shown in Figure 4(c-1). PI staining was also specific to dead MEFs (Figure 4(c-2)) and signals from live cells were not detected, as expected (Figure 4(c-3)).

The control group for MEFs with CaAM and PI double-staining was shown from Figures 5(a-1)–5(a-3). With nil MEFs as the background signal, CaAM and PI specifically stained alive and dead cells, respectively, without significant noise interference. After HDEN-based transfection, viability of each sample group was observed to be over 95% without any detection of dead cells. However, only a small group of cells were detected in CaAM and PI double positive signals, indicating that the cell status was alive but damaged after electroporation. The results have been shown from Figures 5(b-1)–5(b-7). The survival rate of MEFs after transfection measured by using conventional equipment such as the Gene Pulser MXcell Electroporation System (Bio-Rad Laboratories, Inc., USA)²³ and the Neon Transfection System (Life Technologies Co., USA)²⁴ were reported to be about 70%–80%. The experimental results showed that HDEN-based transfection has been successful in achieving higher cell viability than traditional electroporators.

B. HDEN transfection efficiency

Fluorescence observation was further performed after 24 h of HDEN-based gene transfection (Figure 6). It showed that MEFs were healthy in cellular morphology. Furthermore, multiple fluorescent proteins in a single cell were successfully observed, as shown from Figures 6(a)–6(d). Transfection efficiency was further quantified with fluorescence counting and the data have been summarized in Table I. The highest transfection rate in both single- and triple-plasmid DNA occurred at an applied voltage of 150 V, which thereby implies a suitable condition for HDEN-based gene transfection with MEFs. The transfection rate was calculated to be 9.6%–42.4% in single-plasmid DNA transfection for DsRed, EGFP, or ECFP, respectively. The transfection rate of MEFs from previous works has been reported to be 49.6% in chemical transfection (four times of consecutive transfections for 10 days)¹⁶ and 87.8% in viral-induced transfection (single infection for two days).¹⁶ Note that the viral-induced transfection may cause genomic insertion

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FIG. 3. Morphological observation of MEFs after 24 h electroporation. MEFs maintained normal morphology from (a) control group without electroporation, (b) 90 V, (c) 120 V, to (d) 150 V, and then lost normal morphology in the applied voltage of (e) 180 V, and started to de-attach from the surface in the voltages of (f) 210 V, (g) 240 V, and (h) 270 V.

of viral vectors into the host cells.¹⁶ The HDEN-based gene transfection achieved a transfection rate of 42.4% with single-plasmid DNA, which was lower than the viral transfection but nearly comparable with the chemical transfection. Note that the HDEN-based gene transfection was able to guarantee successful gene delivery with a relatively high cell viability. Another unique

FIG. 4. Viability of MEFs was determined by flow cytometry to distinguish the alive and dead cells. (a) MEFs were selected for analysis in P1 group. (b-1) The control group of Nil MEFs was used for threshold setting; (b-2) alive MEFs should be stained with CaAM specifically; (b-3) dead MEFs were not stained with CaAM. (c-1) The control group of Nil MEFs was used for threshold setting; (c-2) PI dye could be specifically stained to dead cells; (c-3) CaAM dye did not interfere with the collection of PI signals.

feature of the HDEN-based gene transfection is the relatively high co-transfection rate with high cell viability. With co-transfection of above three-plasmid DNA, approximate 5.8%–24.5% of cells could be transfected simultaneously. It is the first time that three kinds of exo-gene vectors have been transfected into MEFs via HDEN. Note that the transfection efficiency was determined by quantifying the transcription factors in a cell, and the gene copy number of exo-genes. When multiple exo-genes were sent into a single cell, each gene expression rate might be reduced due to the limitation of transcription factors. Therefore, it is reasonable that gene efficiency of co-transfection was lower than that of single transfection in the same group.

C. Expression of iPSC factor genes OSKM in MEFs

An applied voltage of 150 V was used to perform the transfection experiments. Four iPSCs factor genes OSKM encoded in pCX-OSK-2A and pCX-cmyc, respectively, were further successfully transfected into MEFs by using the HDEN-based gene transfection. These transgenes were steadily expressed in MEFs for at least a period of 96 h with a single electroporation procedure. The highest gene expression of murine *oct4*, *sox2*, and *klf4* was experimentally found to be at 48 h, as shown in Figure 7. High expression of both murine *oct4* and *sox2* has been observed in murine ES cells by using traditional approaches⁸ and were also observed in transfected MEFs by using HDEN-based gene transfection in this study.

To demonstrate the capability of the HDEN device as a potential way of iPSCs induction, the efficiency of transgene expression of single MEFs was calculated and has been presented in Table II. Relatively high expression of murine *oct4* and *sox2* genes in the group of iPSCs has been observed in this work. In the previous study, gene expression level was about $25\text{--}10^4$ of murine *oct4* and *sox2* in a group of MEFs.^{25,26} Our experimental results showed that a single MEFs achieved over 10^5 in gene expression level of murine *oct4* and *sox2*, as shown in Table II. It also demonstrates that the application of the HDEN device achieved higher gene expression level in single host cell.

FIG. 5. Viability of MEFs after HDEN electroporation was determined by PI and CaAM double staining. Four regions were defined as upper right (UR), upper left (UL), lower left (LL), and lower right (LR), respectively. (a-1) Nil MEFs were used to set a threshold value at the LL region; (a-2) alive cells could be stained with CaAM at the LR region; (a-3) dead cells were successfully detected at the UL region. After electroporation while applied with voltages ranging from (b-1) 90 V, (b-2) 120 V, (b-3) 150 V, (b-4) 180 V, (b-5) 210 V, (b-6) 240 V, to (b-7) 270 V, most of MEFs were alive without detection of dead cells. A small group of cells, in a small percentage less than 3.0% at the UR region, were detected by CaAM (which meant alive cells), and detected by PI as well (which meant that the cell membrane was broken when the cell was dead or damaged). It indicated that these small group of cells were alive but might be damaged after electroporation.

FIG. 6. A MEF cell was successfully transfected with three plasmids, expressing three kinds of fluorescence including (a) ECFP, (b) EGFP, and (c) DsRed. (d) A merged image in a bright field.

TABLE I. Transfection efficiency of MEFs by using HDEN-based gene transfection.

Applied voltage (V)	EGFP-N1 (%)	ECFP (%)	DsRed (%)	EGFP-N1 + ECFP + DsRed (%)
90	18.8 $\pm$ 4.8	25.2 $\pm$ 6.9	24.5 $\pm$ 4.7	13.7 $\pm$ 3.8
120	27.1 $\pm$ 3.5	33.1 $\pm$ 4.1	24.5 $\pm$ 4.7	21.8 $\pm$ 1.1
150	42.4 $\pm$ 4.7	35.7 $\pm$ 0.4	36.5 $\pm$ 5.5	24.5 $\pm$ 1.6
180	19.6 $\pm$ 4.9	23.9 $\pm$ 6.5	23.2 $\pm$ 3.4	13.3 $\pm$ 4.2
210	19.2 $\pm$ 7.3	9.6 $\pm$ 3.6	13.5 $\pm$ 6.1	5.8 $\pm$ 1.2
240	14.5 $\pm$ 5.1	13.1 $\pm$ 3.9	24.1 $\pm$ 3.1	6.3 $\pm$ 1.5

FIG. 7. Detecting murine iPSC factor genes, OSKM, in MEFs with HDEN, and transgene stability with time elapse from 24 h to 96 h. Note that fold change represented quantification of gene expression. Murine embryonic stem cells (MES), highly expressed OSKM, were used as the positive control of OSKM detection. The highest ex-gene expression was detected in murine *oct4* and *sox2* at 48 h, and all OSKM genes were successfully expressed in MEFs.

TABLE II. OSKM gene expression efficiency of MEFs by using HDEN-based gene transfection.

	Murine <i>oct4</i>	Murine <i>sox2</i>	Murine <i>klf4</i>	Murine <i>cmyc</i>
MEF Nil	1.00	1.00	1.00	1.00
MEF OSKM 24 h	4.54×10^4	1.27×10^5	1.56×10^2	1.09×10^2
MEF OSKM 48 h	2.48×10^5	6.72×10^5	8.61×10^2	31.06
MEF OSKM 72 h	2.95×10^3	4.47×10^3	46.15	23.72
MEF OSKM 96 h	5.04×10^2	4.18×10^3	6.05	8.10

IV. CONCLUSIONS

This study demonstrated the capability of the HDEN devices in non-viral induction of murine iPSCs. The HDEN device was successfully used to express three kinds of fluorescent proteins in a single cell with reliable efficiency and high cell viability. We also presented the delivery of iPSC factor genes with a single electroporation. Steady transgene expression in host cells with a relatively small consumption of cells ($<1.5 \times 10^4$ cells). Combining the HDEN device with plasmid DNA might be a promising approach for virus-free and highly efficient induction of iPSCs for safe gene therapy in the near future.

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