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Original

Utility of a Single Particle Isolation System in Genome-editing and Gene-transfection Experiments

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SUMMARY

In recent decades, genetic engineering and its application to medical sciences has been rapidly progressing, and gene transfection and genome-editing techniques are generally used even in medical investigations. Although establishing single-cell clones are essential for confirming the cellular phenotypic changes in association with genetic modifications, it is technically hard to dispense single-cell efficiently and correctly. Here, we demonstrate single-cell cloning of genetically modified lung cancer cells using a single particle isolation system, On-chip SPiS, by which bi-allelic gene-knockout cells and highly gene-expressing transfectants can be easily and certainly established. In our experiment, we obtained one bi-allelic knockout clone from only 12 clones (8.3%) and eight highly transgene-expressing clones from 20 clones (40%). Furthermore, we could define that considerable rate of clones without transgene were mixed among selection marker-resistant cell populations. These results suggest that the single-cell dispensing system is a quite useful tool for single-cell cloning in genome-editing and gene-transfection experiments.

Key words : Single particle isolation system, Genome-editing, Gene-transfection, Single-cell cloning

INTRODUCTION

Genome-editing and gene-transfection technologies play important roles in recent advancement in molecular biology. Since these methods can freely modify or add genes in cultured cells, we can precisely investigate the function of specific genes of interest. The most commonly used genome-editing method is a clustered regularly interspaced short palindromic repeats (CRISPR)-Cas9 system. It is known that recombinant Cas9 protein and synthesized guide RNA (gRNA), which is short RNA optimized to oligonucleotide sequences of target gene, can induce mutations efficiently[1)]. In general, Cas9 cDNA is transiently transfected using plasmid-based expression vectors. Since expression vectors contain selection marker antibiotics-resistant genes, transfected cells could be selected by culture media containing appropriate concentration of selection marker antibiotics. In case of transient transfection, expression of transferred cDNA- and selection marker antibiotics-resistant

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gene-derived molecules is limited to several days. Therefore, it is enough for transfected cell selection to culture in selection marker antibiotics-containing media for a short period. On the other hand, stable gene transfection is usually conducted by virus-based expression vectors[2], and it is usually necessary for some weeks of selection marker antibiotics treatment to obtain stable transfectants.

Gene transfection are powerful tools to investigate the function of transfected genes especially in tumor biology and developmental biology. However, investigators have often experienced difficulties obtaining transfectants stably expressing inserted gene products. There are various reasons for the difficulties obtaining enough levels of transgene-expressing transfectants, e.g., growth suppressing function of transgene products, low transformation efficacy, and existence/appearance of selection marker antibiotics-resistant cells. As a methodology for overcoming these issues, single-cell sorting is an essential technique for establishing genome-edited cell clones and gene-transfected cell clones.

Single-cell sorting is generally conducted by a limiting dilution method using 96-well microplates ; cultured cells are commonly diluted to 5 cells/mL, and seeding an average of 0.5 cells/well ensures that some wells receive a single cell. Skillful technique and suitability of used cell lines for single-cell sorting are necessary to establish a monoclonal cell population by limiting dilution. Furthermore, it is very important to keep high viability during trypsinization and breaking up any cell clumps into individual cells by passing several times through a serological pipet or by passing through cell strainer mesh. Some cell lines tend not to grow well in sparse/individual cultures, probably due to insufficient amounts of secreted growth factors.

As a method overcoming difficulties in single-cell sorting, we present here our experiences obtaining favorable genome-editing clones and transgene clones by using a single particle isolation system, On-chip SPiS ™ (On-chip Biotechnologies, Tokyo, Japan). This system could easily, quickly, and precisely sort single-cell into each well of 96-well or 384-well microplates with keeping enough cell viability to culture. We suggest this system will greatly contribute to genome-editing and gene-transfection experiments.

MATERIALS AND METHODS

Cells and cell culture

Two human lung adenocarcinoma cell lines (A549[3] and H1975[4]), a colon cancer cell line (TKB-102[5]), a monkey kidney cell (COS-7[6]), and a human embryonic kidney cell (GP2-293) were used in this study. Cells were cultivated in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% heat-inactivated fetal calf serum (FCS), 100 U/mL penicillin, and 100 μg/mL streptomycin. They were maintained at 37℃ in 5% CO_2, and semiconfluent cells were used for the experiments.

CRISPR-Cas9 system and genome-editing

To knock out retinoblastoma transcriptional corepressor 1 (*RB1*) gene, gRNA for CRISPR-Cas9 was designed by CRISPRdirect (http://crispr.dbcls.jp/). The gRNA sequence targeting exon 2 of *RB1* gene was 5'-GAGAGAGAGCTTGGTTAACT-3'. The gRNA was assembled into pSpCas9 (BB) -2A-Puro (PX459) V2.0 which was a gift from Feng Zhang[7] (Addgene plasmid #62988 ; http://n2t.net/addgene : 62988 ; RRID : Addgene_62988), Watertown, MA, USA). The gRNA-inserted PX459 was transiently transfected into each cell line with Lipofectamine 3000 (Thermo Fisher Scientific, Waltham, MA, USA). Forty-eight hours post transfection, the cells were treated with 1 μg/mL puromycin for positive selection. After 48 hours of selection, single-cell plating into 96-well microplates was performed to obtain single-cell clones, with a single particle isolation system, On-chip SPiS™ (Fig. 1) (On-chip Biotechnologies, Tokyo, Japan). Knock-out of *RB1* gene was confirmed by western blot and DNA sequence analysis.

Construction of retroviral vector and transfection

Total RNA was isolated from TKB-102 cells using TRIzol reagent (Thermo Fisher Scientific), and cDNA synthesis was performed with SuperScript IV reverse transcriptase (Thermo Fisher Scientific) according to the manufacturer's instruction. Human caudal type homeobox 2 (CDX2) cDNA fragment was amplified using PrimeSTAR GXL DNA Polymerase (Takara Bio, Shiga, Japan) and specific primers (forward prim-

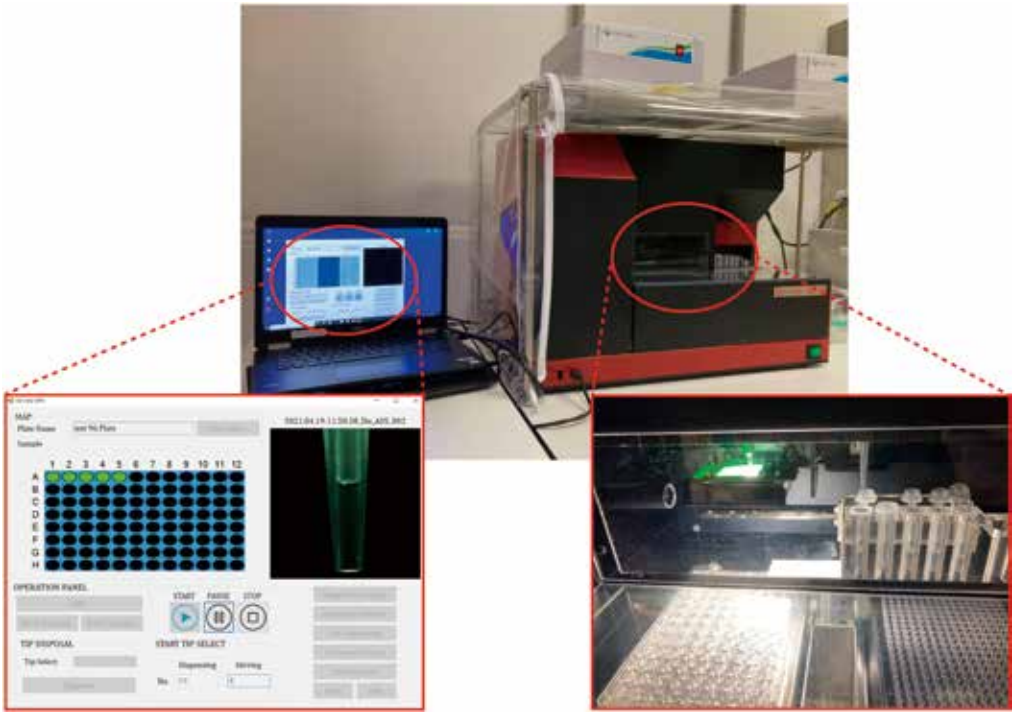

Figure 1

Single particle isolation system, On-chip SPiS, is composed of a main device (right) and a personal computer (left) controlling the main device. The main device is set into the HEPA-filtered air-circulating mobile clean chamber. The number of cells of the aspirated sample solution in the pipette tip is counted using CCD camera, and dispensing is performed only when the cell count is correct.

er : 5'-CCTTAATTAAGGCGCCACCATGTACGTGAGCTA-3', reverse primer : 5'-CGGAATTCCGTTGGCTTCCGCAGTGTAAACC-3'). The PCR product was cloned into T-Vector pMD20 (Takara Bio) and sequenced. CDX2 cDNA-inserted pMD20 was cut out with PacI/EcoRI, and the generated CDX2 cDNA fragment was ligated to pQCXIN Retroviral Vector (Clontech Laboratories, Mountain View, CA, USA). Next, retroviral vector (pQCXIN-CDX2) was transfected into GP2-293 cells with pVSV-G envelope vector using PEI Max (Polysciences, Warrington, PA, USA). After 48 hours, culture supernatant containing recombinant retroviruses was collected and filtrated. The retroviral solution was added to cell culture media of A549 and H1975 cells. The infected cells were positively selected with 500 μg/mL Geneticin (G418) for 2 weeks.

Single-cell seeding by On-chip SPiS

RB1-knockout A549 cells were obtained by transfection of CRISPR/Cas9 vector targeting *RB1* gene through positive selection using G418 as mentioned above. The bulk *RB1*-knockout A549 cells was trypsinized (Sigma-Aldrich, St. Louis, MO, USA) and diluted to 2×10^4 cells/mL. The cell suspension was set to On-chip SPiS, and the cell concentration was automatically optimized for single-cell sorting. Next, the optimized cell suspension was dispensed into 96-well microplates at one cell per well. In this system, single-cell dispensing, which is the most important step, is confirmed by monitoring via CCD camera. If counted cell number in the micropipette content is not one, the content is discarded and dispensing action is repeated until single cell is detected. This automated cell-plating required about 1.5 hours. After 3 weeks of culture, we examined the expression of RB1 protein in each single-cell clone by western blot analysis.

Western blot analysis

Whole cell lysate (28 μg protein/lane) was separated by 10% SDS-PAGE and transferred to Immobilon-P

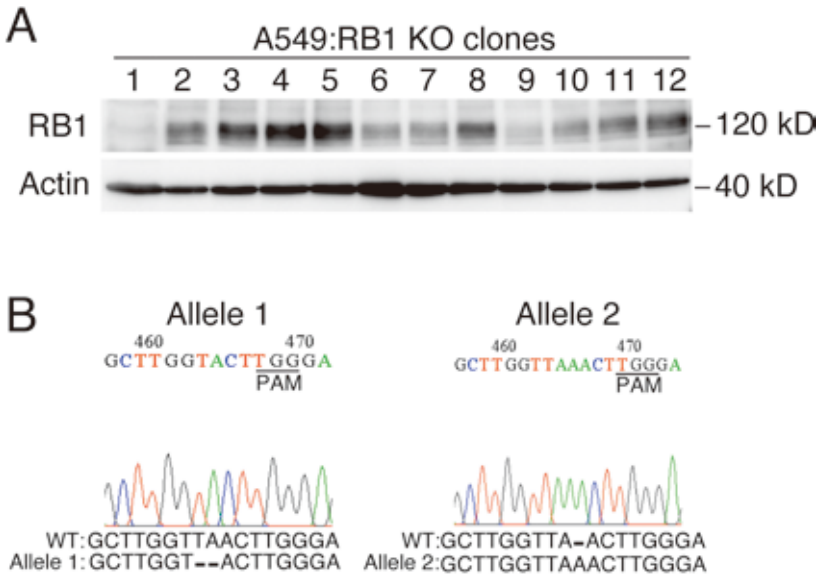

Figure 2

(**A**) The expression of RB1 protein in each A549 clone was confirmed by western blot analysis. β-Actin (Actin) was used as a loading control on the same membrane. (**B**) The sequence of near protospacer adjacent motif (PAM) site in clone #1 was determined by DNA sequence analysis. WT, wild type.

polyvinylidene difluoride membranes (Merck Millipore, Cork, IRL). Membranes were blocked for 1 hour at room temperature with 1% skim milk in PBS containing 0.1% (v/v) Tween 20 (PBS-T) and then incubated with diluted mouse monoclonal anti-RB1 (554136, BD Biosciences, Franklin Lakes, NJ, USA) or rabbit monoclonal anti-CDX2 (ab76541, Abcam, Cambridge, UK) overnight at 4℃. After three washes for 10 minutes with PBS-T at room temperature, membranes were incubated at room temperature for 30 minutes with a diluted peroxidase-labeled secondary antibody against mouse or rabbit IgG (Sigma-Aldrich). The membranes were then washed three times for 10 min with PBS-T at room temperature, and immunopositive signals were visualized using an enhanced chemiluminescence detection kit (EzWestLumi plus, Atto, Tokyo, Japan). Mouse monoclonal anti-β-actin (A5441, Sigma-Aldrich) was used for the internal control.

Sequencing

All sequence analyses were performed using a 3730xl DNA analyzer (Applied Biosystems, Foster, CA, USA). The sequencing region was cloned into pMD20 and used primers were M13M4 (5'-GTTTTC CCAGTCACGAC-3') and M13RV (5'-CAGGAAACAG CTATGAC-3').

RESULTS

Successful cloning of bi-allelic RB1-editing cells by On-chip SPiS

We verified whether single seeding by On-chip SPiS is helpful for establishment of the knockout cell. A549 cells in which PX459 targeting *RB1* gene were transfected were dispensed single-cell into 96-well microplates by On-chip SPiS. After about 3 weeks of culture, the expression of RB1 was confirmed by western blot. As shown in Figure 2A, the clone #1 lacked RB1 protein, and the clone #9 was weakly expressed RB1 protein, suggesting both alleles and one allele editing, respectively. Therefore, we selected clone #1 as a representative sample, and *RB1* locus was subcloned for DNA sequencing. The sequencing data revealed one-base insertion and two-base deletion of the exonal region of *RB1* (Fig. 2B). Since A549 cells possess two *RB1* alleles, the result of DNA sequencing indicated that *RB1* genes of clone #1 was completely knocked out. Consequently, we obtained one clone which both alleles of RB1 was knocked out from twelve clones (8.3%).

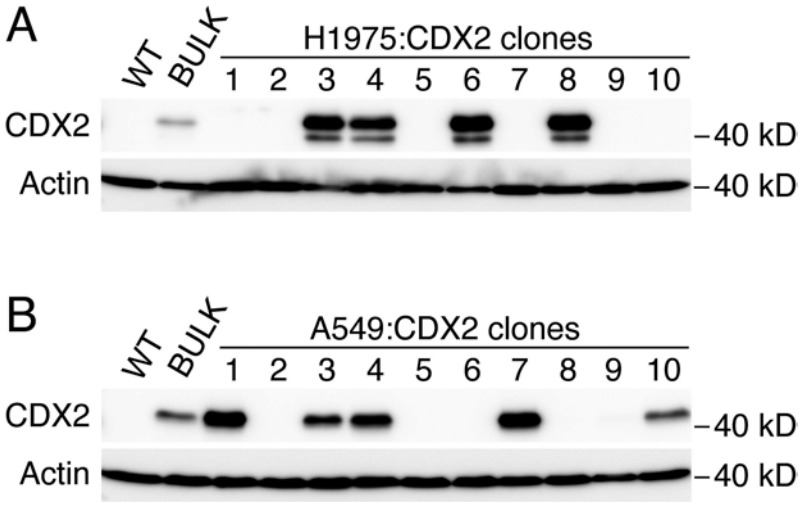

Figure 3

The expression of CDX2 protein of wild type cell (WT), bulk cell (BULK), and single-cell clones in H1975 (**A**) and A549 (**B**) cells was examined by western blot analysis. β-Actin (Actin) was used as a loading control on the same membrane.

Successful establishment of highly CDX2-expressing clones by On-chip SPiS

We also used On-chip SPiS to establish stably CDX2-expressing cell lines. Bulk transfectants of H1975 and A549 were obtained by infection of pQCX-IN-CDX2 and positive selection. Then, we seeded individual cells into 96-well microplates by On-chip SPiS. Each single cell clone was cultured for about 3 weeks, and the expression of CDX2 was validated by western blot. Several clones strongly expressed CDX2 compared with bulk cells of H1975 (Fig. 3A) and A549 (Fig. 3B). Interestingly, approximately 50 to 60 % of clones did not express CDX2, suggesting existence of selection maker-resistant clones without transgene. These results suggested that On-chip SPiS is quite useful for establishing stable transfectant clones in which transfected gene is highly expressed.

DISCUSSION

We had previously used the limiting dilution method for single cell-cloning. This method always has a risk that generated clones were derived from multiple cells. However, after deploying On-chip SPiS, the probability of generating single-cell derived clones significantly increased. Although there are a few possibilities that multiple-cell derived clones are mixed, the frequencies can be minimized by setting appropriate parameters, including cell-size. As the other cell cloning methods, there has been known colony cloning using silicone cylinders or single cell-based clone selection system. Park et al. described that silicone cylinder was more resistant to inward flow from the outside of the cylinder as compared with conventional cloning rings that were settled with silicone grease in culture dish[8]. Nakamura et al. reported the utility of clonal isolation of cultured cells using semi-solid media and a ClonePixFL (Molecular Devices, San Jose, CA, USA) which is the automated colony picker[9]. Since semi-solid media have high viscosity and minimize diffusion of secreted proteins and migration of cells. Since single cells are immobilized in agarose or methylcellulose-based semi-solid medium and form colonies[10,11], single cell-based colony selection could be done by ClonePixFL. Compared with these two methods, single cell-sorting method has advantages in correctness and speed of single-cell dispensation.

In the present study, we succeeded in obtaining *RB1*-knockout A549 cells using On-chip SPiS. Since the dispensing accuracy of lung cancer cells by On-chip SPiS is close to 90% (https://on-chip.co.jp/app-cat/single-cell-assay-atp-accuracy.html), RB1 protein-deficient clone #1, which was confirmed by

western blot, was speculated single-cell clone. As the result of DNA sequencing, clone #1 was ascertained highly purified *RB1*-knockout clone as expected. In contrast, since clone #9 expressed RB1 protein only slightly, it was estimated one-allelic knockout cell clone. Establishing highly purified single-cell clones is particularly important for evaluating the phenotypic alteration in association with gene knockout. Therefore, our results suggest that On-chip SPiS is an extremely useful tool for gene knockout experiments of culture cells.

We also generated H1975 and A549 cell clones expressing CDX2 stably and strongly by single-cell seeding with On-chip SPiS. In the present study, we used the retroviral vector system for CDX2 gene transfection. Since copy numbers of transfected genes and integrated region in the genomic DNA are different in each infected cell, the bulk cells consist of polyclonal cells with various CDX2-expressing levels and selection marker-resistant cells without transgene. Actually, the intensity of CDX2 protein expression in the bulk cells was low level compared with the highly expressing clones (Fig. 3A and B). Our study suggests that performing single-cell cloning with single-particle isolation system is also quite useful to obtain cell clones which transfected gene is highly expressed.

In summary, we established bi-allelic gene-knockout cells and highly gene-expressing transfectants using single-particle isolation system, On-chip SPiS. These results suggest that the high reliability of this system for single-cell sorting is vital for obtaining single-cell clone. A combination of this system and a cell sorter will enhance the quality of single-cell sorting.

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