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A rapid screening and production method using a novel mammalian cell display to isolate human monoclonal antibodies

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ABSTRACT

Antibody display methods are increasingly being used to produce human monoclonal antibodies for disease therapy. Rapid screening and isolation of specific human antibody genes are valuable for producing human monoclonal antibodies showing high specificity and affinity. In this report, we describe a novel mammalian cell display method in which whole human IgG is displayed on the cell surface of CHO cells. Cells expressing antigen-specific human monoclonal IgGs with high affinity on the cell surface after normal folding and posttranscriptional modification were screened using a cell sorter. The membrane-type IgG-expressing CHO cells were then converted to IgG-secreting cells by transfection with a plasmid coding Cre recombinase. This mammalian cell display method was applied to *in vitro* affinity maturation of monoclonal C9 IgG specific to the human high-affinity IgE receptor (FcεRIα). The CDR3 of the C9 heavy chain variable region gene was randomly mutated and inserted into pcDNA5FRT/IgG. A C9 IgG (CDRH3r)-expressing CHO cell display library consisting of 1.1×10^6 independent clones was constructed. IgG-displaying cells showing high reactivity to FcεRIα antigen were screened by the cell sorter, resulting in the establishment of a CHO cell line producing with higher reactivity than the parent C9 IgG.

1. Introduction

Expression of recombinant proteins in mammalian cells is attractive means to produce proteins with proper folding and post-transcriptional modifications suitable for analysis of their character or for therapeutic drugs. Monoclonal antibodies are major proteins produced by mammalian expression system. These are important tools in modern biological research and have great clinical potential, particularly in the analysis and treatment of human diseases.

Several display methods, including phage [1–3], bacterial [4], ribosomal and yeast [5], have been developed to screen for antigen-specific human antibody genes, and many researchers have successfully produced human monoclonal antibodies using these methods. However, because the full-length human monoclonal antibody genes were screened using non-mammalian expression systems with antigen-binding fragments of antibodies such as scFv, the original structures of the intact human antibodies are often lost. Also different codon usages and insertion of mutations by frequent PCR decrease antibody acquisition efficiency. These factors does not ensure the screened antibodies to keep the same structures and characteristics as those when screened after these antibodies are produced on mammalian expression system [6]. To overcome these problems a number of groups tried to develop mammalian cell display systems for antibody generation [7–12]. In most of these methods, antibodies were expressed on the membrane surface of mammalian cells, and antibody genes were recovered after selection and used for secretion of antibody for their characterization. It is more desirable to generate antibodies using identical cell lines for selection and secretion to strictly maintain their characteristics.

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We previously produced human monoclonal antibodies against the human high affinity IgE receptor alpha chain (FcεRIα), an immune receptor and key regulator of allergy [13], by combining phage display with *in vitro* immunization [14]. The resulting antibodies showed specific binding to FcεRIα and sufficient affinity for therapeutic use [15]. In this study, we present a novel method to construct a Chinese hamster ovary (CHO) cell library consisting of independent cell clones expressing a set of human IgG antibody genes to screen clones expressing high-affinity antibody genes. The method was applied to *in vitro* affinity maturation of the human anti-FcεRIα monoclonal antibody. This system enables efficient screening of full-length human IgG antibody genes with high affinity for antigens in a mammalian environment, to rapidly establish IgG-secreting CHO cells.

2. Materials and methods

2.1. Cell lines and cell culture

CHO cells containing the Flp Recombination Target (FRT) site (Flp-In CHO cells) were purchased from Invitrogen (Carlsbad, CA). The Flp-In CHO cell line contains a single integrated FRT site and stably expresses the lacZ-Zeocin fusion gene. Flp-In CHO cells were cultured in Ham's F12 medium (Nissui, Tokyo, Japan) containing 100 U/ml of penicillin (Meiji Seika, Tokyo, Japan), 100 μg/ml of streptomycin (Meiji Seika), 100 μg/ml of Zeocin (Invitrogen) and 10% fetal bovine serum (FBS, company) at 37 °C in a humidified atmosphere consisting of 5% CO_2 in air.

2.2. Recombinant FcεRIα

Recombinant FcεRIα protein was prepared as our previous work [14].

2.3. Plasmid construction

pcDNA5/FRT (Invitrogen) was used to construct the expression system. The coding regions of the leader peptide, variable region (VH, VL) and constant region (CH, Cκ) were derived from C9 human anti-FcεRIα antibodies (C9) in our previous study [14] by PCR with specific primer pairs (Table 1). Internal ribosome entry site (IRES) was amplified from pIRESbleo (Takara bio, Shiga, Japan) by PCR with specific primer pairs (Table 1). The DNA fragment for the transmembrane domain region was synthesized by TaKaRa bio. The fragment consisted of the transmembrane domain between the loxP sequences hidden in the intron and two restriction digestion sites (*BamH* I and *BstX* I), as shown in Supporting Fig. S1. The prepared genes were digested with the relevant restriction enzymes and the DNA fragments were ligated to pcDNA5/FRT yielding the vector pcDNA5/FRT/IgG.

Table 1
Oligonucleotide sequence

Name	Sequence
Not I-Ld	5′-ATAAGAATGCGGCCGCATGGAGACAGACACACTCCT-3′
CL-Apa I	5′-AAAGGGCCCCCTCTAAGACTCTCCCCTGTTG-3′
Nhe I-Ld	5′-AAAGCTAGCATGGAGACAGACACACTCCT-3′
IgGc-BamH I	5′-AAAGGATCCCGGAGACAGGGAGAGGCTCT-3′
BstX I-IRES	5′-CTGCAGAACCAGTGTGGTGGAATTAATTCGCTGTCTGCGA-3′
IRES-NotI	5′-ATAAGAATGCGGCCGCGGTGATCAGATCTGCAGGGC-3′
IgGc-m	5′-GTGGAGTGGGAGAGCAATGGG-3′
IRES-m	5′-CCTCCTGGTTTTTGGAAACTGAC-3′
C9VHCDR3	5′-TCCACCTGAGGAGACGGTGACCAGGGTTCCCAGGCCCCA NNSNNSNNSNNSNNSNNSNNSNNSTCTCGCACAGTAATACAGC-3′
T7-2	5′-AATACGACTCACTATAGGG-3′

2.4. Transfection of Flp-In-CHO cells

Transfection was performed by the lipofection method with cationic amphiphiles [16]. To construct the mammalian cell display library, Flp-In CHO cells were transfected with pcDNA5/FRT-IgG(D) and pOG44. After 48 h of transfection, the culture medium was replaced with Ham's F12 medium containing 10% FBS and hygromycine B for antibiotic selection. Stable clones were collected and used to produce the human monoclonal antibodies. IgG-displaying CHO cells were transfected with pCEP4/Cre coding the nuclear localizing signal and Cre recombinase to remove the transmembrane domain gene by Cre-mediated recombination.

2.5. Flow cytometry

CHO cells (2×10^5 cells) were harvested and incubated with 2 μl of phycoerythrin (PE)-conjugated mouse anti-human IgG (H) antibody (Beckman Coulter, Fullerton, CA) or 2 μl of PE-conjugated mouse anti-human Igκ antibody (Beckman Coulter) in 50 μl of PBS containing 0.5% BSA and 1 mM EDTA (PBE). After incubation for 15 min at 4 °C, the cells were washed with PBE and resuspended in 500 μl of PBE. The fluorescence of living cells was measured using a FACSAria™ Cell Sorter (BD Biosciences, Franklin Lakes, NJ).

2.6. Microscopic observation

CHO cells (2×10^5 cells) were harvested and incubated for 15 min at 4 °C with 2 μl of rabbit anti-human IgG Fc• fragment and 2 μl of mouse anti-human Ig• light chain antibodies in 100 μl of PBE. After incubation, cells were washed and resuspended in 100 μl of PBE with 2 μl of Qdot 525 goat anti-rabbit IgG and 2 μl of Qdot 605 goat anti-mouse IgG. After incubation for 15 min at 4 °C, cells were washed and resuspended in 1 ml of PBE. The co-localization of the heavy and light chain at the plasma membrane of the cell was observed by conforcal laser scanning microscopy LSM 780 (Carl Zeiss, Jena, Germany).

2.7. Confirmation of site-specific recombination

Cre/loxP recombination was confirmed by PCR. Total RNA was isolated from Flp-In-CHO cells by the Fast Pure RNA kit (Takara). cDNA was synthesized using a ReverTra Ace qPCR RT kit (TOYOBO, Osaka, Japan). PCR was performed using the synthesized cDNA or genomic DNA as templates and the specific primer pairs IgGc-m and IRES-m shown in Table 1.

2.8. Measurement of antibody production

The amount of IgG expressed in cells or secreted into the culture supernatant was determined by sandwich enzyme-linked immunosorbent assays (ELISA), as previously described [17].

2.9. ELISA to assess the characteristics of the produced antibodies

The microtiter plates were coated with 1 mg of antigen per well and incubated overnight at 4 °C. After blocking non-specific binding with 1% fish gelatin and washing with PBST, diluted human monoclonal antibodies were added to each well and incubated. After washing with PBST, HRP-conjugated anti-human IgG was added and the plate incubated. The captured IgGs were then incubated with ABTS substrate solution and absorbance was measured at 405 nm using a microplate reader.

2.10. Isolation of CHO cells expressing high-affinity anti-FcεRIα IgG

Mutagenesis of heavy chain CDR3 was performed by PCR using specific primers containing NNS degenerate codons. The amplified C9 VH CDR3 randomized (C9VHCDR3r) DNA was digested and cloned into pcDNA5FRT/C9IgG vector. Flp-In CHO cells were co-transfected with the vector and pOG44 by the lipofection method according to the manufacturer's procedure. Hygromycin was added to the culture medium after 48 h of transfection to select antibiotic-resistant cells. To isolate affinity-optimized antibody-displaying cells, 1×10^6 hygromycin-resistant cells were stained with FITC-conjugated FcεRIα and subjected to single cell sorting using a FACSAria™ Cell Sorter (BD Biosciences). Cells with higher binding potency to the antigen were sorted into a 96-well plate. The sorted cells were then transfected with pCEP4/Cre and maintained in serum-free medium. Antibodies secreted into the culture supernatants were purified by affinity chromatography using a Hi-Trap Protein G HP column (GE healthcare) and their response against antigens and specificities were determined.

3. Results

3.1. Expression of whole human IgG on the surface of mammalian cells

The mammalian cell display system was designed as shown in Fig. 1. Mammalian cells expressed clonal IgG on their cell surface following transfection with the plasmid vector using the Flp-In system. The antigen-specific monoclonal IgG-displaying cells were subsequently screened for IgG expression by FACS. After selection, transfection with the Cre recombinase coding plasmid allowed the cells to secrete the monoclonal IgG molecules previously anchored to the cell surface into the culture supernatant.

First, we constructed the plasmid vector pcDNA5/FRT-IgG that formed the basis for the animal cell display system to facilitate the isolation of human monoclonal antibodies, as shown in Fig. 2A. The expression plasmid contains the cytomegalovirus (CMV) promoter, Igκ chain signal peptide, antibody heavy chain, transmembrane domain (TM), IRES, Igκ chain signal peptide, antibody light chain, FRT site and a polyadenylation signal. The IRES sequence was located between the genes encoding the heavy and light chains to produce whole IgG from a single transcript. The transmembrane domain gene of human γ1 [18] was inserted downstream of the heavy chain constant region gene to localize the expressed IgG to the cell surface. The transmembrane domain coding region was designed to be interleaved with two loxP sequences and hence be removable by Cre/loxP recombination [19,20]. The loxP sequence was hidden in an intron between the heavy chain and the transmembrane domain to avoid inhibiting the effector functions of the produced antibodies by additional amino acids located at the C-terminus of the IgG molecule.

The constructed plasmid, pcDNA5/FRT-IgG, was transfected to CHO cells and the cells labeled with PE-conjugated anti-human IgH or anti-human Igκ were subjected to flowcytometry. The transfected cells expressed the heavy or light chains on the cell surface (Fig. 2B and C). Surface localization of these heavy and light chains was verified by microscopy. The transfected CHO cells labeled with rabbit anti-human IgG Fcγ fragment and mouse anti-human Igκ light chain antibodies followed by Qdot 525 goat anti-rabbit IgG and Qdot 605 goat anti-mouse IgG were examined by confocal laser scanning microscopy. The fluorescent signal of heavy chain (green) and light chain (red) were co-localized on the plasma membrane of non-permeabilized cell (Fig. 2D). These results suggest that CHO cells displayed the whole IgG molecule following transfection with the plasmid vector pcDNA5/FRT-IgG.

3.2. Recombination of the transgene created antibody-secreting cells: secretion of displayed IgG by gene conversion

Cre-mediated recombination of the loxP site was expected to convert the expression form of IgG. Transgene modification by Cre/loxP recombination was analyzed by PCR using the cellular genome or cDNA as templates. Schematic diagrams of the template DNAs and regions amplified by PCR are shown in Fig. 3A. The original and recombined transgene were expected to express fragments consisting of 696 and 464 bp, respectively, using the cellular genome, and 556 and 321 bp, respectively, using the cDNA template. The 464-bp recombined DNA fragment was detected in pCEP4/Cre-transfected cells (Fig. 3B). We also analyzed splicing reactions of these transgenes by PCR. Bands formed by the splicing reactions were confirmed in the PCR products using the cDNA template (white arrow head) but not with genomic DNA (Fig. 3C).

We next performed ELISA to determine whether the cells expressing the recombined transgene could secret the expressed IgG into the culture medium as expected. The expression of

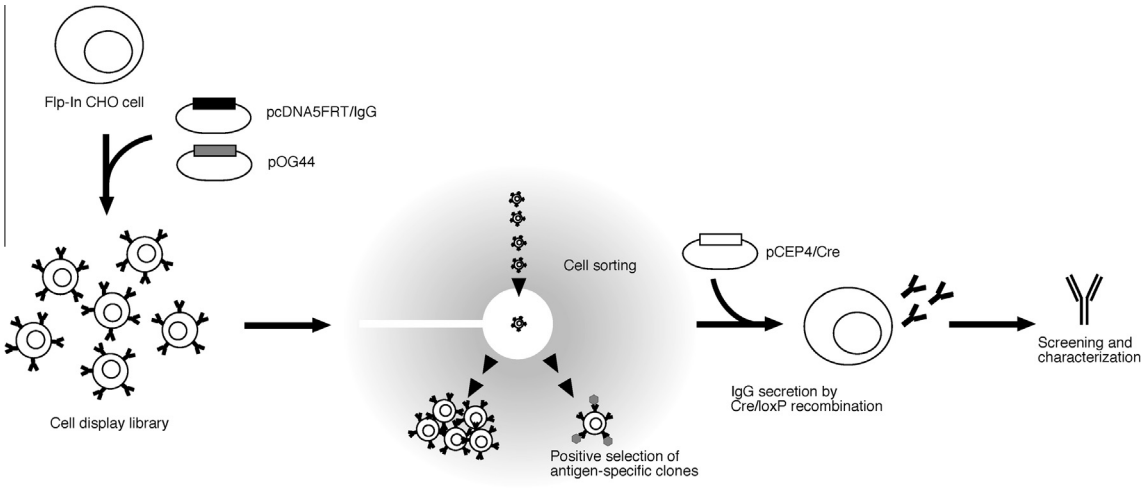

Fig. 1. Schematic diagram of the mammalian cell display system. Flp-In CHO cells are co-transfected with pcDNA5/FRT/IgG and pOG44 to generate the mammalian cell display library. Then, clonal IgG molecules are displayed on each CHO cell by the Flp-In system. Single cells expressing functional IgG that react with a fluorescence-labeled antigen are sorted by FACS. The isolated cells are transfected with a Cre recombinase coding vector, which allows secretion of the antibody into the culture supernatant. The secreted antibodies can then be used in further analyses.

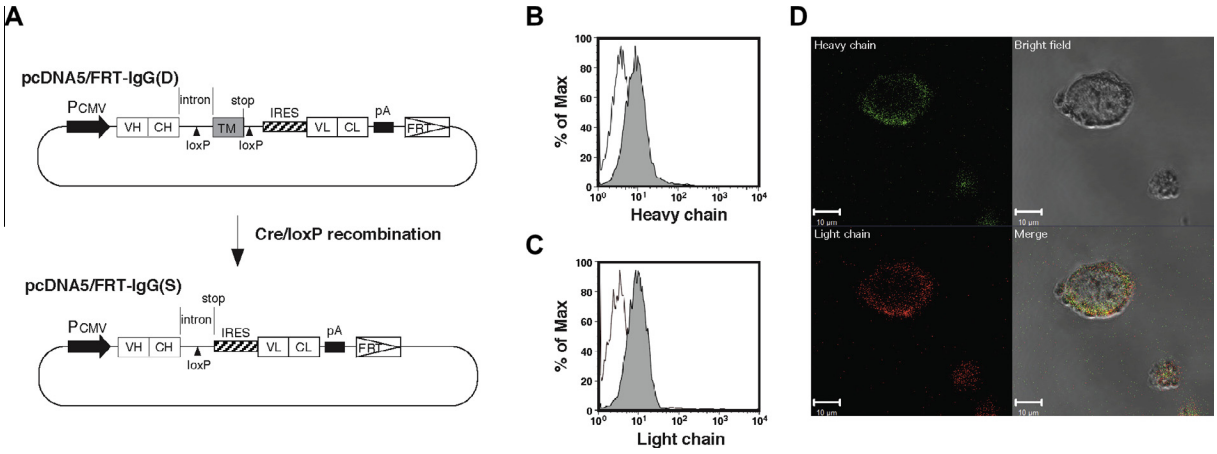

Fig. 2. Expression of human IgG molecule on Flp-In CHO cells. (A) Construction of the plasmid vector pcDNA5/FRT-IgG. The vector is based on pcDNA5/FRT. The TM is located between two loxP sequences and is removed by Cre/loxP recombination. (B and C) Flow cytometric analysis of IgG expressed on CHO cells. Cells expressing IgG molecules on the cell surface were sorted by FACS. Flp-In CHO cells reacted with PE-conjugated mouse anti-human IgH (B) or anti human Igκ (C) antibodies and were subjected to FACS. The solid and open peaks indicate pcDNA5/FRT-IgG-transfected cells and non-transfected cells, respectively. (D) Transfected cells were stained with rabbit anti-human IgG Fcγ fragment and mouse anti-human Igκ antibodies followed by Qdot 605 goat anti-mouse IgG and Qdot 525 goat anti-rabbit IgG, and observed co-localization of the heavy and light chains on the cell surface by using laser scanning microscopy. P_{CMV}, CMV promoter; VH, variable region of the human Ig heavy chain; CH, constant region of the human Ig heavy chain; TM, transmembrane domain; IRES, internal ribosome entry site; VL, variable region of the human Ig light chain; CL constant region of the human Ig light chain; pA, polyadenylation signal; FRT, Flp-In recombination target.

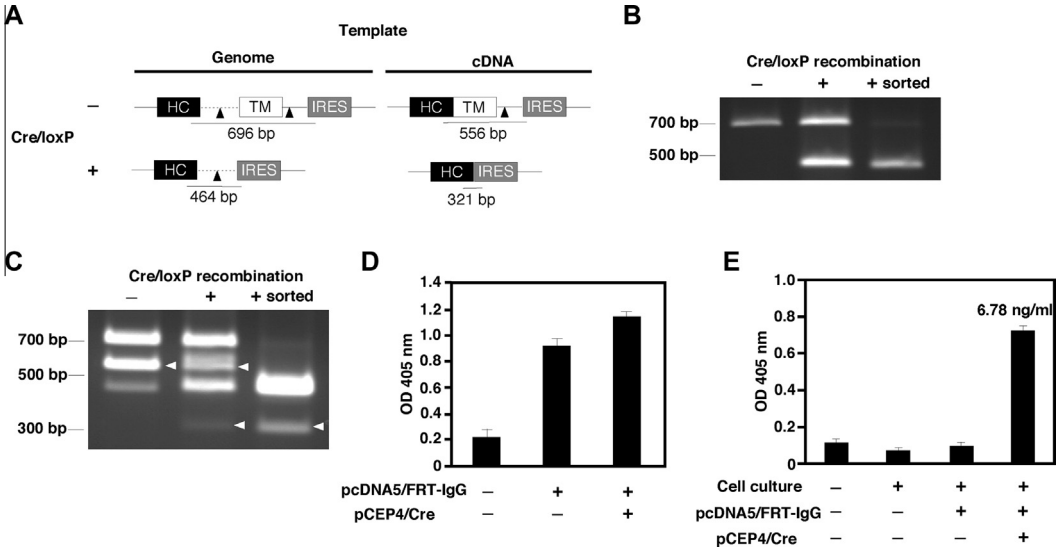

Fig. 3. Detection of gene conversion and IgG secretion. (A) Genomic DNA and cDNA were prepared from the cells in each stage of the process and the sequence between the heavy chain and IRES was amplified by PCR, Dotted lines indicate the intron sequence, underlining indicates the amplified regions and the black arrow head indicates the loxP site. (B and C) The PCR products obtained from genomic DNA or cDNA were electrophoresed and stained with ethidium bromide. (D and E) The intracellular Ig level (D) and IgG secretion from CHO cells (E) were determined by ELISA. Flp-In CHO cells and cells transfected with pcDNA5/FRT/IgG with/without pCEP4/Cre were cultured. The cells and supernatants were collected and the cells were lysed with lysis buffer before the assays.

intracellular IgG was confirmed in pcDNA5FRT/IgG-transfected cells (Fig. 3D). On the other hand, IgG secretion was only detected in the culture supernatant of pCEP4/Cre-transfected cells (Fig. 3E). These results suggest that Cre-mediated recombination is capable of converting antibody-displaying cells to antibody-secreting cells.

3.3. Isolation of affinity improved anti-FcεRIα human monoclonal antibodies by using the animal cell display method

To demonstrate the usefulness of the new cell display system for isolating cells producing specific monoclonal antibodies, the C9 anti-FcεRIα monoclonal antibody was used for *in vitro* affinity maturation. We constructed a CHO cell surface display library, in which an intrinsic antibody hotspot was randomized. There are six CDRs involved in antigen binding in an antibody molecule; three are located on the heavy chain and three on the light chain. CDR3 of C9 VH gene was randomly mutated by PCR using primers to specifically mutate the CDR3 sequence (CACAACTGGTATC ACTTTGACTCC) to NNSNNSNNSNNSNNSNNSNNS and the parental C9 VH gene was replaced with pcDNA5FRT/C9IgG. The CDRH3 randomized (CDRH3r) IgG library was transduced into *Escherichia coli* cells, and the resulting library consisted of 1.1×10^6 independent clones. The library was then transfected into CHO Flp-In cells with pOG44 coding FLP recombinase [21]. After selection, a mammalian cell display library containing 1.6×10^6 independent clones was obtained. These IgG-displaying cells were then stained with FITC-conjugated rFcεRIα and subjected to FACS to select affinity-improved mutants. The cells

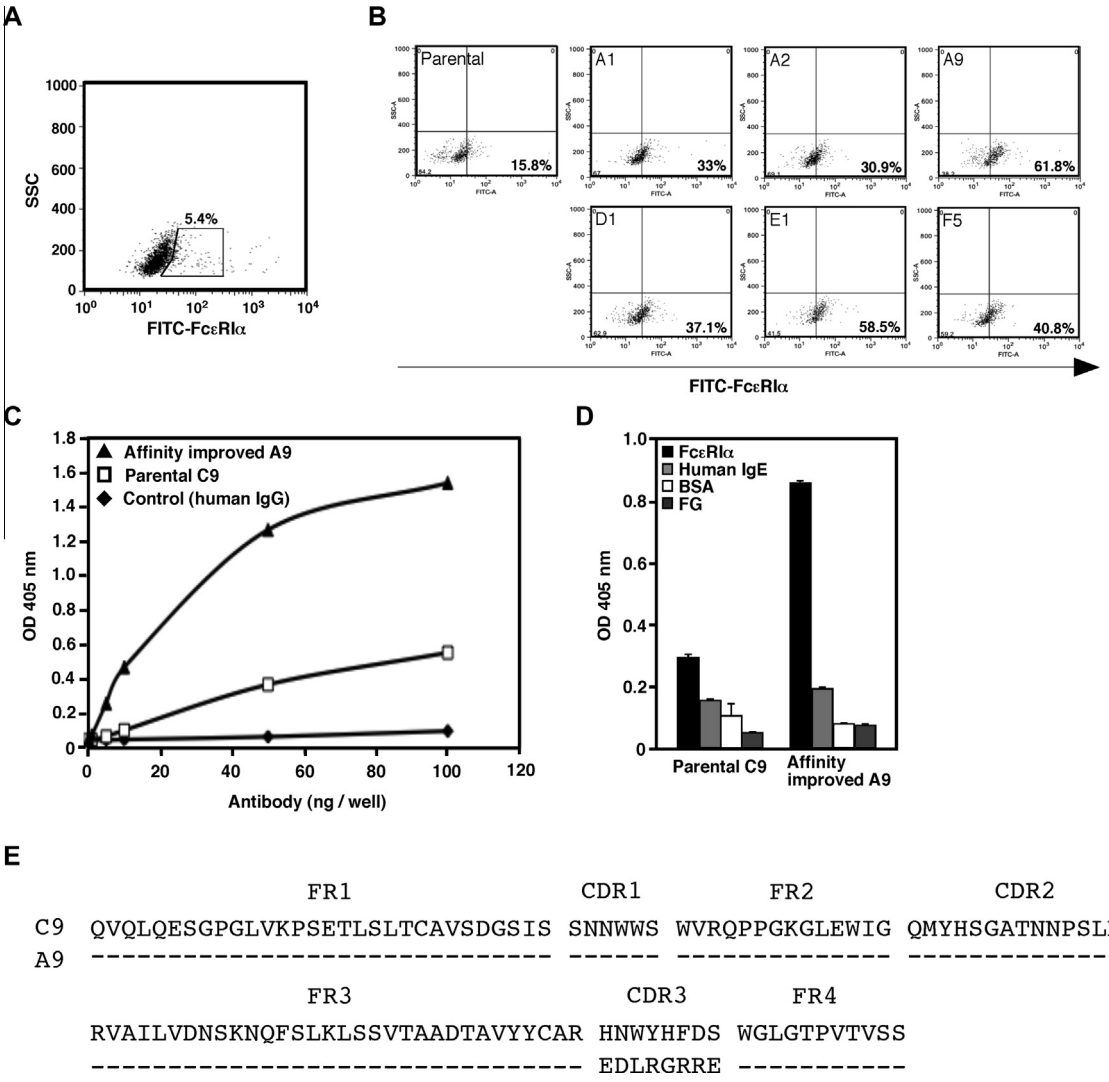

Fig. 4. Isolation of human monoclonal antibody by the method. (A) Cells were stained with FITC-labeled rFcεRIα and subjected to FACS to analyze their reactivity to the antigen. Cells in the gated population with high binding affinity for the antigen were subjected to single cell sorting. (B) The sorted cells were expanded and their reactivity for FcεRIα was re-evaluated. (C) Binding reactivity of isolated antibody to antigen and (D) Specificity of antibodies were assessed by ELISA. (E) Comparison of the amino acid sequences of affinity purified clone A9 and the parental clone.

showing greater affinity to the rFcεRIα antigen than the main population gated as shown in Fig. 4A were single cell sorted to 96-well plates. We then re-analyzed the reactivity of the 49 expanded clones to the antigen by FACS. Overall, 38 of these clones showed greater reactivitties than the parental clone C9 and the top of six clones are shown in Fig. 4B.

Sorted cells were then subjected to Cre/loxP recombination after transfection with pCEP4/Cre to remove the transmembrane domain gene. This procedure enabled secretion of the displayed IgG molecules and the IgG secreting cells were cultured for several weeks. The culture supernatants containing recombinant human IgG were collected and IgG molecules were purified by protein G column affinity chromatography. An ELISA was performed to evaluate the characteristics of the affinity-improved monoclonal antibodies; with clone A9 showing the greatest reaction to FcεRIα. The A9 clone specifically recognized FcεRIα (Fig. 4C) with higher affinity than the parental C9 IgG in a density dependent manner (Fig. 4D). The VH domains of the parental and affinity improved clones were sequenced to compare their amino acid sequences (Fig. 4E). Overall, the only differences in sequences were found in the CDR3 region, which was randomly mutated by PCR.

4. Discussion

In this report, we have described a novel method to isolate human monoclonal IgG. The membrane-bound monoclonal IgG was displayed on a Flp-In CHO strain and could be used for positive screening of monoclonal antibodies against a specific antigen by using the Flp-In system. The selected cells displaying antigen-reactive IgG were transformed to antibody-secreting cells by Cre/loxP recombination via transient expression of Cre recombinase. The characteristics of the resulting antibody were maintained throughout the procedure, which offers a conceptually simple and optimized system.

The system provides some advantages for efficient production of human monoclonal IgG by using mammalian expression environment and artificially designed recombineering system. In terms of codon usage, regulating the rate of transcription and translation is a critical factor for controlling protein expression and there are marked differences between bacteria and mammalian cells [22]. To produce human monoclonal antibodies with the phage display method, antibodies are expressed using gene coding human antibodies via the bacterial expression system,

Table 2
The experimental procedure of monoclonal antibody production after screening

Step	Process
Traditional display method	
1	Plasmid purification
2	PCR amplification for VH and VL genes per clone
3	Restriction digestion
4	Ligation to plasmid for secretion, transformation
5	Insert check
6	Plasmid preparation having insert gene
7	Sequence analysis of VH and VL genes per clone (until obtained plasmid vector having correct sequence)
8	Transfection to mammalian cell for producing IgG
9	Characterization of antibody clones
The novel mammalian cell display method	
1	Transfection of Cre recombinase cording plasmid
2	Characterization of antibody clones

resulting in limitations in the constructed library because of the difficulty of displaying human antibodies on phages. The mammalian cell display using a mammalian expression system allows us to construct a human antibody library with less bias than the other display methods. The efficiency of the method is substantially different from those of other methods. Cell sorting-based positive screening makes it possible to obtain CHO clones expressing monoclonal antibodies specific for the target antigen in a single round of screening. Moreover, the IgG molecules displayed on CHO cells can be secreted following Cre/loxP recombination. Characterization of the selected antibody clones require strategies for gene conversion and transient or stable expression on mammalian cells to produce a secreted form of IgG when using the traditional display methods, including mammalian cell display [7–10,23]. This process involves many steps, as shown in Table 2, for each clone. On the other hand, the mammalian cell display proposed here can induce IgG secretion by transfection with a Cre recombinase coding plasmid. IgG secreting cells could not separate from mixed population after transfection of pCEP4/Cre into the IgG displaying cells on flowcytometric analysis, but the Cre recombinase mediated deletion efficiency of the transmembrane domain was relatively high as shown in Fig. 3 and we could easily obtain IgG secreting clone by random sorting from the mixed population. This simple acquisition process reduces time, cost and effort for selecting candidate monoclonal antibodies from a large number of potential clones.

In conclusion, we have developed a method to produce human monoclonal antibodies using mammalian cells that offer a suitable environment for IgG. The method enables *in vitro* maturation of a human monoclonal antibody and avoids the limitations associated with bacterial expression and PCR recombination. We believe this method will become a powerful tool to produce human monoclonal antibodies for research and therapeutic purposes.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.bbrc.2013.10.007.

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