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Reversible switching of immunoglobulin hypermutation machinery in a chicken B cell line

Naoki Kanayama[a], Kagefumi Todo[a], Michael Reth[b], Hitoshi Ohmori[a,*]

[a] *Department of Biotechnology, Okayama University, Tsushima-Naka 3-1-1, Okayama 700-8530, Japan*
[b] *Department of Molecular Immunobiology, Max-Planck-Institute for Immunobiology, University of Freiburg, D-79108 Freiburg, Germany*

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Abstract

A chicken B lymphoma line, DT40, hypermutates immunoglobulin (Ig) genes spontaneously during culture. Thus, cultured DT 40 cells constitute a useful Ig library for screening antibodies (Abs) in vitro. To fix desirable Ig mutants by stopping hypermutation or to resume mutation for further improvement of Ab affinity, activation-induced cytidine deaminase (AID), a key enzyme responsible for the Ig mutation machinery, must be switched on or off. To this end, we generated a DT40 line whose one AID allele was disrupted, and the other allele was replaced by the loxP-flanked AID construct. In this engineered cell line designated as DT40-SW, AID expression could be switched reversibly by tamoxifen-regulated Cre recombinase. Devices were also introduced to discriminate between the "AID-ON" and the "AID-OFF" cells by GFP expression and puromycin resistance, respectively. Starting from a single DT40-SW cell, Ig gene repertoire was efficiently diversified during culture only when AID expression was on.

Keywords: DT40; Immunoglobulin gene; Hypermutation; Gene conversion; Antibody; Activation-induced cytidine deaminase

Several approaches have been exploited to select an antigen-specific antibody (Ab) in vitro. For instance, single-chain Abs can be selected from a phage-display library [1–3]. To improve the primarily selected clones, these may be diversified by secondary mutagenesis, followed by expression in appropriate host cells to assess their functions. Generally, these processes are laborious and time-consuming. This, however, is successfully accomplished in vivo in the immune system, where somatic hypermutation of immunoglobulin (Ig) genes coupled with selection of B cells expressing high-affinity Abs results in an increase in the affinity of secreted Abs, a process termed affinity maturation [4–6]. In this context, a chicken B lymphoma line, DT40, may be useful to mimic the affinity maturation process in in vitro culture [7], because DT40 cells produce IgM Abs, and undergo hypermutation in the variable region (V) of Ig genes spontaneously during culture [8–10]. Using DT40, mutagenesis in Ig genes and their expression can be sequentially performed in a single cell. Propagated DT40 cells have been shown to generate Ig diversity that was sufficient for selection of Abs against test antigens including protein A, streptavidin, and a rat IgG [11]. In addition, targeted integration of transfected genes occurs with exceptionally high frequency in DT40 [12], thereby making the cell line genetically tractable.

Chicken B cells including DT 40 cells have been shown to diversify Ig genes not only by point mutation, but also by another mechanism called gene conversion, which introduces partial sequences from upstream V pseudogenes into the expressed V gene [13,14]. Recently, activation-induced cytidine deaminase (AID) has been identified as an essential enzyme responsible for point mutation [15–18] and gene conversion [8,9]. DT40 cells have been shown to express AID constitutively

* Corresponding author. Fax: +81 86 251 8197.
E-mail address: hit2224@cc.okayama-u.ac.jp (H. Ohmori).

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[8,9,11]. To isolate a genetically stable clone producing a desirable monoclonal antibody, AID activity must be shut down for avoiding further mutation. On the other hand, AID expression should be switched on again to resume secondary mutation for improving the Ab affinity. To this end, we generated a DT40 line whose hypermutation machinery can be reversibly switched "ON" and "OFF" by controlling AID expression with an exogenous stimulus.

Materials and methods

Oligonucleotides and DNA polymerase. All primers used in this study are listed as follows: AID1, AID4, AID9 [8], ACT2 5′-CACC TCGAGGTGAGCCCCACGTTCTGCTT-3′, ACT4 5′-CCAGATC TTGTCGACATCATCCCAGTTGGTGACAAT-3′, AIDF1 5′-CAC CGTCTGAAACCCAGCAAGAGTAGATAG-3′, AIDR1 5′-CTCC AGGAGGTGAACCATGTGATGCGGTAG-3′ cmvE-R 5′-ACTCC ATATATGGGCTATGAACTAATGACC-3′, CITE1 5′-CGACAGA TCTAAGCTTGTAATACGACTCACTATAGG-3′, CITE2 5′-CAT AGGATCCGTCGACATGGTATTATCATCGTGTT-3′, cLL5 5′-C GGCGTGGGGATCCACAGCTGCTGGGATTC-3′, cCL3 5′-ACT CGGATCCCTTCAGGGTCTTCGTGATAG-3′, GFP1 5′-GCCCT GAGCAAAGACCCCAA-3′, and SV-1 5′-AACTCATCAATGTA TCTTATCATGTCTGG-3′. KOD plus DNA polymerase (TOYOBO) was used for PCR amplification as instructed by the manufacturer.

Plasmid construction. To express the Cre chimeric protein gene [19] under the control of the CMV promoter, a *Hin*dIII fragment from pANMerCreMer [19] was ligated into the *Nhe*I–*Not*I region of pEGFP-N1 that contains the CMV promoter and neomycin resistance gene, after filling up digested sites, to yield pCNMerCreMer.

Genomic DNA and cDNA of the AID gene were amplified by primers AID1 and AID4, cloned into the *Eco*RV site of pBluescript (Stratagene), and confirmed by sequencing. To construct the AID-disrupting vector pAID-KO, the *Bst*XI–*Bam*HI region of the AID genomic DNA, which contains the exon encoding the catalytic domain of AID, was replaced with a loxP-flanked blasticidin S resistance gene (Bsr) in a *Bam*HI fragment of pLoxBsr [20] after filling in the digested ends.

The targeting vector pAID-ctrl was prepared as follows. An IRES amplified from pCITE2a(+) (Novagen) by primers CITE1 and CITE2 was cloned into the *Bam*HI site of pEGFP-N1 (Clontech) after *Bgl*II/*Bam*HI-digestion. The IRES connected with the GFP gene was excised by *Nhe*I/*Hpa*I-digestion and inserted into the *Nhe*I–*Eco*47III region of pLoxPuro [20] to yield pLIG containing one loxP-flanked part. Next, a *Bam*H1–*Eco*RI fragment containing rabbit β-globin poly(A) from pCAGGS [21] was inserted into pPUR (Clontech) with modification of the *Eco*RI site to a *Not*I linker. The puromycin resistance gene (Puro^r) including SV40 poly(A) connected with the inverted β-globin poly(A) was excised by *Hind*III/*Not*I-digestion, and replaced with IRES and GFP genes in pLIG to create pLPsg containing another loxP-flanked part. Two *Spe*I/*Not*I-digested loxP-flanked parts from pLIG and pLPsg were ligated at the *Not*I sites and inserted into the blunted *Nhe*I site of pExpress [20] whose promoter had been replaced with the CAG promoter from pCAGGS [21] by *Spe*I/*Xba*I-digestion, to yield pCLPGIL. A *Pvu*II–*Sma*I fragment of the AID cDNA was inserted into the blunted *Eco*RI site of pCLPGIL to yield pCLPGIAL. Finally, the CAG-driven and loxP-flanked AID gene cassette was excised by *Spe*I/*Eco*RI-digestion and inserted into the *Bst*XI–*Bam*HI region of AID genomic DNA to complete pAID-ctr1 (Fig. 1A).

Cell culture and transfection. The DT40 cell line was obtained from RIKEN Cell Bank (Tsukuba, Japan). DT40 cells were cultured in RPMI 1640 medium (ICN Biomedicals) supplemented with 10% fetal bovine serum (Life Technologies), 1% chicken serum (Sigma), 50 μM 2-mercaptoethanol, 2 mM glutamine, 1 mM pyruvic acid, 100 μg/ml penicillin G, and 50 μg/ml streptomycin at 37 °C in 5% CO_2 and 95% air. DT40 cells were suspended in 250 μl phosphate-buffered saline at 2×10^7 cells/ml and transfected with 15 μg of a linearized vector by electroporation using Gene Pulser Xcell (Bio-Rad) at 550 V and 25 μF in 4 mm cuvette. DT40 cells transfected with pCNMerCreMer were selected in a medium containing 2 mg/ml G-418 (Sigma). A DT40 clone bearing the 4-OHT-regulated Cre recombinase gene was stepwise transfected with pAID-KO or pAID-ctrl and selected in a medium containing 50 μg/ml blasticidin S (Invitrogen) or 0.5 μg/ml puromycin (Sigma), respectively. The targeted integration to AID loci was confirmed by PCR (40 cycles of 15 s at 94 °C, 30 s at 60 °C, and 4 min at 68 °C) using primers AIDF1, AIDR1, SV-1, and cmvE-R for AID loci, or ACT2 and ACT4 for the β-actin gene. Transcription of the endogenous AID genes was assessed by RT-PCR (35 cycles of 15 s at 94 °C, 30 s at 65 °C, and 1 min at 68 °C) using primers AID1 and AID4, or cLL5 and cCL3 for the L chain gene.

Switching of AID expression by 4-OHT-treatment. DT40-SW cells were incubated with 50 nM 4-OHT for 48 h. After washing twice to remove free 4-OHT, the treated cells were cultured for 48 h. GFP expression was analyzed with FACSCalibur (BD Biosciences). GFP$^+$ (AID-ON) DT40-SW cells were sorted by using FACSAria equipped with Auto Cell Deposit Unit for single cell isolation (BD Biosciences). The purity of the sorted cells was more than 95%. To isolate AID-OFF DT40-SW cells, 4-OHT treated cells were cultured in a medium containing 0.5 μg/ml puromycin for at least 48 h. To confirm the inversion of the loxP-flanked AID gene, the GFP$^-$ or GFP$^+$ cells were genotyped by PCR (40 cycles of 15 s at 94 °C and 3 min at 68 °C). AID transcription was compared between wild type DT40 and AID-ON DT40-SW cells by RT-PCR using primers AID9 and AIDR1.

Analysis of gene conversion in V genes. cDNA was synthesized from total RNA of GFP$^-$ or GFP$^+$ DT40-SW cells as described previously [6]. The L chain genes were amplified by primers cLL5 and cCL3, cloned into pCR-Blunt (Invitrogen), and sequenced with ABI PRISM 310 Genetic Analyzer (Applied Biosystems). Sequence changes were assigned to gene conversion or somatic point mutation by comparing mutated sequences with the published Vλ pseudogene sequences that could act as donors for gene conversion [13]. Maximal length of a Vλ1 sequence covering a mutation site that coincided with a donor pseudogene was indicated as a converted region illustrated in Fig. 2C. When a sequence spanning a mutation could not be assigned to any pseudogene, this was regarded as point mutation. Sequence data of the original Vλ1 (Lmn) and mutant (Lm1–Lm13) genes were deposited to DDBJ/EMBL/GenBank (Accession Nos.: AB193002, AB193003, AB193004, AB193005, AB193006, AB193007, AB193008, AB193009, AB193010, AB193011, AB193012, AB193013, AB193014, and AB193015).

Results and discussion

Integration of an AID-switching device into DT40

The strategy for switching AID expression is based on the replacement of the endogenous AID gene with the loxP-flanked counterpart whose orientation can be inverted by Cre recombinase [22,23]. First, DT40 cells were transfected with a modified pANMerCreMer vector that bears the gene encoding a chimeric protein between the Cre protein and a mutated hormone-binding domain of the murine estrogen receptor [19]. In this transfectant, Cre recombinase activity can be turned on by exposing the cells to an anti-estrogen drug, 4-hydroxytamoxifen (4-OHT) [19,20].

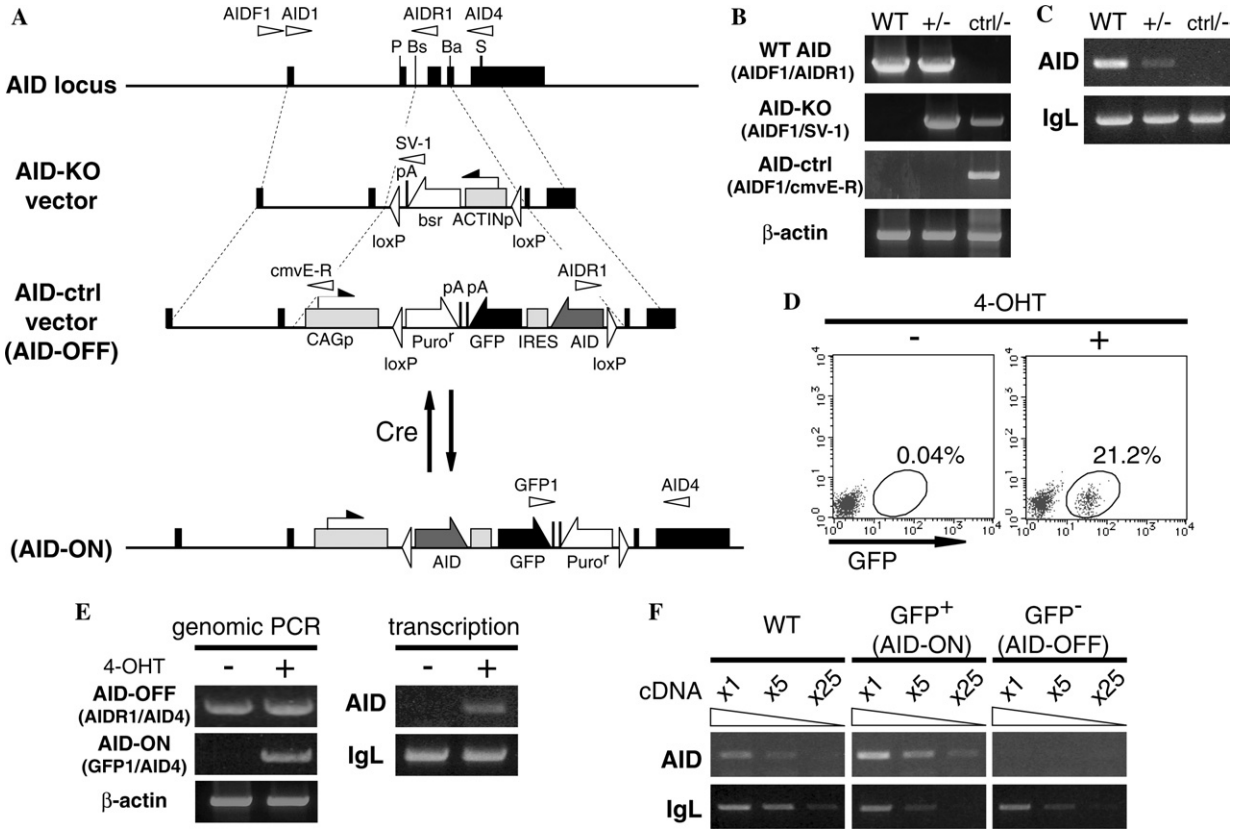

Fig. 1. Generation of DT40-SW, an engineered cell line whose AID expression can be switched on and off. (A) The strategy for disruption of one AID locus with the AID-KO vector and subsequent replacement of another AID locus with the Cre-regulated AID construct by targeting with the AID-ctrl vector. P, Bs, Ba, S, and pA indicate *Pvu*II, *Bst*XI, *Bam*HI, *Sca*I sites, and poly(A) site, respectively. (B) Confirmation of endogenous AID disruption and the targeted insertion of AID-ctrl in DT40-SW by genomic PCR. Primer pairs (see panel A) used for confirming the targeted insertion are shown in parentheses. The wild type (WT) and the heterozygously AID-knockout (+/−) DT40 cells, and the AID-knockout and AID-ctrl-knockin (ctrl/−) DT40-SW cells were examined. (C) AID mRNA expression in WT, +/− and ctrl/− cells. (D) Generation of GFP^+ (AID-ON) cells after treatment of GFP^- (AID-OFF) DT40-SW cells with 4-OHT. (E) The inversion of the AID construct from the "OFF" to the "ON" orientation (left panel) and the induction of AID transcription (right panel) after treatment of GFP^- (AID-OFF) DT40-SW cells with 4-OHT. Primer pairs (see panel A) used for confirming the inversion are shown in parentheses. (F) Comparison of the expression level of AID mRNA between WT DT40 and GFP^+ (AID-ON) DT40-SW cells. As a reference, GFP^- (AID-OFF) DT40-SW cells were also examined. Serially 5-fold diluted cDNA samples were used for RT-PCR. IgL cDNA was amplified as the DNA-loading control.

To disrupt one AID locus in the Cre-introduced DT40 line, the targeting vector pAID-KO containing the blasticidin S-resistance gene (Bsr) (Fig. 1A) was flanked by two loxP sites to remove the drug-resistance gene if necessary. To replace another AID locus with the AID construct bearing a switching device, an AID(+/−) line thus obtained was next transfected with the targeting vector pAID-ctrl, which contains the CAG promoter [21] followed by a cluster of genes flanked by loxP sites in opposite orientation, in which the puromycin-resistance gene ($Puro^r$) and the reversed GFP and AID genes were arranged in this order (Fig. 1A). In a transfected clone designated as DT40-SW, genomic PCR using primers shown in Fig. 1A revealed that pAID-KO and pAID-ctrl were inserted to the respective targeted sites in AID loci correctly (Fig. 1B). In contrast to the wild type DT40, DT40-SW thus generated did not express AID because the orientation of the AID gene was initially opposite to the CAG promoter (Figs. 1A and C). All these genetic manipulations had no significant effects on the growth and viability of the cells.

Inversion of the loxP-flanked AID gene by 4-OHT-treatment

Next, we examined whether AID expression in DT40-SW cells was switched on after activation of Cre recombinase by the treatment with 4-OHT. Inversion of the AID gene can be monitored by the expression of the GFP gene that is inserted in tandem adjacent to the AID gene (Fig. 1A). As expected, more than 20% of the cells became GFP^+ after 4-OHT-treatment for 48 h (Fig. 1D). Further, PCR using primer pairs AIDR1/AID4 and GFP1/AID4 that amplify AID- and GFP-derived segments, respectively, showed that the AID-ON configuration was detected only in

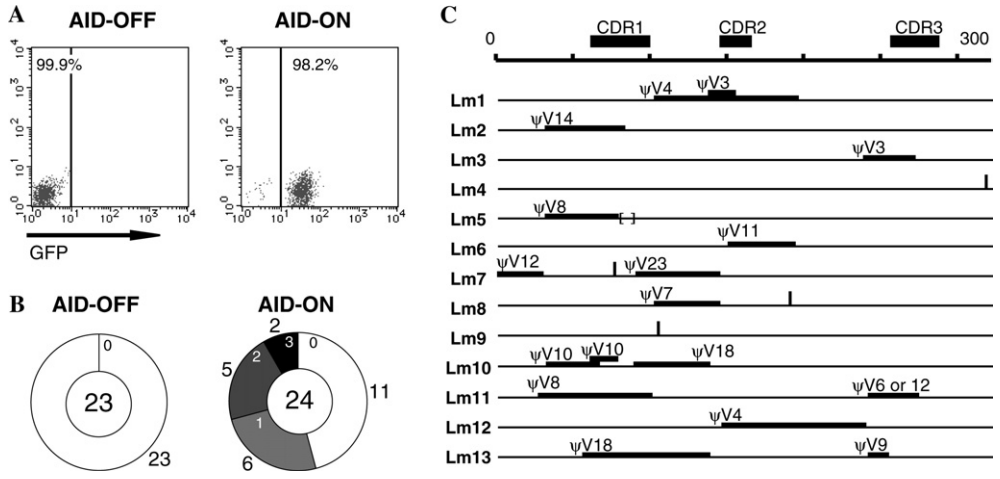

Fig. 2. Gene conversion of the L chain gene in DT40-SW cells occurred only when AID expression was on. (A) Expression status of GFP was maintained in AID-ON and AID-OFF DT40-SW cells after 2 months culture. A single cell from each population (GFP^+ (AID-ON) or GFP^- (AID-OFF)) was cultured for 2 months, followed by flow cytometric analysis of GFP expression. (B) The frequency of gene conversion in the L chain genes in the AID-ON or AID-OFF cells shown in (A). The total number of analyzed L chain genes is indicated in the center of the pie chart. Segment sizes are proportional to the number of sequences (indicated around the chart) that contained 0, 1, 2, or 3 mutations (shown within each segment). (C) Mutated V region sequences shown in (B). These are designated as Lm1–Lm13. Thick lines indicate the sites of gene conversion in which assigned V pseudogene donors are indicated. Short vertical lines show the sites of point mutation.

the 4-OHT-treated cells, in which AID mRNA expression was also induced (Figs. 1A and E). All these results support the fact that AID expression was switched on through the inversion of the loxP-flanked gene by 4-OHT-activated Cre recombinase. The expression level of the AID gene in the sorted GFP^+ DT40-SW cells was comparable to or a little higher than that in the wild type DT40 cells (Fig. 1F), suggesting the effectiveness of the CAG promoter.

Switching of hypermutation machinery in DT40-SW

It has been reported that cultured DT40 cells continue to undergo gene conversion, which did not occur in AID-deficient mutants [8,9]. Then, is the AID switching device able to control the mutation machinery in DT40-SW? Starting from a single GFP^- (AID-OFF) DT40-SW cell, the propagated cells were divided into two parts. One-half was cultured for 2 months without any treatment. The other half was initially treated with 4-OHT to switch on AID expression. A single GFP^+(AID-ON) cell thus induced was sorted and cultured for 2 months. Flow cytometric analysis shows that the expression status of the GFP gene was stably maintained in each clone after 2 months of culture (Fig. 2A). The same was true as for the expression of the AID gene, positive in GFP^+ cells and negative in GFP^- cells (data not shown). The AID-ON and the AID-OFF populations were examined for the nucleotide sequence of V region in the L chain gene. No mutation was found in all of 23 sequences examined that derived from the AID-OFF cells, while mutations were found to occur in 13 out of 24 sequences derived from the AID-ON cells (Fig. 2B). Interestingly, all of the 13 mutated sequences (designated as Lm1–Lm13) were not identical with one another (Fig. 2C), suggesting a high potency to diversify Ig genes in the AID-ON cells. Seventeen mutations found in Lm1–3, Lm5–8, and Lm10–13 were all assigned to published V pseudogene donors, thus suggesting the result of gene conversion. On the other hand, four mutations found in Lm4, Lm7, Lm8, and Lm9 may be due to point mutation because they could not be assigned to the known V pseudogenes (Fig. 2C). All these point mutations are regarded as C to T (Lm4 and Lm9) or G to A (Lm7 and Lm8) transition mutation that may be the result of AID-mediated deoxycytidine deamination [18,24].

Repeated switching of AID expression

When the GFP^+ (AID-ON) cells were treated with 4-OHT, approximately a half of the cells became GFP^- (AID-OFF) (Figs. 3A and B). The GFP^- (AID-OFF) cells could be separated by passage through the puromycin-containing medium because the $Puro^r$ gene had been inverted to the productive orientation in these cells (Fig. 3C). The GFP^- cells thus selected were rendered GFP^+ again after the second 4-OHT-treatment (Fig. 3D), showing that repeated switching of AID expression is successful. In this system, GFP^+ and GFP^- cells can be separated by the use of the cell sorter and the puromycin-containing medium, respectively. Rate of the change from GFP^- to GFP^+ was apparently lower than that of the reverse process. This might be due to an equilibrium characteristic of the loxP-flanked construct or to the imprecise gene inversion in generating GFP^+ cells. However, this inversion rate may not be too low to resume secondary mutation in the selected primary clones.

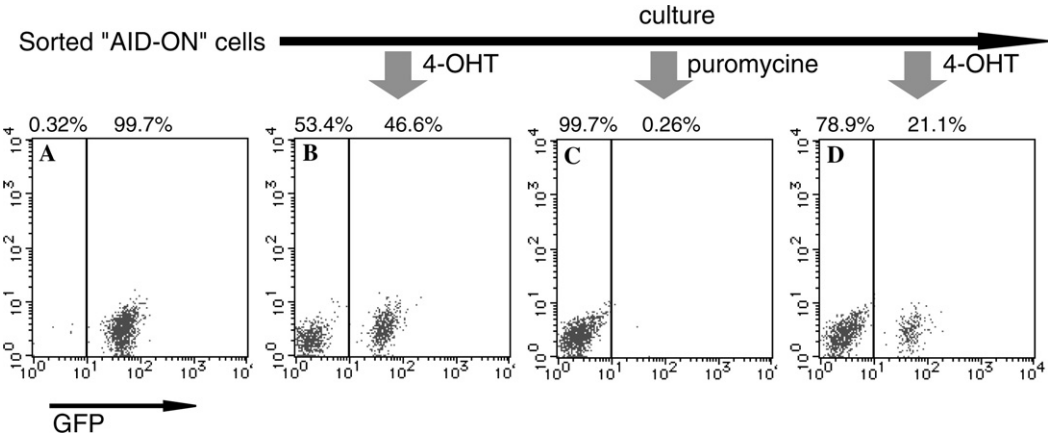

Fig. 3. Repeated switching of AID expression in DT40-SW cells. Treatments proceeded from (A) to (D). Sorted GFP^+(AID-ON) cells (A) were treated with 4-OHT, resulting in the generation of the GFP^- (AID-OFF) cells (B). The GFP^- (AID-OFF) cells were separated by passage through the puromycin-containing medium (C). Then, GFP^+ (AID-ON) cells were regenerated after the second 4-OHT-treatment of puromycin-selected GFP^- (AID-OFF) cells (D).

In conclusion, Ig hypermutation machinery could be reversibly switched in DT40-SW cells. By switching on or off AID expression, this engineered cell line will provide a useful means to select and evolve Abs in vitro. This may also be applied to the molecular evolution of proteins other than Abs since somatic mutation has been reported to target a heterologous gene in place of the Ig gene [17].

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