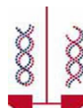
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Efficient affinity maturation of antibodies in an engineered chicken B cell line DT40-SW by increasing point mutation

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The chicken B cell line DT40 undergoes hypermutation of immunoglobulin variable region (IgV) genes during culture, thereby constituting an antibody (Ab) library. We previously established an *in vitro* Ab generation system using an engineered line DT40-SW whose hypermutation machinery can be switched on and off. Abs for various antigens (Ags) can be obtained from the DT40-SW library and the specificity of the Ag-specific clones can be stabilized by stopping hypermutation. Furthermore, the affinity of obtained monoclonal Abs (mAbs) can be improved through further mutation followed by selection, a process analogous to "affinity maturation" that occurs *in vivo*. Although gene conversion dominantly diversifies the IgV genes in DT40 cells, point mutation is considered to be more favorable for fine-tuning Ab properties during affinity maturation. Here, we examined whether affinity maturation occurs more efficiently when the hypermutation pattern was transformed from gene conversion into point mutation in DT40-SW cells. To this end, we disrupted the *XRCC3* gene that is essential for gene conversion. It was found that hemizygous disruption of the *XRCC3* gene was sufficient to increase the point mutation frequency. Since hemizygous disruption is conducted more easily, we tested whether the *XRCC3* (+/−) mutant generates high-affinity Abs through affinity maturation more efficiently than the wild type. Using this affinity maturation technique, we generated an improved 4-hydroxy-3-nitrophenylacetyl-specific mAb with ~600-fold lower K_D than that of the original mAb. Taken together, hemizygous disruption of the *XRCC3* gene is considered to be useful for obtaining high-affinity mAbs from DT40-SW cells though affinity maturation.

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[**Key words:** Antibody; Affinity maturation; Point mutation; Gene conversion; XRCC3; Chicken B cell line DT40]

In vitro screening systems have been developed to obtain desired monoclonal antibodies (mAbs) efficiently with avoiding the limitation of immunologic tolerance, which is a mechanism restricting generation of self-reactive Abs *in vivo*. For instance, a variety of single-chain Fv Abs have been selected from phage-display libraries (1–3). However, in immunized animals, antigen (Ag)-stimulated B cells undergo somatic hypermutation in the immunoglobulin variable region (IgV) genes and only B cells producing mutated Abs with high affinity are positively selected, a process termed affinity maturation (4–6), and thereby the *in vivo* affinity maturation is efficiently accomplished. On the other hand, the affinity of the primarily selected Abs from phage-display libraries can be also improved by diversifying the Ab clones with error-prone PCR, followed by expression in appropriate host cells to assess their biological activities. Generally, these processes of the *in vitro* affinity maturation are laborious and time-consuming. In this context, a chicken B cell line DT40 is considered to be useful to mimic the *in vivo* affinity maturation in *in vitro* culture. DT40 cells produce full-length Abs both in secreted and membrane-bound forms, and spontaneously introduce the activation-induced cytidine deaminase (AID)-dependent hypermutation in the IgV genes during culture (7–11), while the phage display system expresses Ab fragments and is not equipped with an intrinsic mutation machinery. Consequently, only a long-term culture of DT40 cells results in the formation of an Ab-displaying cell library, which would hold a wide variety of Ag specificities, in a cell-autonomous manner (7,8), whereas the other *in vitro* systems require separate processes for constructing libraries (e.g. error-prone PCR for mutation and phage DNA packaging for expression and display). It has been reported that several Ag-specific clones were successfully selected from the DT40 cell library (12–14). Thus, an Ab selection system using DT40 cells could be an alternative to conventional *in vitro* methods.

However, clones that are selected from the DT40 library may alter their Ag-specificity during expansion culture unless the hypermutation machinery is shut down. To overcome this problem, we have established an engineered DT40 line named DT40-SW, in which AID expression can be reversibly switched on and off using the Cre-loxP system driven by an exogenous estrogen derivative, 4-hydroxytamoxifen (4-OHT) (15–18). We have shown that Ag-specific mAb-producing clones were efficiently isolated from the DT40-SW library,

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and their Ag-specificity was genetically stabilized by switching off AID expression (14).

Another advantage of the DT40-SW system is that selected mAbs can be improved in their affinity for target Ags through further rounds of cell-based mutation and selection (14). Basically, this procedure mimics the affinity maturation that occurs *in vivo*. In the chicken, the IgV genes in Ag-stimulated B cells are diversified by gene conversion and point mutation, both of which have been shown to be AID-dependent (19). Gene conversion is a mutation mechanism that introduces partial sequences from upstream pseudo-V genes into the homologous regions of expressed IgV genes (20). In contrast, point mutation does not depend on pseudo-V genes (11). In young chickens, expansion of B cell repertoire during B cell development occurs predominantly through gene conversion in the specified organ called the bursa of Fabricius (21). On the other hand, during immune responses, Ag-stimulated B cells undergo hypermutation preferentially by point mutation in germinal centers (22). Thus it is suggested that gene conversion is effective in generating a wide variety of B cell repertoire, while point mutation that may enable to tune Ag-specificity finely is more favorable in mutating the IgV genes for affinity maturation (19, 22). Gene conversion has been shown to be a dominant mutation mechanism in cultured DT40 cells (7, 8). It has been reported that Rad51 paralogues, including XRCC2 and XRCC3, which are involved in homologous recombination during DNA damage repair, are essential in gene conversion (23). Interestingly, it has been shown that disruption of one of these genes led to the transformation of the mutation pattern from gene conversion into point mutation in DT40 cells (23, 24).

To explore whether point mutation is more favorable for affinity maturation of Abs in the DT40-SW system, we generated DT40-SW mutants in which the *XRCC3* gene was disrupted by gene targeting hemizygously (*XRCC3* (+/−)) or homozygously (*XRCC3* (−/−)). Interestingly, we found that the *XRCC3* (+/−) mutant that can be easily generated by a single gene targeting operation accumulated a comparable level of point mutations during culture with that observed in the *XRCC3* (−/−) mutant. Here, we examine whether the *XRCC3* (+/−) mutant is more useful than the wild type in obtaining high-affinity mAbs through affinity maturation in the DT40-SW system.

MATERIALS AND METHODS

Cell culture and transfection The DT40 cell line was obtained from RIKEN Cell Bank (Tsukuba, Japan). An engineered DT40 line, DT40-SW, whose AID expression can be switched on or off by Cre-mediated recombination, was established as reported previously (17, 18). DT40 cells were cultured in RPMI-1640 medium (MP Biomedicals, Irvine, CA, USA) supplemented with 10% fetal bovine serum (JRH Biosciences, Lenexa, Kansas, USA), 1% chicken serum (Sigma, St. Louis, MO, USA), 50 μM 2-mercaptethanol, 2 mM glutamine, 1 mM pyruvic acid, 100 μg/ml penicillin G, and 50 μg/ml streptomycin at 40 °C in 5% CO_2 and 95% air.

In transfection experiments, DT40-SW cells were suspended in 250 μl of phosphate-buffered saline (PBS) at 2×10^7 cells/ml and transfected with 15 μg of a linearized targeting vector by electroporation using Gene Pulser Xcell (Bio-Rad Laboratories, Hercules, CA, USA) at 550 V and 25 μF in 4 mm cuvette. The *XRCC3* alleles were disrupted by pXRCC3-his and pXRCC3-bsr vectors containing the histidinol dehydrogenase (*his-D*)

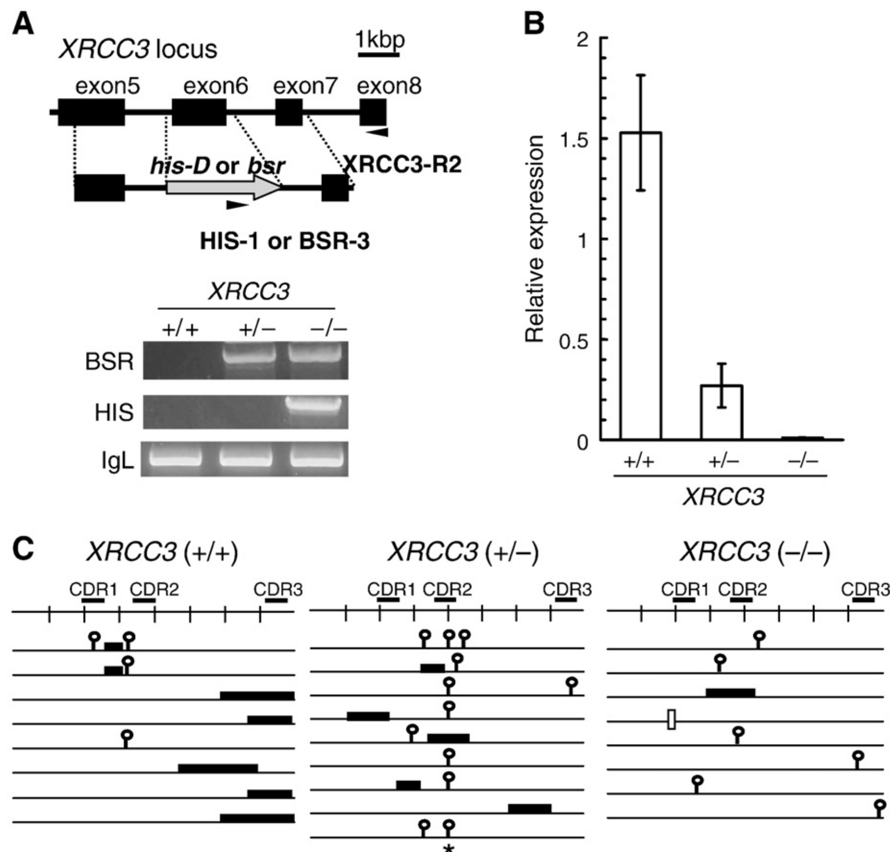

FIG. 1. Mutation pattern in the IgVL genes of the wild type and XRCC3-knockout DT40-SW cells. (A) Hemizygous (+/−) and homozygous (−/−) disruption of the *XRCC3* gene in DT40-SW were confirmed by genomic PCR. Primer pairs used are shown as arrowheads. The genomic DNA of the IgVL gene was amplified using primes CVLF-6 and CVLR-3 as a control. (B) Transcription of the *XRCC3* gene in the wild-type, *XRCC3* (+/−), and *XRCC3* (−/−) cells. The level of the XRCC3 transcript analyzed by quantitative RT-PCR is expressed as the normalized value (ΔCt/ΔCt) for that of the control β-actin transcript. Data are indicated as means ± standard deviations from triplicate assays. (C) Comparison of mutation pattern in the IgVL genes from XRCC3 wild type (+/+), hemizygous (+/−), and homozygous (−/−) DT40-SW cells. Thin horizontal lines represent the IgVL genes with mutations. Point mutation, gene conversion tract, and deletion are indicated as lollipop shape, thick horizontal bar above line, and hollow box, respectively. An asterisk indicates a common mutation found in 6 out of 9 mutated *XRCC3* (+/−) clones.

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