A novel strategy for the negative selection in mouse embryonic stem cells operated with immunotoxin-mediated cell targeting

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ABSTRACT

Immunotoxin-mediated cell targeting (IMCT) is a technique for conditionally ablating specific cell types based on the cytotoxic activity of a recombinant immunotoxin anti-Tac(Fv)-PE40. To examine the feasibility of this technique for the negative selection in mouse embryonic stem (ES) cells, we investigated the responsiveness of cells expressing human interleukin-2 receptor α subunit to anti-Tac(Fv)-PE40. The immunotoxin treatment efficiently eliminated only ES cells bearing the receptor as a consequence of the target specificity of anti-Tac(Fv)-PE40, indicating that IMCT can be used as a novel strategy for positive and negative selection to enrich ES cell clones with a targeted mutation.

Gene targeting in embryonic stem (ES) cells is a powerful technique for generating mice that carry null mutations into the germ line. The technique requires suitable selection procedures to enrich the rare ES cell clones in which the exogenous DNA sequence is introduced into the genetic locus of interest by eliminating the majority of the clones containing the random integration (1,2). A general method for negatively selecting the non-homologous recombinants is to use the herpes simplex virus thymidine kinase (tk) gene (1). Cells containing a functional tk gene become sensitive to nucleoside analogs, whereas homologous recombinants are resistant to these drugs due to the removal of the negative selection marker. Another method is the use of diphtheria toxin A-fragment (DT-A) gene that makes it possible to enrich homologous recombinants by a single G418 selection (3). To introduce subtle mutations into the target locus, several improved techniques have been designed which include a two-step recombination approach termed ‘hit and run’ (4) and double replacement (5,6). In these techniques, the ES cell clones with desired mutations are negatively selected on the basis of removal of the tk gene in the second step of homologous recombination. However, the DT-A gene cannot be used in this negative selection because of the cytotoxic activity of the gene per se.

Immunotoxin-mediated cell targeting (IMCT) has been developed to conditionally ablate the specific cell types of transgenic mice (7). The strategy of IMCT is based on the target specificity of a recombinant immunotoxin, anti-Tac(Fv)-PE40, which is composed of the variable chains of the anti-Tac antibody against human interleukin-2 receptor α-subunit (IL-2Rα) fused to a truncated form of Pseudomonas exotoxin (8,9). The immunotoxin treatment induces degeneration of the specific cell types engineered to express IL-2Rα in transgenic mice.

The IMCT strategy provides an approach for the negative selection of ES cells that could be used instead of the tk or DT-A gene. To examine the feasibility of this approach, we have introduced a vector containing the neo and IL-2Rα expression cassettes into ES cells, and examined the responsiveness of cells bearing the receptor to anti-Tac(Fv)-PE40. We show that IMCT can be used as a novel technology for the positive–negative selection to enrich homologous recombinants.

The plasmid vector termed pPGK-neo/IL-2Rα (Fig. 1A) contains the IL-2Rα expression cassette (7) driven by the pgk-1 gene promoter (10) as a negative selection marker, placed downstream of the positive selection marker in the PGK-neo gene cassette (11) with the same direction of transcription. The plasmid containing PGK-neo/IL-2Rα or PGK-neo gene cassette was linearized, electroporated into E14 ES cells and positively selected with G418 (400 µg/ml). Out of several drug-resistant ES clones, the cells carrying a single copy of plasmid per genome were identified on the basis of Southern blotting data. Poly (A)* RNA prepared from the cells carrying the two kinds of plasmids was subjected to reverse transcription–PCR analysis. A single band of 0.8 kb containing the coding region of IL-2Rα was only detected in the PGK-neo/IL-2Rα transfected cells (Fig. 1B). Also, immunofluorescence analysis with anti-Tac antibody (12) demonstrated the expression of the IL-2Rα receptor in colonies derived from the same cells (Fig. 1C).

To investigate the responsiveness of the ES cells expressing IL-2Rα to anti-Tac(Fv)-PE40, the colony forming efficiency of ES cells carrying a single copy of PGK-neo was compared with a clone carrying the PGK-neo/IL-2Rα gene cassette (Fig. 2). (The

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anti-Tac(Fv)-PE40 is available from Dr Pastan on request.) Immuno toxin treatment was not cytotoxic to the ES cells carrying the PGK-neo gene over a concentration range from 0.5 to 200 ng/ml; also the growth and morphology of these cells appeared to be normal. In contrast, the plating efficiency of the cells carrying the PGK-neo/IL-2Rα gene was decreased depending on the immunotoxin concentration used. The ID₅₀ value was ~1.3 ng/ml. Also, a large number of cell expressing the receptor (3 x 10⁶ cells) were plated and selected with 100 ng/ml of the immunotoxin. As a result of treatment, the plating efficiency fell to ~5 x 10⁻². These data indicate that the anti-Tac(Fv)-PE40 can efficiently kill ES cells bearing the IL-2Rα receptor under conditions where the growth and morphology of ES cells not expressing the receptor is not affected. This result is a consequence of the target specificity of anti-Tac(Fv)-PE40.

Our results show that IMCT can be used as a novel strategy for positive and negative selection of mouse ES cells to efficiently isolate cell clones with a targeted mutation. In our preliminary studies, the IMCT selection was used for introducing a mutation into several genetic loci with the targeting vector that includes the IL-2Rα expression cassette in the 5'- or 3'-end. The number of the G418-resistant and immunotoxin-sensitive cell clones was decreased by a factor of 10 as compared with that of the cell clones obtained from only G418 selection, and the enrichment factor was of the same order of magnitude as with tk or DT-A negative selections (data not shown).

The IMCT selection can be also used for negative selection in order to introduce subtle mutation in the two-step recombination procedures (4–6) instead of the tk gene. The initial targeted clones obtained from procedures that introduce a targeted mutation by exchange using the neo and tk selection markers cannot be used to produce knock-out mice since it is known that ES cells having a functional tk gene cannot be normally transmitted into the germ line due to disruption of sperm development (13).
IMCT as a negative selection might overcome the limitation of the \( th \) gene selection because expression of human IL-2R\( \alpha \) should not affect normal development of germ line cells. This possibility is now being examined by producing chimeric mice with the ES cells carrying the IL-2R\( \alpha \) gene cassette.

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