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Correction of a Genetic Defect by Nuclear Transplantation and Combined Cell and Gene Therapy

William M. Rideout III,[1,6] Konrad Hochedlinger,[1,2,6] Michael Kyba,[1,6] George Q. Daley,[1,3] and Rudolf Jaenisch[1,4,5]
[1]Whitehead Institute for Biomedical Research
9 Cambridge Center
Cambridge, Massachusetts 02142
[2]University of Vienna
A-1010 Vienna
Austria
[3]Division of Hematology/Oncology
Massachusetts General Hospital
Harvard Medical School
Boston, Massachusetts 02115
[4]Department of Biology
Massachusetts Institute of Technology
Cambridge, Massachusetts 02142

Summary

Immune-deficient Rag2$^{-/-}$ mice were used as nuclear donors for transfer into enucleated oocytes, and the resulting blastocysts were cultured to isolate an isogenic embryonic stem cell line. One of the mutated alleles in the Rag2$^{-/-}$ ES cells was repaired by homologous recombination, thereby restoring normal Rag2 gene structure. Mutant mice were treated with the repaired ES cells in two ways. (1) Immune-competent mice were generated from the repaired ES cells by tetraploid embryo complementation and were used as bone marrow donors for transplantation. (2) Hematopoietic precursors were derived by in vitro differentiation from the repaired ES cells and engrafted into mutant mice. Mature myeloid and lymphoid cells as well as immunoglobulins became detectable 3–4 weeks after transplantation. Our results establish a paradigm for the treatment of a genetic disorder by combining therapeutic cloning with gene therapy.

Introduction

The development of somatic cell nuclear transfer (NT) techniques to produce viable cloned mammals (Wakayama et al., 1998; Wilmut et al., 1997) demonstrated the ability of oocyte cytoplasm to reprogram a somatic donor nucleus to a pluripotent state (Rideout et al., 2001). Additionally, embryonic stem (ES) cells have been derived from blastocysts generated by transfer of somatic cell nuclei (Kawase et al., 2000; Munsie et al., 2000; Wakayama et al., 2001; Hochedlinger and Jaenisch, 2002). These "NT ES" cells have been shown to differentiate in vitro into cells of several different developmental lineages including neurons, blood, and cardiac muscle. In addition, NT ES cells were shown to contribute extensively to diploid chimeras (Wakayama et al., 2001) and to generate fertile mice following tetraploid embryo complementation (Hochedlinger and Jaenisch, 2002). Because somatic nuclear transfer allows the isolation of ES cells genetically matched to diseased individuals, this "therapeutic cloning" or "nuclear transplantation therapy" (Vogelstein et al., 2002) approach has been suggested as an attractive possibility to treat a host of medical problems such as hematopoietic and cardiac disorders and diseases such as diabetes, Alzheimer's, and Parkinson's (Colman and Kind, 2000). In addition, the availability of ES cells opens the prospect for repairing a gene defect by homologous recombination, which has been shown to be effective in correcting a spontaneous mutation in a wild-type (wt) ES cell line (Doetschman et al., 1987). This report provides the proof of principle for therapeutic cloning combined with gene therapy to treat a form of severe combined immune deficiency in mice.

We chose a mouse strain with a defined genetic disorder to develop a model that combines therapeutic cloning with gene and cell therapy (Figure 1). The basic steps involve (1) nuclear transfer of a somatic cell nucleus from the affected donor mouse into an enucleated oocyte, (2) activation and cultivation of the NT embryo to the blastocyst stage, (3) isolation and culture of ES cells (ntES) from the blastocyst, (4) repair of the genetic defect by homologous recombination, (5) differentiation of the repaired ntES cells in vivo, via tetraploid embryo complementation, or in vitro into hematopoietic stem cells (HSCs), and (6) transplantation of the "repaired" HSCs into affected donor mice. As a model we selected the severe combined immune deficiency caused by inactivation of the Rag2 recombinase resulting in the complete absence of mature B and T cells in the lymphoid organs and absence of immunoglobulins from the serum of the mouse (Shinkai et al., 1992). The immune deficiency in Rag1 and 2 knockout mice resembles Omenn syndrome and the severe combined immune deficiency seen in humans homozygous for mutations at either RAG1 or RAG2 (Notarangelo et al., 1999). Rag2 null mice remain viable and have a normal lifespan when housed in a clean animal facility. Importantly, their lymphoid system can be restored by transplantation of isogenic bone marrow or fetal liver hematopoietic stem cells from wild-type mice. Therefore, the Rag2 mutant mice provide a sensitive experimental system to detect functional engraftment of hematopoietic stem cells derived from genetically modified ES cells.

Therapeutic cloning for treating an immune deficiency depends on the in vitro differentiation of ntES cells into functional hematopoietic cells that are able to provide long-term repopulation of the lymphoid compartment after transplantation. ES cells can be differentiated in vitro into hematopoietic precursors, as demonstrated by the appearance of blood islands in embryoid bodies (EB) and the isolation of several types of primitive hematopoietic colonies from EBs (Keller et al., 1993; Wiles and Keller, 1991). While there have been reports of repopulation of hematopoietic lineages in lethally irradiated mice, efficient methods for functional, long-term, and multilineage hematopoietic engraftment from ES

[5]Correspondence: jaenisch@wi.mit.edu
[6]These authors contributed equally to this work.

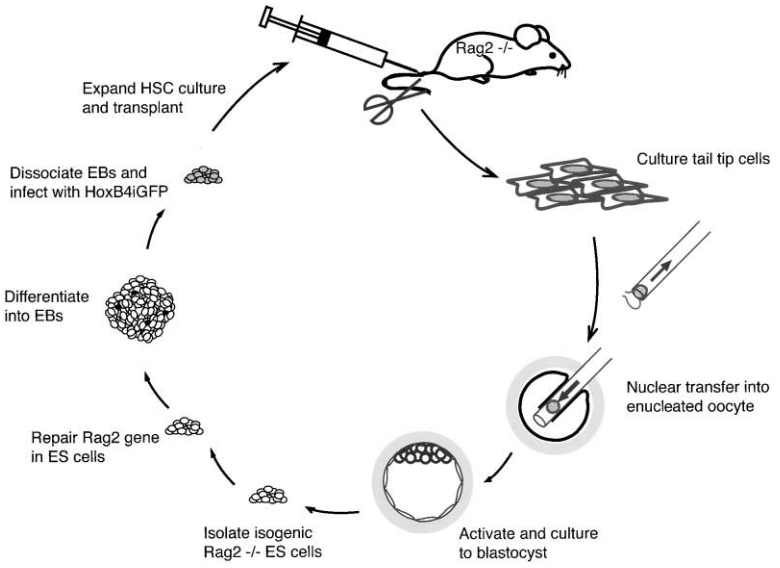


Figure 1. Scheme for Therapeutic Cloning Combined with Gene and Cell Therapy

A piece of tail from a mouse homozygous for the recombination-activating gene 2 (Rag2) mutation was removed and cultured. After fibroblast-like cells grew out, they were used as donors for nuclear transfer by direct injection into enucleated MII oocytes using a Piezo-electric-driven micromanipulator. Embryonic stem (ES) cells isolated from the NT-derived blastocysts were genetically repaired by homologous recombination. After repair, the ntES cells were differentiated in vitro into embryoid bodies (EBs), infected with the HoxB4iGFP retrovirus, expanded, and injected into the tail vein of irradiated, Rag2-deficient mice.

cells have been lacking (Hole et al., 1996; Muller and Dzierzak, 1993; Potocnik et al., 1997). Recently, we showed that expression of the leukemia-associated BCR/ABL oncogene in differentiating ES cells enabled engraftment of mice with leukemic lymphoid and myeloid elements (Perlingeiro et al., 2001). The BCR/ABL experiments also demonstrated that the target cell required for our purpose, the lymphoid-myeloid HSC, was present by day 5 in embryoid bodies derived from ES cells (Perlingeiro et al., 2001). In the current study, we used an improved method for deriving normal hematopoietic progenitors by genetic modification of ES cells with the Homeobox gene HoxB4, which provides a means for functional hematopoietic reconstitution of lethally irradiated mice (Kyba et al., 2002 [this issue of *Cell*]). Here we show that "repaired" ES cells derived from a Rag2-deficient mouse can be differentiated into functional hematopoietic stem cells that restore immune function when transplanted into adult Rag2 mutant mice.

Results

NT and Pluripotent ntES Cell Derivation

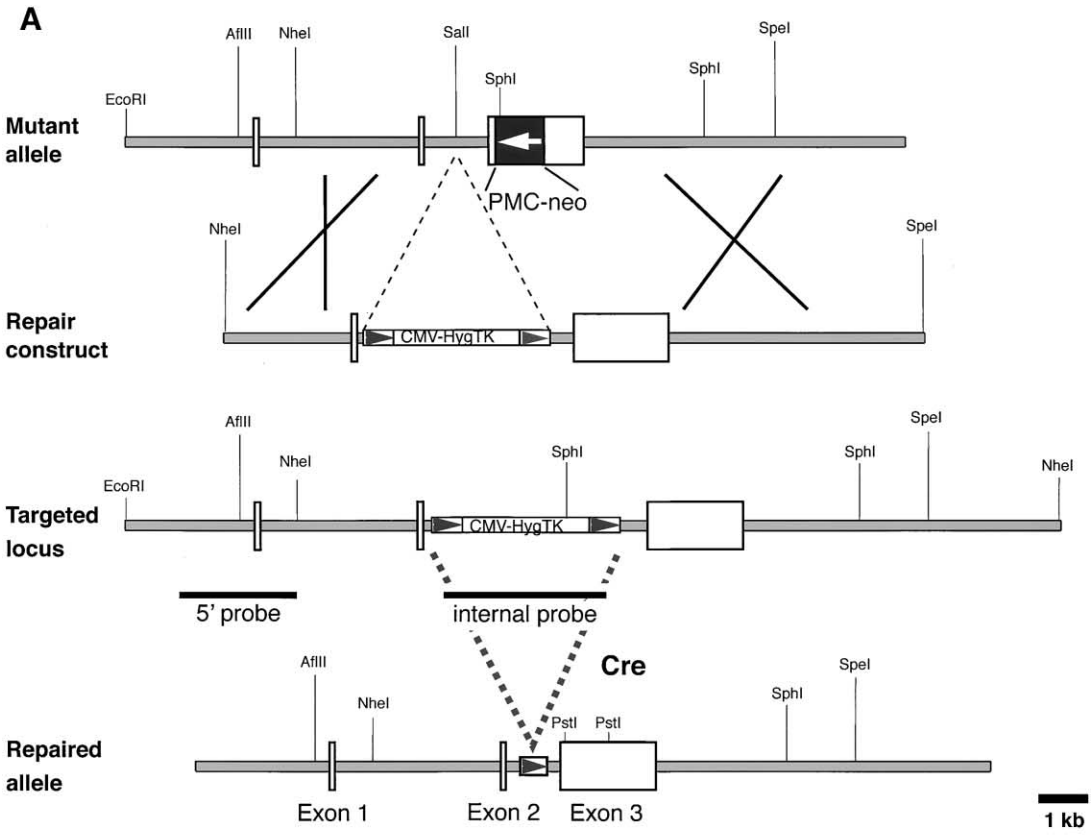
The immunodeficient mouse model for Rag2 deficiency has previously been generated by deletion of part of the third coding exon and the insertion of a pMCneo cassette transcribed in the opposite orientation (Figure 2A; Shinkai et al., 1992). Tail-tip cells from a Rag2$^{-/-}$ male mouse (129Sv/Ev X C57Bl/6 [129B6F1]) were used as nuclear donors for transfer into enucleated MII (metaphase II) oocytes by the "Honolulu" method (Wakayama et al., 1998). The development of the tail-tip NT embryos to the blastocyst stage was usually delayed compared to in vitro activated and cultured parthenogenetic embryos (4.5 and 3.5 dpc, respectively). Approximately 13% (27 of 202) of the reconstructed oocytes developed into blastocysts, and of these one generated an ES cell line (Rag2$^{-/-}$ ntES). While this rate of blastocyst formation following nuclear transfer from tail-tip cells was lower than that reported by others (13% versus 38%; Wakayama et al., 2001), the rate of ES cell derivation from the cloned blastocysts was comparable (3% versus 6%).

The resulting cell line, Rag2$^{-/-}$ ntES, was tested for pluripotency by the most stringent method available, namely, tetraploid embryo complementation (Eggan et al., 2001; Hochedlinger and Jaenisch, 2002; Nagy et al., 1993). ES cell complementation of tetraploid host blastocysts results in the embryo being completely derived from the injected ES cells while the tetraploid host cells contribute to the placenta (Wang et al., 1997). We previously showed that injection of wild-type F1 ES cells into tetraploid blastocysts resulted in viable mice from 4%–10% of the manipulated embryos (Eggan et al., 2001) and that injection of ntES cells derived from F1 lymphocytes also gave rise to viable mice (Hochedlinger and Jaenisch, 2002). Similarly, the Rag2$^{-/-}$ ntES line generated four viable pups out of 14 reconstituted tetraploid blastocysts (28%), indicating that this line was pluripotent and able to efficiently generate all somatic cell types (Table 1).

Repair of the Rag2 Mutation in the Rag2$^{-/-}$ ntES Line

We restored Rag2 function in the Rag2$^{-/-}$ ntES line by homologous recombination followed by cre recombinase-mediated removal of the loxP-flanked selectable marker (Hygtk) (Figure 2A). Because the selectable marker was positioned close to the site of the insertion/deletion mutation of the Rag2 null allele (approximately 0.5 kb), recombination occurring between the site of the Hygtk cassette and pMC-Neo insertion in the mutant allele was unlikely. Southern analysis of DNA from Hyg-resistant subclones was performed with a 5′ probe (Figure 2B) to check for homologous recombination and by an internal probe to exclude random integrations (data not shown). Correct targeting was found in 58/288 (20%) of the subclones, demonstrating that NT-derived ES cells can be effectively targeted by homologous recombination like normal mouse ES cells.

Two targeted subclones (#4 and #132) were transiently



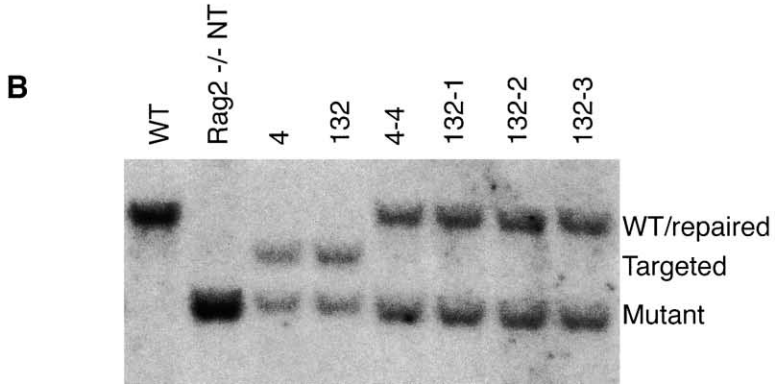


Figure 2. Repair of the Rag2 Mutant Allele

(A) Scheme for repairing the knockout allele of Rag2. The top line illustrates the mutant allele, showing the replacement of much of Exon 3 by the selectable pMCNeo cassette. The repair contruct for targeting is shown below with the LoxP-flanked selectable CMV Hygtk cassette inserted into a SalI site between exons 2 and 3 (CMV, cytomegalovirus promoter; Hygtk, hygromycin resistance/thymidine kinase fusion gene). The next two lines illustrate the structure of the targeted allele and the repaired allele (after Cre recombinase-mediated loop-out of CMV-Hygtk). Relevant restriction sites and 5′ and internal probes for Southern analysis are shown. Exons are shown as open rectangles (exons 1 and 2 are not to scale). The scale is as shown (kb, kilobase).

(B) Southern analysis of ES cell DNA. Ten micrograms of each DNA was digested by AflII and SphI overnight, electrophoresed on a 0.85% agarose gel, blotted to nylon membrane, and probed with ^{32}P-labeled 5′ probe. (WT, wild-type; Rag2$^{-/-}$ ntES, nuclear transfer-derived ES cells; 4, 132, targeted subclones; 4-4, 132-1, 132-2, and 132-3, repaired subclones).

transfected with a cre-expressing plasmid (pCrePAC) (Taniguchi et al., 1998) and selected with gancyclovir for loop-out of the Hygtk-selectable marker (Figure 2A). Southern analysis with the 5′ probe on DNA from gancyclovir-resistant subclones detected the loss of the selectable Hygtk marker and no additional gene rearrange-

Table 1. Mice Derived from Tetraploid Embryo Complementation with Rag2$^{-/-}$ and Rag2$^{+R/-}$ ntES Cell Lines

ES Line	# 4n Blastocysts Injected	Live Pups (Number of Neonatal Death)
Rag2$^{-/-}$	14	4 (0)
Rag2$^{+R/-}$	226	38 (9)

ments in the repaired allele (Figure 2B). This restored normal Rag2 gene structure on one allele and left a single loxP site in the second intron (this allele was designated Rag2^{+R}). In order to assess whether the gene targeting had restored proper gene function, mice were generated from the repaired ES cells.

Tetraploid Embryo Complementation with the Rag2$^{+R/-}$ ES Lines

The genetically repaired ntES cells were used to generate mice by tetraploid embryo complementation (Hochedlinger and Jaenisch, 2002) in which the embryo proper is entirely derived from the ntES cells, and the extraembryonic lineages are derived from the tetraploid host blastocyst. Therefore, successful correction of the Rag2 mutation can be directly assessed by analyzing immune function in the "repaired" ES cell-derived animals. Furthermore, neonate blood (analogous to cord blood transplants) or adult bone marrow harvested from the animals can be transplanted into adult Rag2 mutant recipients to evaluate their potential to colonize the lymphoid compartment and correct the immune deficiency. We injected 226 tetraploid blastocysts with four different repaired subclones (4-4, 132-1, 132-2, and 132-3) and obtained 38 live pups (16%) delivered by C-section (Table 1). Of these, nine died shortly after delivery, but the rest were viable, healthy, and fertile. Thus, the repaired Rag2$^{+R/-}$ ntES cells remained fully pluripotent, with no loss of developmental potential.

We analyzed the lymphoid cells of the Rag2$^{+R/-}$ ntES mice derived by tetraploid embryo complementation to determine whether the repaired allele was functional. PCR analysis to detect rearrangements at the immunoglobulin heavy chain and T cell receptor β loci showed the presence of multiple rearranged alleles in the thymus and spleen of Rag2$^{+R/-}$ ntES-derived mice, while no rearranged alleles and only the germline allele were seen in mice derived from the original Rag2$^{-/-}$ ntES line (Figures 3A and 4A). In addition, peripheral blood from Rag2$^{-/-}$ ntES- and Rag2$^{+R/-}$ ntES-derived mice was compared to blood from a wild-type mouse by flourescence-activated cell sorting (FACS) with antibodies against markers for B cells (B220 and IgM) and T cells (CD4 and CD8) (Figure 3B). The relative numbers of B and T cells detected in Rag2$^{+R/-}$ ntES mice were comparable to the B and T cell populations in wild-type mice; in contrast, blood from mice derived from the parental Rag2$^{-/-}$ ntES line showed essentially no mature B and T cells. This proved that the repaired Rag2 allele could restore normal TCR and immunoglobulin rearrangements and enable B and T cell production during normal development of mice derived by tetraploid embryo complementation.

The mice derived from the repaired ES cells were used as HSC donors (from neonate peripheral blood and bone marrow from 1-month-old mice) for transplantation back into sublethally irradiated Rag2 null mice. After two to three months the mice were bled and analyzed by FACS, which showed the presence of mature B and T cells (Figure 3C). The relative level of mature B and T cells (30.3% ± 9.2% and 27.7% ± 6.1% [n = 6], respectively) in total peripheral blood mononuclear cells (PBMCs) was similar to that of normal mice. This indicates that the repaired ntES cells gave rise to normal bone marrow that restored the lymphoid system after transplantation into Rag2 mutant mice. The donor HSCs in these experiments were generated during the course of normal mouse development in the tetraploid complementation embryos. The restoration of immune function in the recipients indicated that bone marrow cells derived from the "repaired" ES cell mice were able to fully function after transplantation into Rag2 mutant host animals.

We next assessed whether in vitro differentiation of the repaired ES cells instead of in vivo formation of normal bone marrow would allow the generation of definitive hematopoietic stem cells that could be used for transplantation into mutant animals.

In Vitro Differentiation of Rag2$^{+R/-}$ ntES Cells and Transplantation into Rag2 Mutant Mice

Therapeutic cloning requires that the ntES cells be differentiated in vitro into the relevant tissue or cell types followed by transplantation into affected nuclear donors. We recently established a method for in vitro differentiation of ES cells into embryonic hematopoietic stem cells that could be used for long-term lymphoid and myeloid engraftment of lethally irradiated mice (Kyba et al., 2002 [this issue of *Cell*]). We used this system to attempt restoration of immune function in immunodeficient Rag2$^{-/-}$ mice with the Rag2$^{+R/-}$ ntES cells. After differentiating the Rag2$^{+R/-}$ ntES cells into EBs for 6 days, we introduced the HoxB4 and GFP (green fluorescent protein) genes by retroviral transduction with the MSCVHoxB4iGFP vector (Kyba et al., 2002 [this issue of *Cell*]). The infected cells were then cultured for 14 days on OP9 stromal cells in the presence of hematopoietic cytokines to promote formation of HSCs that could be used for transplantation into Rag2$^{-/-}$ isogenic mice. Our initial transplantation of hematopoietic derivatives of Rag2$^{+R/-}$ ntES cells into Rag2$^{-/-}$ mice showed little to no chimerism of the hematopoietic compartment as assessed by the numbers of GFP-positive cells in the peripheral blood of recipient mice (data not shown). This suggested that resistance to engraftment was a property of the Rag2-deficient recipients and not the ntES cells, because HoxB4-modified embryonic hematopoietic stem cells engrafted in isogenic wild-type but not Rag2-deficient recipients (not shown).

Host NK Cells Present a Barrier to Engraftment of Hematopoietic Progeny of the ntES Cells

Yolk sac hematopoietic progenitors, which are closely related to EB-derived progenitors, have lower major histocompatability complex (MHC) expression than bone marrow-derived HSCs (Cumano et al., 2001; Huang and Auerbach, 1993), and it is known that hematopoietic cells with low MHC expression are a target for NK cell-

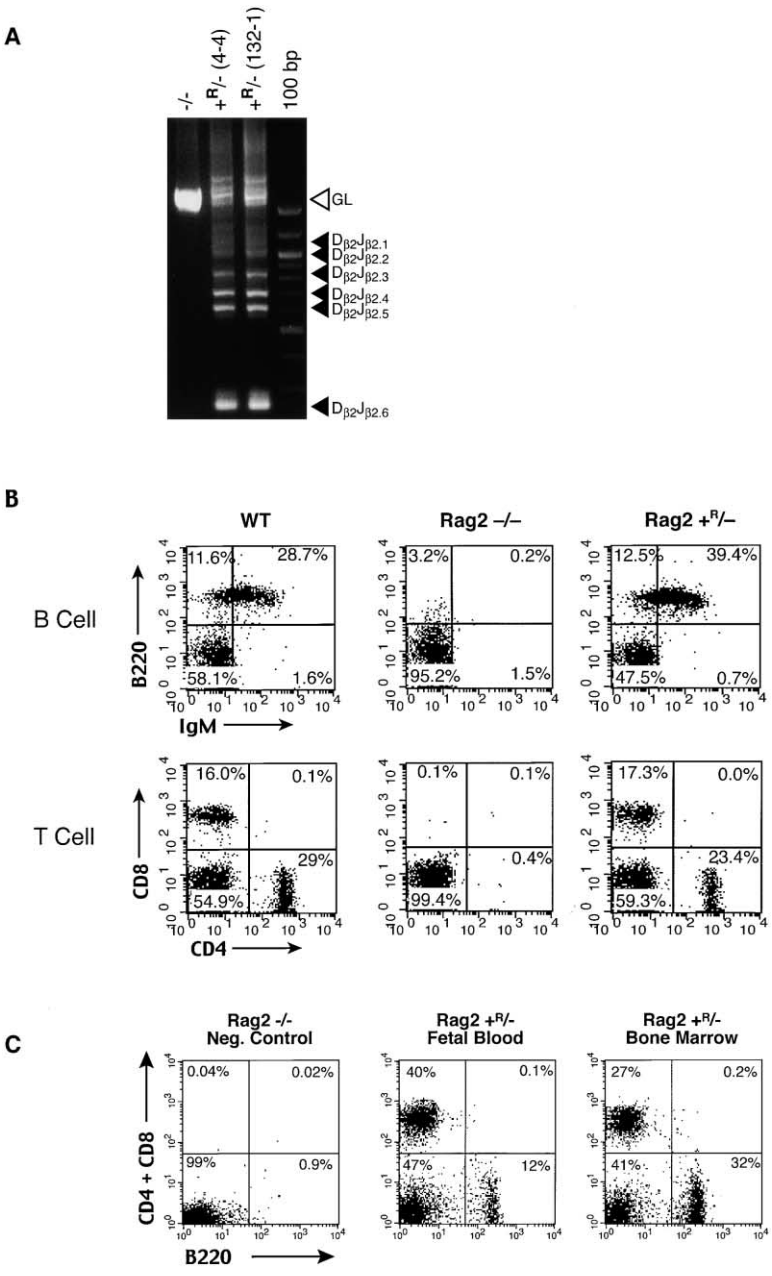


Figure 3. Analysis of Lymphoid Cells from "Repaired" Mice and Mice Engrafted from "Repaired" Donors

(A) PCR analysis for rearrangments at the TCR β locus. PCR products amplified from thymus DNA from tetraploid embryo complementation-derived neonatal mice were electrophoresed on a 1.5% agarose gel, stained, and photographed. Recombination products of TCR Dβ2Jβ2 elements are shown using primers 5 and 7 (Whitehurst et al., 1999). Filled triangles indicate rearranged alleles; open triangle indicates germline unrearranged allele. Last lane shows 100 bp ladder (New England Biolabs).

(B) FACS analysis of mature B and T cells in peripheral blood. Peripheral blood was obtained from a wild-type mouse (WT) and ntES cell-derived mice and was immunostained with antibodies against B cell markers, PE-B220 and FITC-IgM (top row, as indicated), or against T cell markers, FITC-CD4 and PE-CD8 (bottom row, as indicated). The percentage of gated lymphoctes in each quadrant is shown.

(C) FACS analysis of peripheral blood from 129B6F1 Rag2$^{-/-}$ mice engrafted with neonate blood or bone marrow (from a 28-day-old mouse) from Rag2$^{+R/-}$ ntES-derived mice. The samples were stained with FITC-B220, PE-CD4, and PE-CD8 antibodies. The FACS plots show the percentages of gated peripheral blood mononuclear cells (PBMCs).

mediated rejection (Bix et al., 1991). Indeed, expression of the two class I MHC genes (H2-K^{B} and H2-D^{B}) was significantly lower in the Rag2$^{+R/-}$ ntES-derived HSCs than in bone marrow (not shown). Therefore, it appeared possible that enhanced NK activity in Rag2 mutant recipients was preventing engraftment of ES cell-derived HSCs.

We tested this hypothesis using two different approaches. First, pretreatment of the Rag2$^{-/-}$ mice with an anti-NK1.1 antibody that depletes NK cells responsible for the phenomenon of hybrid resistance prior to transplantation (Kung and Miller, 1995; Lee et al., 1996) resulted in low-level reconstitution of hematopoietic cells in the peripheral blood as assessed by FACS (≈1.5%; Figure 4A, upper left). A small minority of these cells stained with B220 antibody (0.13% of total PBMC), demonstrating that the repaired ntES cells contributed to the B cell lineage. However, staining for IgM-positive B cells and mature CD4- and CD8-positive T cells was essentially negative (data not shown). Analysis of these mice showed persistence of NK1.1-positive cells in peripheral blood, suggesting that immunodepletion was inefficient and incomplete. Therefore, in the second approach, we engrafted hematopoietic derivatives of the Rag2$^{+R/-}$ ntES cells into Rag2$^{-/-}$ recipients with a complete absence of NK cells due to deletion of the IL2 common cytokine receptor γ chain (γC) (Mazurier et al., 1999). This strategy has recently been shown to enhance engaftment of definitive intraembryonic populations of hematopoietic progenitors (Cumano et al., 2001). FACS analysis of PBMCs from these double-mutant animals transplanted with the Rag2$^{+R/-}$ ntES cells showed es-

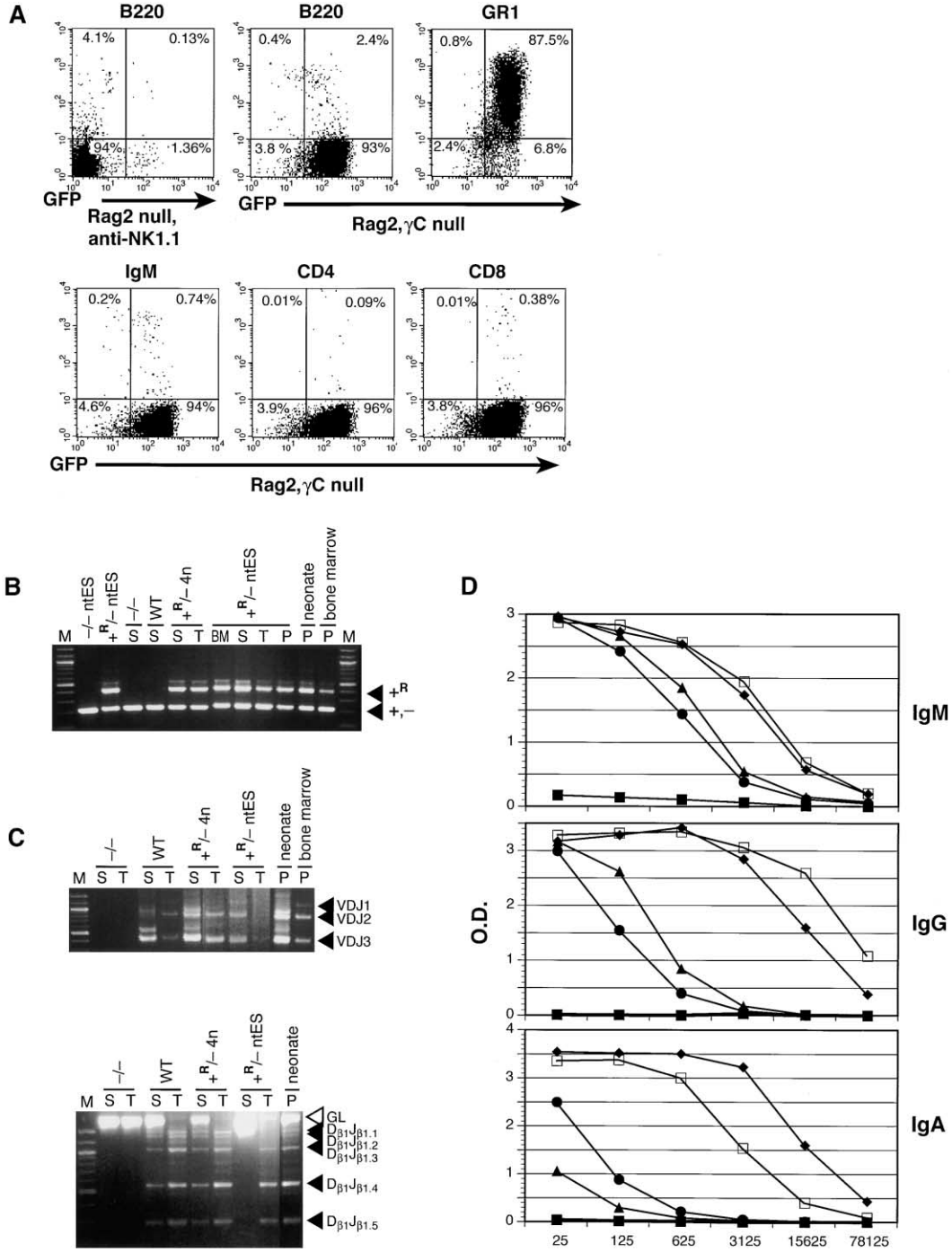


Figure 4. Functional Analysis of ntES Cell-Engrafted Mice

(A) FACS analysis of peripheral blood. GFP intensity is shown along the *x* axis, and the PE conjugated B220, GR1, IgM, CD4, or CD8 antibody fluorescence is shown on the *y* axis. Percentage of gated mononuclear cells is shown in each quadrant.

(B) PCR was performed to detect the repaired Rag2^{+R} allele. The primers KH1 and KH2 flank the SalI site where the loxP-flanked selectable marker CMVHygtk was inserted (Figure 1). The wild-type (+) and unrepaired (–) Rag2 alleles give a 200 bp product, while the repaired allele (+^{R}) gives a 400 bp band. The left two sample lanes show controls from ES cell DNA (–/– ntES; +^{R}/–) followed by spleen (S), thymus (T), bone marrow (BM), and peripheral blood (P) DNA samples isolated from Rag2 null (−/−), wild-type (WT), and repaired ntES tetraploid embryo complementation (+^{R}/− 4n) mice; the next four lanes show samples from a Rag2$^{-/-}$, γC$^{-/-}$ mouse engrafted with repaired ES cells (+^{R}/− ntES); and the last two lanes show samples from Rag2$^{-/-}$ mice engrafted with neonate blood or bone marrow donated from a repaired ntES tetraploid complementation mouse.

(C) PCRs were performed to detect IgH (upper gel) (Schlissel et al., 1991) and TCRβ (primers 1 and 4; lower gel) (Whitehurst et al., 1999) rearrangments in spleen (S), thymus (T), or peripheral blood (P) DNA isolated from Rag2 null (−/−), wild-type (WT), and Rag2$^{+R/-}$ (ntES tetraploid embryo complementation) mice and a Rag2$^{-/-}$, γC$^{-/-}$ mouse engrafted with repaired ES cells (+^{R}/− ntES). The last lanes show samples from Rag2$^{-/-}$ mice engrafted with neonate blood or bone marrow donated from repaired ntES tetraploid complementation mice. DNA (50 ng) was

sentially complete donor chimerism (95% GFP-positive PBMC; Figure 4A, Rag2, γC null graphs), with predominantly myeloid repopulation as shown by extensive staining with GR1, a marker for granulocytes (94%; Figure 4A, third graph), and limited staining with B220, a marker for B cells (2.4%; Figure 4A, second graph). FACS analysis detected a low level of GFP-positive, mature B cells by IgM staining (0.74%) and GFP-positive, mature T cells by CD4 and CD8 staining (0.09% and 0.38%, respectively). The detection of lymphocytes in the peripheral blood suggested that some lymphoid progenitors derived from the ntES cells were maturing in the engrafted mice.

To ensure that the engrafted cells in the double-mutant mice were derived from the repaired ntES cells, we performed PCR analysis to detect the $Rag2^{+R}$ allele. The repaired allele was detected in DNA isolated from hematopoietic tissues of mice transplanted with in vitro differentiated ES cells or neonate blood/bone marrow from ntES-derived mice by tetraploid embryo complementation (Figure 4B). In contrast, the repaired allele was absent in DNAs from wt and $Rag2^{-/-}$ control animals (Figure 4B).

To confirm that the proper rearrangements necessary for B and T cell function had occurred in transplanted double-mutant mice, PCR analyses of IgH and TCR loci were performed on lymphoid organs of a mouse 3.5 weeks after transplantation. Multiple rearranged alleles were detected, indicating that the transplanted ntES cell derivatives gave rise to polyclonal reconstitution of the B and T cell compartments (Figure 4C, ntES graft). The level of TCR β gene rearrangement was about 20% of that seen in $Rag2^{+R/-}$ ntES mice, derived by tetraploid embryo complementation, or wt mice. Levels of detectable IgH gene rearrangement in the spleen were much lower, approximately 2% of wt. Moreover, we tested serum of ntES cell-engrafted animals and controls for the presence of immunoglobulins of the IgM, IgG, and IgA classes (Figure 4D). In contrast to the untreated $Rag2^{-/-}$, $\gamma C^{-/-}$ control, all treated mice demonstrated the presence of serum IgM, IgG, and IgA. In agreement with the fewer peripheral blood lymphocytes in the ntES-treated mice, serum Ig levels, particularly IgA, were lower than in controls. IgM levels were 10- to 15-fold lower in the engrafted mice compared to wt, and IgG and IgA were approximately 125-fold lower. Thus, despite low levels of B and T cells in the peripheral blood of the $Rag2^{-/-}$, $\gamma C^{-/-}$ mice, some immune function was restored in the mice engrafted with in vitro repaired and differentiated ntES.

Discussion

The goal of our work was to demonstrate the feasibility of correcting a genetic defect in somatic cells of an affected individual using a combination of reprogrammed somatic cell therapy, often designated as "therapeutic cloning" or "nuclear transplantation therapy" (Vogelstein et al., 2002), and gene therapy. The procedure involved the isolation of tail-tip cells from mutant mice that are severely immune deficient due to the mutation of the Rag2 recombinase gene. Nuclei from mutant tail tip cells were transferred into enucleated oocytes, and ES cells were derived from one of the cloned blastocysts. Standard homologous recombination was used to correct the gene defect in the ntES cells. To assess whether the genetic manipulation restored recombinase function, we derived mice from the repaired ntES cells by tetraploid embryo complementation. The lymphoid compartment of these animals consisted entirely of the repaired ES cells and was normal, as demonstrated by B and T cell numbers that are typical for wt mice. As expected, Rag2 mutant mice that were transplanted with bone marrow from the ntES cell-derived mice showed a complete restoration of immune function. Thus, homologous recombination in the ntES cells corrected the genetic defect in the donor Rag2 mutant mouse strain.

A critical step in nuclear transplantation therapy is the derivation in vitro of functional somatic cells from the cloned ES cells that can be used for transplantation into the diseased individual. As shown in the accompanying paper, expression of the homeobox gene HoxB4 enables embryonic hematopoietic stem cells to stably engraft and chimerize long-term the lymphoid and myeloid lineages of transplanted mice (Kyba et al., 2002 [this issue of *Cell*]). Here we have applied the principles defined in the accompanying report to generate HSCs from the repaired ntES cells. As discussed below, we have confronted two challenges in treating the immunodeficiency in our model. First, our attempts at hematopoietic repopulation were hindered by an engraftment barrier peculiar to the Rag2-deficient recipients, which we have linked to NK cell function. Second, the repaired cells preferentially engraft the myeloid lineages and show a relative block to T cell maturation by an as yet undefined mechanism. Therefore, while our initial attempts at therapeutic cloning have succeeded in restoring a modest degree of immune function, we have uncovered interesting and unanticipated biological principles that must be more fully defined to make therapeutic cloning more successful in this system. Our current state of understanding of these challenges is outlined below.

The ntES cell-derived HSCs express low levels of MHC (not shown). Because it has been well established that low MHC expression on HSCs can lead to NK cell-mediated graft rejection (Bix et al., 1991) and that Rag2-deficient mice retain NK cell function, we tested whether inhibition of NK activity would improve engraftment of ES donor cell-derived HSCs into Rag2 recipients. Our initial pilot experiments with immunodepletion of NK cells in the $Rag2^{-/-}$ mice prior to transplantation re-

used as template for each reaction, except for the amplification of IgH alleles from the ntES-transplanted mouse (500 ng). Filled triangles indicate rearranged alleles; open triangle indicates germline unrearranged allele; M, 100 bp ladder (New England Biolabs).

(D) Enzyme-linked immunosorbent assays (ELISA) for IgM, IgG, and IgA. Fold dilution of serum is shown on the *x* axis; optical density (OD) is shown on the *y* axis; each point represents the average of duplicates. ■, $Rag2^{-/-}$, $\gamma C^{-/-}$ mouse; ●, ▲, $Rag2^{-/-}$, $\gamma C^{-/-}$ mice engrafted with repaired ntES cells; ◆, $Rag2^{-/-}$ mouse engrafted with neonate blood from a repaired ntES tetraploid complementation mouse; □, wt B6129F1 mouse).

sulted in low-level engraftment of the in vitro derived HSCs (1.5% chimerism in PBMCs). In contrast, engraftment was essentially complete (95% peripheral blood chimerism) in a Rag2 null strain rendered devoid of NK cells by virtue of genetic deletion in the IL2 common cytokine receptor γ chain (γC) knockout. Our results raise the provocative possibility that even genetically matched cells derived by therapeutic cloning may still face barriers to effective transplantation for some disorders.

Despite high-level chimerism in the reconstituted Rag2$^{-/-}$, γC$^{-/-}$ double-mutant mice, we have observed a predominance of myeloid cells and a paucity of lymphoid cells in the peripheral blood. Analysis of lymphoid organs has shown extensive chimerism of the thymus and spleen and evidence of TCR and IgH gene rearrangement, respectively, suggesting a blockade to release of the lymphoid populations into the peripheral circulation. For several reasons, we believe that this relative block to lymphoid differentiation is due to the retroviral-mediated constitutive expression of HoxB4 and not to any specific defect in the repaired ntES cells. The capacity for mature lymphoid development from the repaired ntES cells is clear from our observation of functional lymphoid reconstitution in the animals derived from tetraploid embryo complementation. Furthermore, it has been shown previously that a fully functional lymphoid system can be reconstituted, albeit transiently, from in vitro differentiated ES cells (Potocnik et al., 1997).

Though original reports employing retroviral transduction of murine bone marrow with a HoxB4 retrovirus showed no disruption in hematopoiesis (Sauvageau et al., 1995), more recent data suggests that high-level expression of HoxB4 by adenoviral transduction enhances myeloid differentiation in a concentration-dependent manner (Brun et al., 2001), and retroviral expression of the related HoxB3 protein has been linked directly to inhibition of lymphoid differentiation (Sauvageau et al., 1997). These reports corroborate our own experience that shows the retroviral expression of HoxB4 in the in vitro culture system to yield less consistent lymphoid reconstitution than the inducible expression system (Kyba et al., 2002 [this issue of *Cell*]). High-level constitutive expression of HoxB4 may therefore drive hematopoietic engraftment but skew differentiation away from the very lymphoid populations we were attempting to restore. Though enough maturation of lymphocytes occurs to reconstitute some level of immunoglobulin in serum, clearly the immune reconstitution is incomplete. Overcoming this problem might require engineering the inducible system for HoxB4 expression into the Rag2$^{+R/-}$ ntES cells or devising a differentiation protocol not dependant on HoxB4.

The ability to derive pluripotent cells by NT is not limited to a single species (Cibelli et al., 1998) and suggests that derivation of human NT ES cells might be possible. It is of interest that while the efficiency of deriving ES cells from NT embryos is low (approximately 2.2% from tail-tip cells [Wakayama et al., 2001]), it appears to be greater than the efficiency of obtaining viable clones from NT embryos (0.5% from mouse tail-tip cells [Wakayama et al., 1999]). The more efficient derivation of ntES cells than of viable animals may result from the in vitro expansion of a few successfully reprogrammed cells in an otherwise failing blastocyst. The ntES cells derived from somatic cells have regained complete developmental potential (pluripotency), as evidenced by the ability to derive mice through tetraploid embryo complementation (Hochedlinger and Jaenisch, 2002). The pluripotency of the ntES cells did not appear to be impacted by the genetic manipulation and the substantial time in tissue culture required to execute their genetic repair. Thus, murine ES cells derived from "therapeutic cloning" are highly proliferative and as facile to genetic manipulation as wt ES cells, making them an integral tool in studying cell replacement-based gene therapies.

Our results constitute comprehensive proof of principle approach that combines therapeutic cloning with gene and cell therapy to repair a genetic disorder. This methodology could be adapted to a number of genetic disorders of the hematopoietic system that are currently treated by allogeneic marrow transplantation, including severe forms of hemoglobinopathy (sickle cell anemia, thalassemia) or marrow failure syndromes (Fanconi's anemia) in which the underlying genetic lesion is known. Because ES cells can be differentiated into many therapeutically relevant tissue types including neurons (Lee et al., 2000), cardiac myocytes (Doevendans et al., 2000), and pancreatic β cells (Lumelsky et al., 2001), the strategy employed here is applicable to a variety of genetic diseases that can be corrected by cell transplantation. Similarly, human ES cells have been shown to be highly proliferative and to differentiate in vitro into cells such as neurons (Reubinoff et al., 2001; Zhang et al., 2001), hematopoietic precursors (Kaufman et al., 2001), and cardiac myocytes (Kehat et al., 2001).

The public debate over therapeutic cloning has emphasized the theoretical potential to derive genetically matched pluripotent cells from the somatic cells of a donor by nuclear transfer into enucleated oocytes. Generating genetically matched pluripotent stem cells for in vitro differentiation into the desired cell type has several potential benefits: (1) no requirement of long-term administration of immunosuppressive drugs to prevent rejection of the transplanted cells, (2) the opportunity to repair genetic defects within stem cells to treat or cure inherited diseases, and (3) the possibility to repeatedly expand and differentiate the ntES cells into the desired cell type for continued therapy as needed. For future attempts using reprogrammed somatic cell therapy combined with gene therapy, it is crucial that the biological and methodological constraints be defined that may limit this method's effectiveness for medical applications.

Experimental Procedures

NT and ES Cell Derivation

Tail-tip donor cells were cultured for 1–2 weeks from skinned and macerated 1 cm pieces of 1-month-old male mice, Rag2$^{-/-}$ (129B6F1). NT was performed as described (Wakayama et al., 1998). The reconstituted embryos were cultured in mCZB media until they reach the blastocyst stage (generally 4 days), when they were transferred into cultures of mouse embryonic fibroblasts in ES cell media supplemented with 1000 U/ml LIF and 50 μM of the MEK1 inhibitor, PD098059. PD098059 has been shown to promote stem cell renewal (Burdon et al., 1999). After 2–3 days in culture, most of these tail-

tip cell-derived blastocysts remained unhatched and were treated with acid tyrode's solution to remove the Zona pelucida. After another 4–5 days in culture, the proliferating ICM was dissociated and placed in a fresh well. After the cell line was passaged, PD098059 was no longer added to the media.

Gene Manipulation Methods

We obtained the wild-type Rag2 locus by probing a BAC library (RPCI-22 female 129Sv/EvTAC) with a Rag1 cDNA probe (the Rag1 and 2 loci are closely linked, approximately 10 kb apart). The NheI-SpeI fragment (9.3 kb) containing the second and third exons of Rag2 (from BAC clone 390 L-13) was subcloned, and a loxP-flanked Hygtk selection cassette was inserted into a unique SalI site in the second intron. This targeting construct had 5′ and 3′ homologous arms of 3.2 and 6.1 kb, respectively.

Targeting was carried out as described. Briefly, 50 μg of construct was linearized (NotI) and electroporated into the ntES cells in HEPES-buffered saline (0.4 cm gap cuvette) with a single pulse of 600V, 25 μF. Hygromycin selection (140 μg/ml) was started 24 hr after electroporation. Cre loop-out of the selectable marker was done by electroporating 10 μg of pCrePAC plasmid into several targeted ntES cell lines. Gancyclovir (2 μM final concentration) was added 24 hr later.

DNA from ntES cell subclones was isolated as described (Laird et al., 1991). Restriction enzyme digestions were done according to the suppliers' guidelines (NEB) on 10 μg of DNA overnight. Digestions were electrophoresed on 0.85% agarose gels in 0.5 × TBE, blotted to nylon membranes (GenescreenPlus), and probed in Church buffer (Church and Gilbert, 1984).

Tetraploid Embryo Complementation

Tetraploid embryo complementation was performed as described (Eggan et al., 2001), using B6D2F2 zygotes from C57Bl/6 X DBA/2 F1 mice mated together after standard hormone priming. ntES cells (15–20) were injected per tetraploid blastocyst. After transfer into the uterus of pseudopregnant Swiss females (2.5 dpc), Caesarian sections were performed at 19.5 dpc and live pups were fostered.

Mouse Strains

The $Rag2^{-/-}$ mice were F1s of 129Sv/EvTac X C57Bl/6. $Rag2^{-/-}$, $\gamma C^{-/-}$ mice were a mixed background of C57Bl/6 and C57Bl/10.

ES Cell Propagation and Differentiation

ES cells were grown on primary irradiated mouse embryonic fibroblasts in standard ES cell media, high-glucose DMEM (GIBCO-BRL) containing 15% fetal calf serum (Hyclone), 1× penicillan/streptomycin, 1× nonessential amino acids (GIBCO-BRL), 4 μl/500 mls beta-mercaptoethanol, and 1000 U/ml LIF. They were induced to differentiate into hematopoietic progenitors as described (Kyba et al., 2002 [this issue of *Cell*]). Day 6 EBs were disrupted with collagenase and plated into 6-well dishes of semiconfluent OP9 stromal cells at 10^5 cells per well for retroviral infection with MSCVHoxB4iGFP as described (Kyba et al., 2002 [this issue of *Cell*]). The MSCVHoxB4iGFP retrovirus was the best of several constructs in inducing the primitive to definitive hematopoietic transition in yolk sac-derived cells (Kyba et al., 2002 [this issue of *Cell*], and unpublished data). Colonies that arose were expanded by transferring each well's contents (adherant and nonadherent cells) by trypsinization onto a T175 flask with semiconfluent OP9 cells. Differentiated ntES cells were used for transplantation 14 days after retroviral infection.

Transplantation Procedures

$Rag2^{-/-}$ mice receiving neonatal blood or bone marrow grafts were given a single dose of 450 Rads prior to lateral tail vein injection of neonatal blood or bone marrow from ntES-derived mice from tetraploid embryo complementation. $Rag2^{-/-}$, $\gamma C^{-/-}$ mice were given 950 Rads fractionated into two doses separated by 4 hr. Each animal was injected with 2×10^6 differentiated ntES cells in IMDM/10% IFS.

FACS Sorting and ELISA

Peripheral blood lymphocytes (PBLs) and splenocytes were treated with ACK lysing buffer (0.15 mM NH_4Cl, 10 mM $KHCO_3$, 0.1 mM Na_2EDTA [pH 7.2]) prior to FACS analysis to remove red blood cells. To detect B cells, 1×10^6 cells were stained with PE-B220 and FITC-IgM or PE-IgM antibodies, and to detect T cells, with FITC-CD4 or PE-CD4 and PE-CD8 antibodies. Propidium iodide was added to exclude dead cells. All antibodies were purchased from Pharmingen. FACS analyses were performed on a Becton-Dickinson cell sorter.

ELISA was done using the clonotyping kit from Southern Biotechnology according to manufacturer's specifications.

PCR Analysis

Primers for detecting the $Rag2^{+R}$ allele (KH1, TGCGAAGGGACTA GATGGAC; KH2, CAACCATACGGGCTAGAAGC) were designed by the Primer 3 program (Rozen and Skaletsky, 1998), and amplifications were performed on 50 ng of sample DNA in standard PCR conditions for Taq (GIBCO) for 34 cycles of 95°C, 30 s; 60°C, 30 s, 72°C, 30 s, followed by 72°C for 5 min. The residual sequences left behind after Cre-mediated loop-out of the selectable marker result in a 400 bp product for the repaired allele, while wt and mutant Rag2 alleles give a 200 bp band.

Primers for TCRβ rearrangements were as described (Whitehurst et al., 1999) using primer pairs 1 and 4, 1 and 7, or 5 and 7. Primers for PCR of IgH rearrangments (V to DJ) were as described (Schlissel et al., 1991); a mixture of three degenerate oligonucleotides (V_H7183, V_H558, and V_HQ52) and the J3 primer. TCRβ and IgH PCRs were performed in standard Taq conditions (GIBCO) for 35 cycles of 95°C, 1 min; 62°C, 1 min; and 72°C for 2.5 min. All PCR products were anaylzed by gel electrophoresis in 1.5% agarose, 0.5× TBE and stained with ethidium bromide.

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