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Kyoto University
**Generation and Characterization of Severe Combined Immunodeficiency Rats**

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**SUMMARY**

Severe combined immunodeficiency (SCID) mice, the most widely used animal model of DNA-PKcs (Prkdc) deficiency, have contributed enormously to our understanding of immunodeficiency, lymphocyte development, and DNA-repair mechanisms, and they are ideal hosts for allogeneic and xenogeneic tissue transplantation. Here, we use zinc-finger nucleases to generate rats that lack either the Prkdc gene (SCID) or the Prkdc and Il2rg genes (referred to as F344-scid gamma [FSG] rats). SCID rats show several phenotypic differences from SCID mice, including growth retardation, premature senescence, and a more severe immunodeficiency without “leaky” phenotypes. Double-knockout FSG rats show an even more immunocompromised phenotype, such as the obliteration of natural killer cells. Finally, xenotransplantation of human induced pluripotent stem cells, ovarian cancer cells, and hepatocytes shows that SCID and FSG rats can act as hosts for xenogeneic tissue grafts and stem cell transplantation and may be useful for preclinical testing of new drugs.

**INTRODUCTION**

DNA-dependent protein kinase catalytic subunits (DNA-PKcs) are critical components of the nonhomologous end-joining (NHEJ) pathway of the DNA double-strand break (DSB) repair system. DSBs are usually generated by environmental influences such as ionizing radiation (IR) or by chemical mutagens, or are created during programmed processes such as V(D)J recombination or class switch recombination (CSR), which occur during lymphocyte development (Franco et al., 2006; Mahaney et al., 2009; Shrivastav et al., 2008; Yan et al., 2007). The Ku70/80 heterodimer first binds to the ends of the DSBs and recruits DNA-PKcs to form the active DNA-PK complex. Subsequently, together with Artimis, DNA-PKcs stimulate the processing of the DNA ends. Finally, the LIG4 complex, comprising LIG4, XRCC4, and XLF, seals the DSBs generated during NHEJ. Humans and several types of mammals with a defect in the genes involved in NHEJ cannot complete V(D)J recombination. This blocks lymphocyte development, resulting in severe combined immunodeficiency (SCID) (Bosma et al., 1983; O’Driscoll and Jeggo, 2006; Perryman, 2004; van der Burg et al., 2009). SCID mice, which arose spontaneously due to the defective DNA-PKcs gene (Prkdc), show an immunodeficient phenotype and increased sensitivity to IR (Bosma et al., 1983). In contrast, no PRKDC mutations had been reported in humans until recently, when a hypomorphic mutation with PRKDC kinase activity was identified in a patient with SCID with sensitivity to IR (RS-SCID; van der Burg et al., 2009). Although complete PRKDC deficiency is expected to be lethal in humans, spontaneous null mutations in the PRKDC gene were reported in Arabian horses and Jack Russell terriers, highlighting the fact that PRKDC deficiency is not tolerated equally in all species (Perryman, 2004).

SCID animals are widely used in biomedical research as hosts for allogeneic and xenogeneic tissue grafts. Humanized mice (i.e., immunodeficient mice engrafted with human cells or tissues, such as human hematopoietic stem cells [hHSCs], hepatocytes, or tumor cells) are powerful tools that have enabled scientists to gain greater insights into many human diseases (Azuma et al., 2007; Baiocchi et al., 2010; Brehm et al., 2010; Denton and García, 2011; Ito et al., 2008; Katoh et al., 2008; Kteman and Mercer, 2005; Leonard, 2001; Meuleman et al., 2005; Pearson et al., 2008; Quintana et al., 2008; Shultz et al., 2007; Wege et al., 2008). Although the laboratory rat is an ideal model for physiological, pharmacological, toxicological, and transplantation studies, there are no reports of spontaneous, or gene-targeted, SCID rats. Recently, several strategies have been developed to produce a wide variety of genomic alterations in rats (Geurts et al., 2009; Izsává et al., 2010; Mashimo et al., 2008; Tesson et al., 2011), including embryonic-stem-cell- derived p53 knockout rats (Tong et al., 2010). Other investigators
and our group have also shown the successful application of zinc-finger nucleases (ZFNs) as a gene-targeting technology in rats, which is faster and more efficient than embryonic-stem-cell-mediated knockout technology (Cui et al., 2011; Geurts et al., 2009; Mashimo et al., 2010).

Here, we report the generation and characterization of single-knockout Prkdc (SCID) and double-knockout Prkdc and Il2rg (F344-scid gamma [FSG]) rats.

RESULTS

Generation of SCID Rats Using ZFNs

The design and validation of ZFN reagents targeting the first exon of rat Prkdc gene were described previously (Mashimo et al., 2010) (Figure 1A; Figure S1). The validated ZFN mRNA was microinjected into fertilized F344/Stm or Crlj:WI oocytes, which were then transferred into the oviducts of pseudopregnant Crlj:WI female rats (Figure 1B). Screening of 39 newborn animals revealed that 26 of them (66.7%) carried mutations, comprising deletions from 1 bp to 919 bp and a 1-bp insertion (Figures 1C and 1D; Figure S2). The rate and variation of the ZFN-induced mutations were similar to those reported in previous studies (Cui et al., 2011; Geurts et al., 2009; Mashimo et al., 2010). The majority of these were frame-shift mutations resulting in the complete loss of mRNA expression as confirmed by reverse transcriptase (RT)-PCR (Figure 1E) and protein expression as confirmed by western blotting (Figure 1F). Furthermore, double injection of Prkdc and
interleukin 2 receptor-gamma (Il2rg) ZFN-encoding mRNA into 219 fertilized F344/Stm oocytes resulted in one male carrying 7- and 46-bp deletions in Prkdc, and one female carrying 227- and 716-bp deletions in Prkdc and a 3-bp deletion in Il2rg (Figure 1B).

To clarify whether the ZFNs only induced mutations in the targeted region, we checked 12 sites that showed a high rate of similarity to the targeted site at the sequence level with no targeted region, we checked 12 sites that showed a high rate of similarity to the targeted site at the sequence level with no targeted region, we checked 12 sites that showed a high rate of similarity to the targeted site at the sequence level with no targeted region, we checked 12 sites that showed a high rate of similarity to the targeted site at the sequence level with no targeted region, we checked 12 sites that showed a high rate of similarity to the targeted site at the sequence level with no targeted region, we checked 12 sites that showed a high rate of similarity to the targeted site at the sequence level with no targeted region, we checked 12 sites that showed a high rate of similarity to the targeted site at the sequence level with no targeted region, we checked 12 sites that showed a high rate of similarity to the targeted site at the sequence level with no targeted region, we checked 12 sites that showed a high rate of similarity to the targeted site at the sequence level with no targeted region, we checked 12 sites that showed a high rate of similarity to the targeted site at the sequence level with no targeted region, we checked 12 sites that showed a high rate of similarity to the targeted site at the sequence level with no targeted region, we checked 12 sites that showed a high rate of similarity to the targeted site at the sequence level with no targeted region.

Growth Retardation in SCID Rats
Heterozygous Prkdc<sup>+/−</sup> rats were indistinguishable from their WT littermates in all respects. Approximately 25% of the offspring born to Prkdc<sup>+/−</sup> × Prkdc<sup>+/−</sup> crosses were homozygous Prkdc<sup>−/−</sup> rats, and were significantly smaller than their WT and heterozygous littermates (Figure 2A). When the embryos from Prkdc<sup>+/−</sup> × Prkdc<sup>+/−</sup> crosses were examined and weighed, a difference in size was observed at embryonic day 14.5 (E14.5), which became statistically significant at E17.5 (Figure 2B). During the 6 month observation period, the Prkdc<sup>−/−</sup> rats grew and maintained a body weight that was 70% of that of the controls (Figure 2C). Prkdc<sup>−/−</sup> SCID rats normally survive for at least 1 year under specific pathogen-free conditions. Both male and female Prkdc<sup>−/−</sup> rats were fertile, but the average litter size was small (4.7 ± 2.0 [n = 9] versus 9.1 ± 1.6 of F344 rats [n = 12]). Newly generated F344-Prkdc<sup>+/−</sup>/Il2rg<sup>−/−</sup> rats (FSG rats) showed phenotypes similar to those of SCID rats for growth, survival, and reproducibility (Figure 2C).

To further characterize the growth deficiency, we derived primary fibroblasts from WT (+/+) and heterozygous (+/−), and homozygous (−/−) rat embryos (rat embryonic fibroblasts [REFs]), and monitored their growth in vitro (Figure 2D). Early-passage Prkdc<sup>+/−</sup> REFs grew slowly, at a rate 70% of that shown by the Prkdc<sup>+/−</sup> and Prkdc<sup>+/+</sup> REFs. This difference was partly due to a decrease in the number of dividing cells within the Prkdc<sup>+/−</sup> cultures, as determined by incorporating bromodeoxyuridine (BrdU) into chromosomal DNA during an 18 hr labeling period (Figure 2E). Proliferation decreased with passage number, and by passage 4, Prkdc<sup>+/−</sup> REF cultures contained nondividing giant cells, suggesting premature senescence (Figure 2F). Senescence-associated β-galactosidase (SA-β-Gal) activity assays showed significantly higher numbers of SA-β-Gal-positive cells within Prkdc<sup>+/−</sup> REF cultures compared with Prkdc<sup>+/+</sup> or Prkdc<sup>+/+</sup> REF cultures (Figure 2G). To the best of our knowledge, neither growth retardation nor premature senescence has been reported in SCID mice (Bosma et al., 1983; Gao et al., 1998; Jhappan et al., 1997; Taccioli et al., 1998).

IR Sensitivity and DSB-Repair Defects in SCID REFs
Mouse embryonic fibroblasts (MEFs) from SCID mice or Prkdc-deficient mice, and the Prkdc-deficient human glioma cell line M059J are all IR-sensitive, although the level of sensitivity varies. When we used a colony survival assay to test IR sensitivity in REF cells, Prkdc<sup>−/−</sup> REFs were significantly more sensitive than Prkdc<sup>+/+</sup> or Prkdc<sup>+/−</sup> REFs (Figure 2H). Accordingly, Prkdc<sup>−/−</sup> REF cells accumulated foci comprising histone H2AX (γH2AX), a surrogate marker for DSBs, after an exposure to 1 Gy of irradiation (Figure 2I).

We next used a pEJ assay (Kobayashi et al., 2010) and a DR-GFP (Pierce and Jasin, 2005) assay to further examine the effects of Prkdc-deficiency on the NHEJ and homologous recombination (HR) pathways, respectively. After the generation of DSBs using I-SceI, the number of GFP-positive Prkdc<sup>−/−</sup> REFs in the pEJ assay significantly decreased compared with that of Prkdc<sup>+/+</sup> REFs (Figure 2J), clearly indicating a severe deficiency in the NHEJ pathway in these cells. In contrast, the HR pathway was significantly increased in Prkdc<sup>−/−</sup> REFs (Figure 2K), suggesting that a deficiency in the NHEJ pathway induces a more active HR pathway as a compensatory mechanism.

Impaired Lymphoid Development in SCID Rats
Gross and microscopic analyses of SCID and FSG rats revealed abnormal lymphoid development (Figures 3A–3D). The thymuses from SCID and FSG rats were extremely hypoplastic (Figure 3A) and comprised an epithelial rudiment without any lymphocytes (Figure 3C). The spleens were also smaller (Figure 3B), with severely hypoplastic white pulp, and red pulp containing myeloid cells (Figure 3D). Serum immunoglobulin (Ig) levels (IgA and IgM) were undetectable in 5-week-old Prkdc<sup>−/−</sup> rats, whereas IgG levels in Prkdc<sup>−/−</sup> rats nursed by Prkdc<sup>+/−</sup>-heterozygous mothers were detected at half the levels seen in control Prkdc<sup>+/+</sup> rats (Figures 3E–3G), confirming the postnatal transfer of maternal IgG previously described in rodents (Gustafsson et al., 1994). IgG levels were lowest in 8-week-old Prkdc<sup>−/−</sup> rats, and undetectable in 5-week-old Prkdc<sup>−/−</sup> rats nursed by Prkdc<sup>−/−</sup>-homozygous mothers (Figure 3E). Approximately 20% of young adult (or of the majority of old SCID) mice known to have a “leaky” phenotype showed detectable Ig levels, generated by a few clones of functional B cells (Bosma et al., 1983). To date, none of the SCID rats examined (n = 9, until 1 year of age) have shown a leaky phenotype for serum Ig (Figures 3E–3G).

Consistent with the histology, the number of thymocytes and splenocytes was markedly reduced in SCID and FSG rats compared with F344 rats (Table S2). In the peripheral blood (PB) profile, the number of white blood cells (WBCs) was reduced in SCID and FSG rats compared with F344 rats (Table S3). Differential counts of WBCs showed a dramatic decrease in leucocytes and relative increases in neutrophils and monocytes in SCID and FSG rats (Table S4).

To further characterize the immunological deficiency in SCID rats, we examined cell populations isolated from the thymus, spleen, and bone marrow (BM) using flow cytometry (Figures 3H–3J). CD4<sup>+</sup> or CD8<sup>+</sup> single-positive (SP), and CD4<sup>+</sup>CD8<sup>+</sup> double-positive (DP) T cells were completely absent from SCID
thymuses but were abundant in control thymuses (Figure 3H).

This was clearly different from SCID mice, in which DP T cells
are present in the thymus (Gao et al., 1998; Taccioli et al.,
1998). CD3⁺CD45RA⁺B cells were completely absent from
SCID spleens and BM, whereas CD3⁺CD161a⁺ natural killer
(NK) cells were present or even increased (Figures 3I and 3J).
NK cell numbers were mostly depleted in the BM and spleens
of 5-week-old FSG rats (Figures 3I and 3J).

Figure 2. Prkdc Deficiency in Rats Results in Growth Retardation, Premature Senescence, and Radiation Sensitivity
(A) Photograph of newborn for Prkdc⁻⁻, Prkdc⁺⁺, and WT Prkdc⁺⁺ littersmates.
(B) Development of Prkdc (+/+, n = 3; +/-, n = 6; −/−, n = 3) embryos, as measured by weight at E14.5, E17.5, and E20.5.
(C) Postnatal growth of Prkdc (+/+, n = 4; +/-, n = 8; −/−, n = 4) and Prkdc⁻⁻ Il2rg⁻⁻ FSG (n = 5) rats.
(D) Proliferation of primary fibroblasts from Prkdc (+/+, n = 3; +/-, n = 3; −/−, n = 3) E14.5 rats. Second-passage REFS were plated in 60 mm dishes and counted
every 2 days.
(E) Division of REFS from Prkdc (+/+, n = 3; +/-, n = 3; −/−, n = 3) rats. Incorporation of BrdU into chromosomal DNA was measured after an 18 hr labeling period.
(F) Proliferation of REFS from Prkdc (+/+, n = 3; +/-, n = 3; −/−, n = 3) rats at each passage.
(G) Cell staining for senescence-associated SA-β-galactosidase activity. The percentage of SA-β-galactosidase-positive cells in the REF (+/+, n = 3; +/-, n = 3; −/−, n = 3) cells was calculated from the average from three experiments.
(H) Radiation sensitivity of Prkdc (+/+, +/-, −/−) REF cell lines. Cells were irradiated with the indicated dose of γ-rays, and viability was analyzed using colony
formation assays.
(I) γ-H2AX focus formation assay in Prkdc (+/+, +/-, −/−) REF cell lines after exposure to 1 Gy of γ-rays. Cells were stained with anti-γ-H2AX antibody as a marker
for DSBs.
(J and K) NHEJ activity and HR activity of Prkdc (+/+, +/-, −/−) REF cell lines. I-SceI expression plasmids were introduced into each REF cell line by electroporation.
After 2 days, GFP-positive cells induced through the NHEJ pathway (J) or the HR pathway (K) were analyzed by flow cytometry.
Error bars indicate the mean ± SEM; *p < 0.05 for each genotype by one-way analysis of variance (C–H) or Student’s t test (J and K). See also Figure S2.
Xenotransplantation of Human Induced Pluripotent Stem Cells and Tumor Cells

SCID mice can accept transplanted tissues from other species, including humans. We used SCID rats in a teratoma formation assay. Teratomas were induced by inoculation of human induced pluripotent stem (iPS) cells beneath the testis capsule (Takahashi et al., 2007). All SCID (n = 4/4) and FSG (n = 3/3) rats developed tumors, and in most cases, both testes were affected (Figure 4A). Histological examination showed that the lesion had displaced the normal testis and contained solid areas of teratoma. All teratomas contained differentiated tissues representing all three germ layers, including columnar epithelium, pseudostratified ciliated epithelium (endoderm), neural rosettes (ectoderm), cartilage, and adipose tissue (mesoderm; Figures 4B–4F).

To further evaluate the immunological defects, we used SCID and FSG rats as hosts for the xenotransplantation of human ovarian cancer cells (Mashimo et al., 2010). All SCID rats (n = 6/6) developed tumors within 14 days after injection of ovarian cancer cells, whereas control F344 rats showed no evidence of tumor growth.
Of interest, human cancer cells proliferated more rapidly in FSG rats, presumably due to the lack of NK cell activity in these animals. The tumors were confirmed by histological analysis, and by PCR using primers to amplify the human MHC class II DQB2 region. These observations illustrate the impaired immune system of SCID and FSG rats, and clearly show that these animals will be useful models for cancer and stem cell research.

Transplantation of Human Hepatocytes and hHSCs

The different metabolic enzyme profiles in human and rat livers are a major limitation for toxicology and drug testing. We generated liver-humanized rats by injecting human hepatocytes into the livers of infant FSG rats pretreated with a pyrrolizidine alkaloid (retrorsine) that is toxic to hepatocytes. In this study we used FSG rats because they have more immunocompromised states compared with SCID rats, as observed in the tumor cell engraftment. Human albumin (hAlb) was detectable in the blood of all transplanted FSG rats. hAlb was detected in the blood of all transplanted FSG rats. The detection limit for hAlb (156.3 ng/ml) is indicated by the dotted line. Error bars indicate the mean ± SEM (H and J). *p < 0.05 for SCID versus FSG (H) and for F344 versus FSG (I) by Student’s t test.

Liver sections stained with human cytokeratin 8/18 (hCK8/18) show engraftment and repopulation of donor human hepatocytes in the recipient rat livers. The sections were counterstained with hema-toxylin. The region enclosed by the square in (J) is magnified in (K).

DISCUSSION

In this study, we successfully generated Prkdc-deficient SCID rats by using the ZFN technology. The SCID rats were significantly different from SCID mice, in that they showed growth retardation, defects in fibroblast proliferation, and a more severe immunodeficient phenotype. This suggests that DNA-PKcs have...
distinct functions in mice and rats. Although few studies have examined spontaneous null mutations in human DNA-PKcs, it has been reported that gene-targeting disruption of human somatic cells results in profound growth retardation, IR sensitivity, and increased genetic instability (Ruis et al., 2008). The most reasonable explanation for the phenotypic differences between mice and humans is that human cells express 50 times more DNA-PK activity than rodent cells (Finnie et al., 1995). Our expression analysis of spleen and fibroblast cells showed that humans express many more DNA-PKcs compared with rodents, and rats express three times more compared with mice (Figure S2). Although DNA-PKcs play a major role in DSB repair (as do other members of the phosphatidylinositol 3-kinase-related kinase [PIKK] family, including ATM and ATR), their exact role is still not completely understood. There are at least three DSB repair pathways: NHEJ, HR, and the alternative NHEJ pathway (Mahaney et al., 2009; Shrivastav et al., 2008; Zha et al., 2011). The pathway used for DSB repair seems to differ between species. NHEJ, in which DNA-PKcs play a key role, is considered to be an error-prone pathway, whereas HR (the major process in lower eukaryotes that lack a DNA-PKcs enzyme) is error-free. Considering that much of the genome in higher eukaryotes comprises less-well-conserved noncoding DNA, NHEJ may have evolved along with DNA-PK activity, particularly in higher mammalian species.

Although SCID mice are the most commonly used experimental animal model for xenograft transplantation, the normal NK cell activity observed in these animals contributes to the limited longevity and function of transplanted human cells (Shultz et al., 2007). Therefore, we additionally generated FSG rats, which show no such NK cell activity. Although both SCID and FSG rats could serve as hosts for xenogenic cell grafts such as human iPSCs and ovarian cancer cells, the FSG rats showed higher proliferation of human tumor cells than the SCID rats (Figure 4H), indicating a more severely immunocompromised state similar to that reported in Prkdc<sup>-/</sup>- SIRPa mice (Takado et al., 2008) or human blood cells (Ishikawa et al., 2005). Further transplantation studies using diverse human cancer cells, such as preclincial cancer cells or cancer stem cells, will be interesting and will improve our understanding of severely immunocompromised hosts.

In addition, we engrafted human hepatocytes into the livers of retorsine-treated FSG rats (Figure 4I–4M). HA1 was secreted in the blood of transplanted FSG rats, and clusters of human hepatocytes, will be interesting and will improve our understanding of severely immunocompromised hosts.

In conclusion, the newly developed SCID and FSG rats described in this study can be a valuable resource in various fields, such as stem cell research and translational research, and serve as an important experimental model for preclinical drug testing. These humanized models will also allow preclinical evaluation of stem-cell-based therapies and expand the options for translational research. This is particularly important in the field of regenerative medicine, because humanized rats can be used to evaluate not only the ability of the cells to engraft but also their therapeutic efficiency. However, additional genetic modifications may be required to permit transplantation of human cells or tissues, such as human hepatocytes and hHSCs.

**EXPERIMENTAL PROCEDURES**

**Generation of Knockout Rats Using ZFNs**

Custom-designed ZFN plasmids for the rat Prkdc gene and the Il2rg gene were obtained from Sigma-Aldrich (St. Louis, MO). The design, cloning, and validation of the ZFNs were performed as previously described (Mashimo et al., 2010). In brief, ZFNs were designed to recognize a site-specific sequence within the first exon of the rat Prkdc gene (Figure S4). Approximately 2–3 pl of ZFN mRNA (10 ng/pl) were injected into the pronuclei of embryos collected from F344/Stm or Crl:WI females as previously described (Mashimo et al., 2010). The cultured embryos were then transferred to the oviducts of pseudo-pregnant females (Crl:WI, 8–10 weeks). To edit the ZFN cleavage site in the genome at the Prkdc locus, two primer sets were designed to amplify small (309 bp) and large (1,321 bp) fragments as shown in Figure S4. The PCR products were directly sequenced using the BigDye terminator v3.1 cycle sequencing mix and the standard protocol for an Applied Biosystems 3130 DNA Sequencer (Carlsbad, CA).
All animal care and experiments conformed to the Guidelines for Animal Experiments of Kyoto University, and were approved by the Animal Research Committee of Kyoto University. Transplantation studies using human hepatocytes were approved by the Ethics Board of PhoenixBio Co., Ltd. (Higashihiroshima, Japan). All SCID rats were maintained under specific pathogen-free conditions. The SCID rats are deposited in the National BioResource Project–Rat in Japan (http://www.anim.med.kyoto-u.ac.jp/rbr).

RT-PCR and Western Blotting
Total RNA was extracted from the spleens of 5-week-old rats using Isogen reagent (Nippon Gene, Tokyo, Japan). RT-PCR was performed using the primers for Prkdc described in Figure S2, and with Gapdh 5′-GGCACAGT CAAGGCTGAGAATG-3′ and 5′-ATGTTGTTGAAGACGCCGTA-3′. Western blotting was carried out using cell lysates from the spleens of 5-week-old rats according to standard methods. Signals were detected with antibodies against rat PKRDC (H-183; Santa Cruz Biotechnology, Santa Cruz, CA) and β-actin (AC-40; Sigma Aldrich).

REF Culture, Proliferation Assays, and SA-β-galactosidase Assay
REFs were isolated from embryos after 14.5 days of gestation from the female partners of intercrossed Prkdc+/− rats. To obtain a growth curve, passage 2 REFs (2 × 10^5) were plated on six-well plates in triplicate. The cells were trypsinized, stained with trypan blue, and counted every other day for a total of 8 days. For the BrdU incorporation assay, cells were plated with BrdU (100 μM) and labeled for 48 hr in 96-well plates. The cells were stained with the anti-BrdU-POD antibody and quantified by measuring the absorbance with an enzyme-linked immunosorbent assay (ELISA) reader (BrdU Labeling and Detection Kit III; Roche Applied Science, Indianapolis, IN). To assess SA-β-galactosidase activity, cells were plated 60 mm dishes and stained for 24 hr. The percentage of SA-β-galactosidase-positive cells was determined by manually counting the number of blue cells (Senescence Detection Kit; BioVision, Mountain View, CA) within the total cell population.

Preparation of Immortalized REF Cells
Generation of immortalized REF cells by human telomerase reverse transcriptase (hTERT) was performed as previously described (Nakamura et al., 2002). Briefly, rat primary fibroblasts (+/+, +/−, or −/−) were infected with an hTERT-introduced retrovirus and then continuously cultured with G418. After a few weeks, viable cells were infected with SV40 and then continuously cultured for >1 month. These transformed cells (REF-hTERT/SV) were used for radiation sensitivity assays, and HR and NHEJ assays.

Radiation Sensitivity Assay and Immunofluorescence Staining for γ-H2AX Foci
For the radiation sensitivity assays, the cells were trypsinized and irradiated with 3 or 5 Gy of 60Co γ-rays at a dose rate of 1.1 Gy/min. Immediately after irradiation, the cells were plated into 100 mm dishes at a density such that 50–200 cells would survive, and then incubated for 10 days. The dishes were then fixed with ethanol and stained with 3% Giemsa, and the number of colonies was counted. The survival fractions were calculated by comparing the number of colonies formed by irradiated cells with the number of colonies formed by nonirradiated control cells. Each result represents an average value from three independent experiments.

Immunostaining for γ-H2AX foci was performed as previously described (Kobayashi et al., 2010). Cells grown on a glass slide were fixed with cold methanol for 15 min, rinsed with cold acetone several times, and then air-dried. Anti-γ-H2AX antibody (Upstate: #06-636) and Alexa-566-conjugated anti-mouse IgG antibodies (Molecular Probes, Carlsbad, CA) were used to visualize the γ-H2AX foci.

NHEJ and HR Assays
NHEJ and HR assays were performed as previously described (Kobayashi et al., 2010; Pierce and Jasin, 2005). The cells were generated from REF-hTERT/SV cells via introduction of a pEJ construct for the NHEJ assay or a DR-GFP construct for the HR assay. To measure the repair of 1-Scle-generated DSBs, 50 μg of the I-SceI expression vector (pCBASce) was introduced into 1 × 10⁶ cells by electroporation (GenePulser; Bio-Rad, Hercules, CA). To determine the level of NHEJ or HR repair, the percentage of GFP-positive cells was quantified by flow cytometry (FACS Calibur; Becton Dickinson, Franklin Lakes, NJ) 3 days after electroporation.

Immunofluorescence and Fluorescence-Activated Cell-Sorting Analysis
PB specimens were collected from the caudal vena cava. Serum Ig levels were measured by ELISA using Rat IgG, IgA, and IgM ELISA quantitation kits (Bethyl Laboratories, Montgomery, TX). For histopathology, tissues were fixed in Bouin’s fluid and embedded in paraffin. The embedded tissues were then sectioned (6–7 μm thick) at room temperature and stained with hematoxylin and eosin (H&E) to permit evaluation by light microscopy.

Flow cytometric analysis of cell populations isolated from thymus, BM, and spleen were carried out using IOTest Anti-Rat CD3 FITC/CD45RA-PC7/CD161a-APC (Beckman Coulter, Fullerton, CA) to differentiate the T cell, B cell, and NK cell subpopulations, and IOTest Anti-Rat CD3 FITC/CD4/P-CD8-APC (Beckman Coulter) to enumerate the T cell subpopulations. Anti-CD45 monoclonal antibodies (Beckman Coulter) were used for the intracellular staining of lymphocytes. Mouse IgM, IgG1, and IgG2a antibodies (Beckman Coulter) were used as isotype-matched controls. The cell samples were treated with FcR-blocking reagent (Milenyi Biotec, Auburn, CA) for 10 min, stained with the fluorochrome-conjugated antibodies for 30 min, and washed three times with PBS/10% FCS. Stained cell samples were analyzed with the use of a four-color fluorescence-activated cell-sorting (FACS) flow cytometer (FACSCalibur; Becton Dickinson) and the data were analyzed with CellQuest software (Becton Dickinson).

Teratoma Formation by Human iPS Cells
Human iPS cells (201B7) were supplied by the Center for iPS Cell Research and Application, Kyoto University (Kyoto, Japan). Clumps of ~2 × 10⁶ human iPS cells with an undifferentiated morphology were harvested at the time of routine passage as described previously (Takahashi et al., 2007), and injected into the testis of 6- to 8-week-old rats. Six to 8 weeks later, when testicular lesions developed and were palpable, the resulting tumors were dissected, fixed in 10% neutral buffered formalin, embedded in paraffin, and examined histologically after H&E staining.

Tumor Cell Xenotransplantation
The human ovarian cancer cell line A2780 was purchased from the European Collection of Cell Cultures (ECACC, Wiltshire, UK). Cells were cultured in RPMI 1640 medium (GIBCO, Fort Worth, TX) with 10% heat-inactivated FBS (Hyclone, Logan, UT). Subcutaneous injections of 2 × 10⁶ A2780 cells plus Matrigel (Becton Dickinson) were performed on 5-week-old female rats. Tumors were measured (length [a] and width [b]) in millimeters using calipers, and tumor volumes (V) were calculated using the formula V = ab²/2, where a is the longer of the two measurements. Human-specific PCR primers were designed to amplify major histocompatibility complex class II DQ beta 2 (HLA-DQB2) at exon 4 as follows: 5′-CCTAGGGTGTCGAAGCTGGA-3′ and 5′-AAATCCGGCCAAAAGAAGG-3′.

Transplantation of Human Hepatocytes
Human hepatocytes were isolated from human-hepatoctye chimeric mice (PXB mice, http://www.phoenixbio.co.jp) using the two-step collagenase perfusion method as described previously (Yamazaki et al., 2010). The donor cells (cryopreserved human hepatocytes derived from a 5-year-old boy) were purchased from BD Gentest (Becton Dickinson). Two-week-old rats were given intraperitoneal injections of retorsine (Sigma-Aldrich) at 10 mg/kg body weight. Seven days after retorsine treatment, the isolated human hepatocytes (5 × 10⁵ to 1 × 10⁶ viable cells) were transplanted into the animals via the portal vein. To deplete Kupffer cells, the rats were injected intraperitoneally with 10 ml/kg of liposome-encapsulated clodronate 2 days before and 3 days after transplantation. Plasma samples were collected weekly and hAb levels were measured by ELISA (Human SerumAlbumin ELISA Quantitation Kit; Bethyl Laboratories). The rat livers were harvested 6 weeks after transplantation. When necessary, BrdU (50 mg/kg; Sigma-Aldrich) was injected intraperitoneally 1 hr before sacrifice. Paraffin and frozen sections (5 μm thick) were prepared from the liver tissues and subjected to H&E or...
immunohistochemical staining using human-specific hCK8/18 mouse monoclonal antibodies (NCL3; MP Biomedicals, Aurora, OH) or BrdU mouse monoclonal antibodies (Bu20a; Dako Cytomation, Glostrup, Denmark). The antibodies were visualized with a Vectastain ABC Kit (Vector Laboratories, Burlingame, CA) using DAB substrates.

SUPPLEMENTAL INFORMATION
Supplemental Information includes Extended Experimental Procedures, four figures, and five tables and can be found with this article online at http://dx.doi.org/10.1016/j.celrep.2012.08.009.

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