in vivo *28*: 779-784 (2014)

Establishment of Nude Mice with Complete Loss of Lymphocytes and NK Cells and Application for *In Vivo* Bio-imaging

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Abstract. *Background: Nude mice are used in human xenograft research; however, only 25-35% of human tumors have been successfully transplanted into nude mice and their application is limited due to high natural killer (NK) cell activity. More severely immunodeficient mice with loss of NK activity are needed to overcome this limitation. Materials and Methods: Balb/c nude Rag-2$^{-/-}$Jak3$^{-/-}$ (Nude-RJ) mice were established by crossing Rag-2$^{-/-}$Jak3$^{-/-}$ mice and nude mice. The K562 cell line was implanted subcutaneously to compare tumorigenicity between Nude-RJ mice and Nude mice. The cholangiocarcinoma mCherry expressing cell line (KKU-M213) was implanted subcutaneously, and fluorescence intensity and tumor weight were measured. Results: Nude R/J mice showed complete loss of lymphocytes and NK cells. Xeno-transplantation of K562 cells showed higher proliferation in Nude R/J mice than nude mice. Subcutaneously-transplanted mCherry-transduced KKU-M213 cells were successfully detected with a fluorescence imager. Conclusion: Nude-R/J mice are valuable tools for in vivo imaging studies in biomedical research.*

The discovery of nude atymic (Nude) mice that were T-cell deficient allowed the routine and efficient transplantation and propagation of human tumor tissues (xenograft) in mice (1). Nude mice allow established *in vitro* cell lines to be propagated subcutaneously, reconstituting a solid tumor (2). Human tumor tissue explants obtained from biopsy or autopsy can also be transplanted directly into nude mice. However, only 25-35% of human tumors obtained from patients have been successfully transplanted into nude mice (3). These findings are thought to be related to the elevated natural killer (NK) cell activity in nude mice, and nude mice with additional immunodeficiency have been established to improve the take rate of human tumors in nude mice. Lasat mice (asplenic athymic mice), NIH type 2 nude mice, CBA/N nude mice with X-linked partial B cell deficiencies, nude-beige mice and NOD/Scid nude mice have been established; however, since NK activity is reduced but retained by these mice, the take rate of human tumors is not obviously improved.

Human cancer xenograft models of immunodeficient mice have been widely used in various cancer studies, including pre-clinical drug evaluation, metastasis, and biomarker discovery. Recent approaches have involved the use of severe immunodeficient mice genetically modified to be NK-defective (4-7), which markedly improved the efficiency of xeno-transplantation. We previously generated Rag-2/Jak3 double-deficient mice with a Balb/c genetic background (Balb/c R/J mice) (8). These mice showed a lack of mature T and B lymphocytes and NK cells, and showed high efficiency of both human CD34$^+$ hematopoietic stem cell (HSC) and peripheral blood mononuclear cell (PBMC) transplantation, and human tumor xeno-transplantation (8). In the present study, we established Balb/c Nude mice with Rag-2 and Jak3 double deficiency (Nude-RJ mice) and evaluated them for use in fluorescence bio-imaging.

Materials and Methods

Mice. Balb/c Rag-2 deficient (Rag-2$^{-/-}$) mice and Balb/c Jak3-deficient (Jak3$^{-/-}$) mice were established by crossing Rag-2$^{-/-}$ mice (10) or Jak3$^{-/-}$ mice (11) (Center for Animal Resources and Development, Kumamoto University, Japan) with the Balb/c strain for 10 generations, respectively. Balb/c Rag-2/Jak3 double-deficient (Rag-2$^{-/-}$Jak3$^{-/-}$) mice were established by crossing Balb/c Rag-2$^{-/-}$ mice and Balb/c Jak3$^{-/-}$ mice. Balb/c Nude Rag-2$^{-/-}$Jak3$^{-/-}$ (Nude-RJ) mice were then established by crossing Balb/c Rag-2$^{-/-}$Jak3$^{-/-}$ mice and Balb/c Nude mice (purchased from Japan Clea, Tokyo,

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Key Words: In vivo imaging, nude mice, immunodeficient mice, xeno-transplantation.

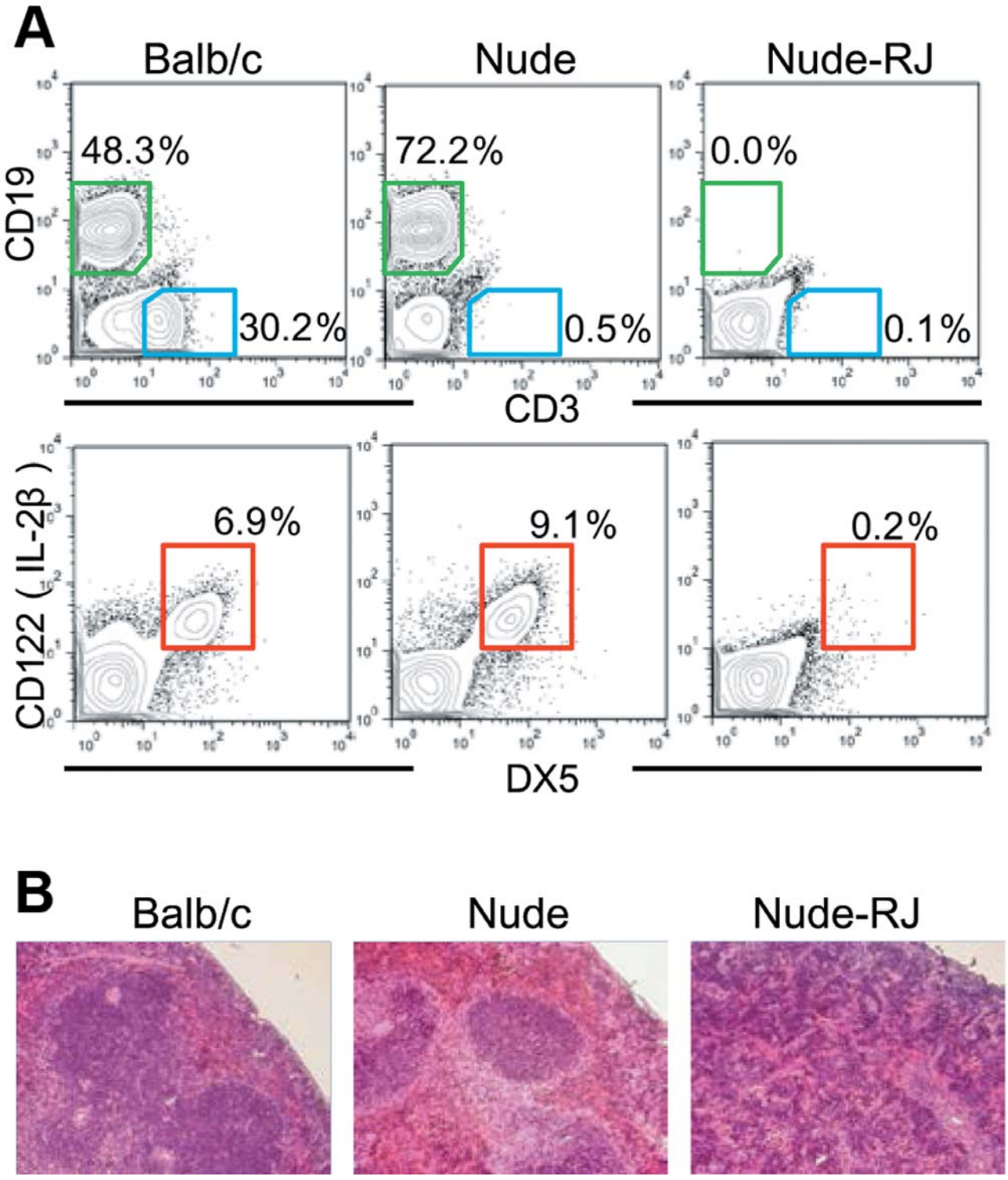

Figure 1. *Lack of mature lymphocytes and NK cells in Nude-RJ mice. A. Spleen cells from Balb/c wild-type mice, Nude mice and Nude-RJ mice, were stained with CD19-APC (B cell marker), CD3-PE/Cy7 (T cell marker), DX5-FITC (pan NK marker), and CD122 (IL-2Rβ). No T and B lymphocytes or NK cells were observed in the spleen of Nude-RJ mice, whereas T lymphocytes and NK cells were observed in Nude mice. B. Hematoxylin & eosin staining of the spleen. There is marked reduction in cellularity in follicles from Nude-RJ mice in contrast to well-developed normal follicles in Balb/c wild-type mice.*

Japan), and were housed and monitored in our animal research facility according to institutional guidelines. All experimental procedures and protocols were approved by the Institutional Animal Care and Use Committee of Kumamoto University.

Cell lines. The human cholangiocarcinoma cell line, KKU-M213, was cultured in Dulbecco's Modified Eagle Medium (DMEM) (Wako Pure Chemical, Osaka, Japan) supplemented with 10% (v/v) heat-inactivated fetal bovine serum (FBS; JRH Bioscience, Lenexa, KS, USA), 100 U/ml penicillin and 100 μg/ml streptomycin (12). The human erythroleukemia cell line, K562, was obtained from RIKEN Cell Bank (Tsukuba, Japan), and was cultured in Roswell Park Memorial Institute (RPMI)1640 Medium (Wako Pure Chemical) supplemented with 10% (v/v) heat-inactivated FBS, 100 U/ml penicillin and 100 μg/ml streptomycin. mCherry-transfected KKU-M213 (M213-mCherry) was established with the pmCherry-N1 Vector (Clontech, Mountain View, CA, USA) and the transfection reagent Lipofectamine 2000 (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's instructions. Transfected cells were selected in media containing neomycin (G418; Carbiochem, Darmstadt, Germany), followed by limiting dilution to isolate stable clones.

Flow cytometry. Mouse spleen cells were stained with DX5-FITC (pan NK marker), mCD122 (IL-2Rβ)-PE, mCD19-APC and mCD3-PE/Cy7 (eBiosciences, San Diego, CA, USA), and analyzed using LSR II (BD Biosciences, San Diego, CA, USA) to detect murine lymphocytes (9). Data were analyzed with FlowJo (Tree Star, San Carlos, CA, USA).

Histological analysis. Spleens were fixed with 10% neutral-buffered formalin immediately after removal, embedded in paraffin, cut into 4 μm sections, and stained with hematoxylin and eosin.

Xenograft mouse model. Balb/c Nude-RJ mice or Balb/c Nude mice (8-10 weeks old) were subcutaneously inoculated with 5×10^6 K562 cells or M213-mCherry suspended in 100μl phosphate-buffered saline (PBS) in both flank sides. On day 16, the xeno-transplanted mice were sacrificed, and the tumors were removed and weighed.

Image acquisition. We confirmed that organs and cells obtained from Nude-RJ mice could be fluorescently visualized. In brief, after euthanizing Nude-RJ mice, internal organs were placed on a tray and imaged using a Maestro *in vivo* fluorescence imaging system (Cambridge Research & Instrumentation, MA, USA).

Statistical analysis. The statistical significance of differences observed between experimental groups was determined using the Student's *t*-test. *p*-Values less than 0.05 were considered significant.

Results

Characterization of Nude-RJ mice. The generated Nude-RJ mice survived and bred well under the specific pathogen-free conditions. To confirm the predicted immunophenotype of Nude-RJ mice, single-cell suspensions from spleen cells were labeled with fluorescent antibodies against mouse DX-5 (pan NK marker), CD122 (IL-2Rβ), CD3 (T-cell marker) and CD19 (B cell marker). Nude mice showed CD3-positive mature T lymphocyte deficiency, whereas B lymphocytes (CD20-positive) and NK cells (DX-5 and CD122 double-

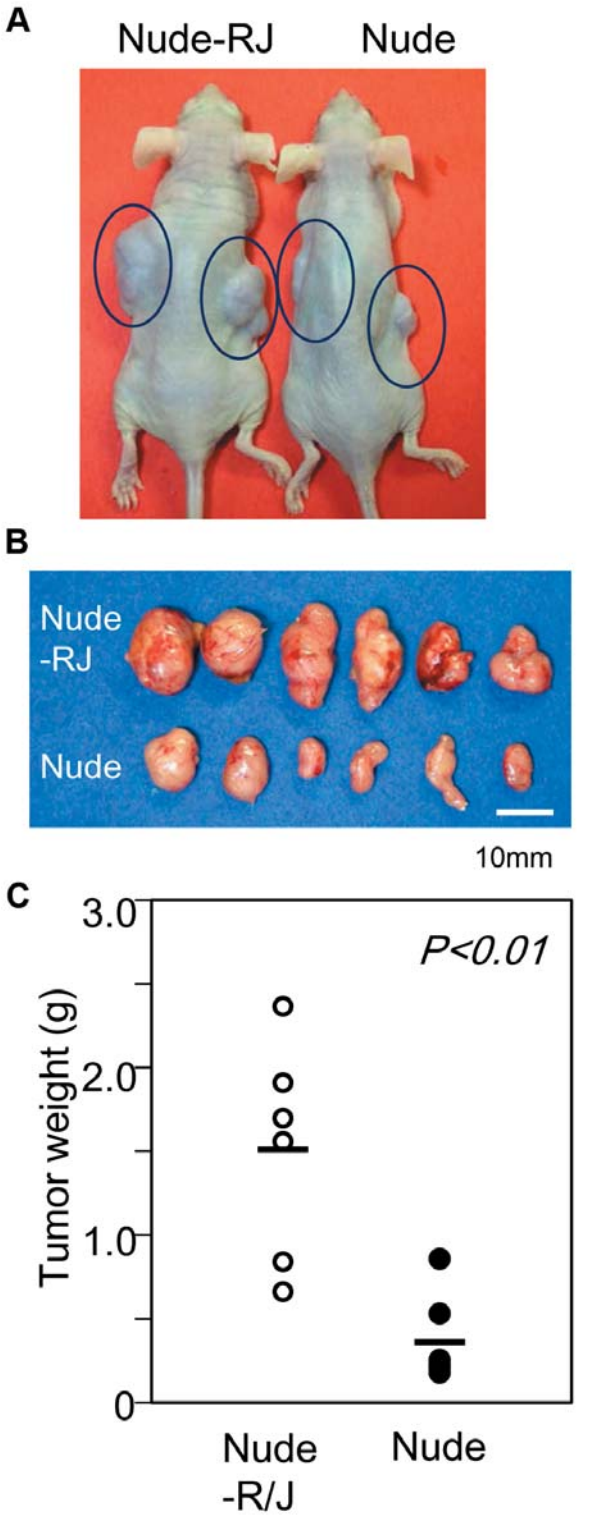

Figure 2. *Better tumorigenicity: Nude-RJ mice showed better tumor growth than Nude mice. K562 erythroleukemia cell line was injected subcutaneously into Nude-RJ mice and Nude mice and tumorigenicity was compared. A, Photograph of Nude-RJ mice and Nude mice 16 days after inoculation; B, Photograph of tumor tissue; C, Comparison of tumor weight obtained from Nude-RJ mice and Nude mice.*

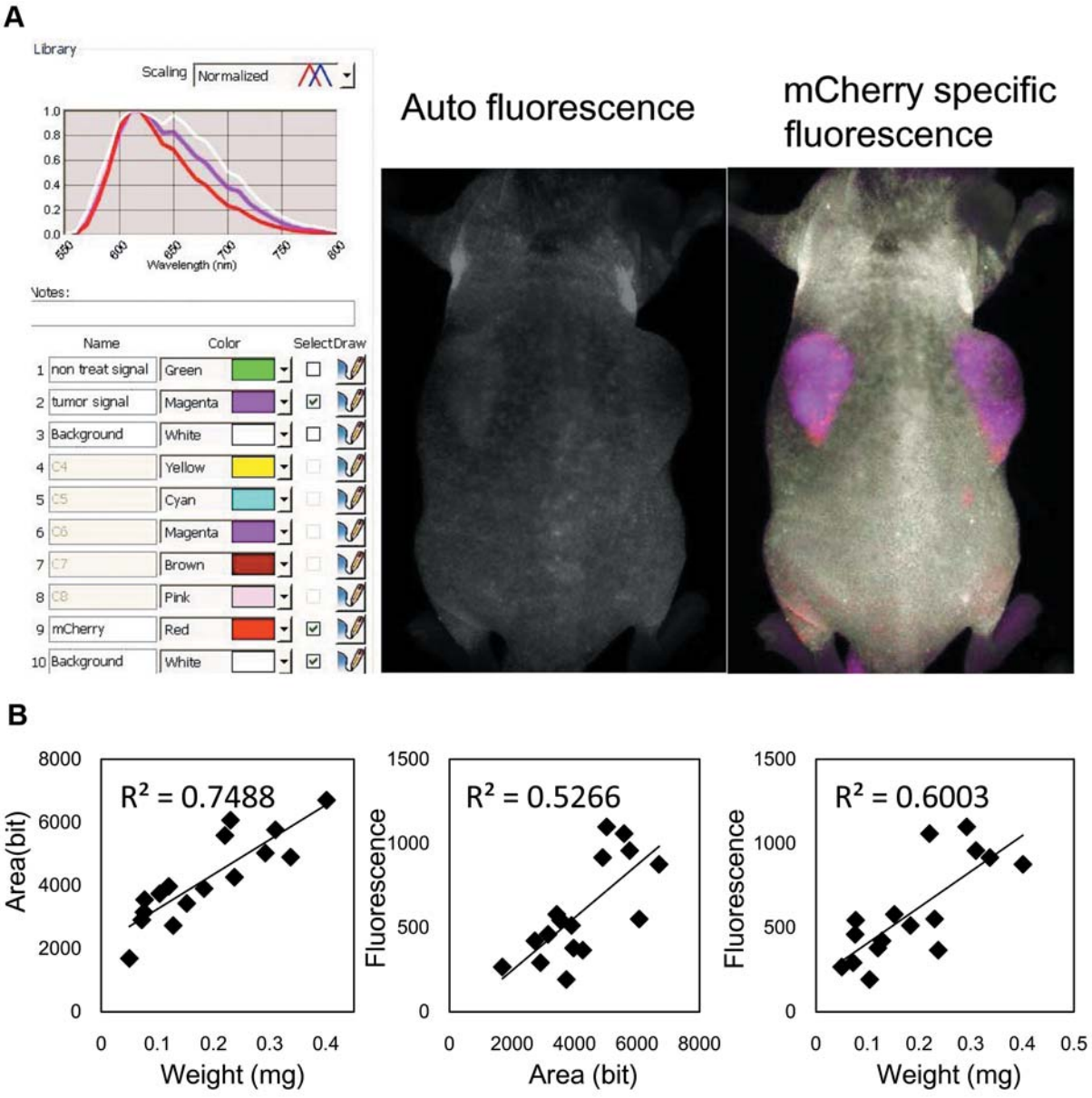

Figure 3. *Correlation of fluorescence intensity, fluorescence area and tumor weight. M213 cholangiocarcinoma cell line expressing mCherry was subcutaneously injected into Nude-RJ mice. On day 12, fluorescence intensity and area were measured with an in vivo fluorescence imaging system (Maestro). Mice were sacrificed and tumors were weighed. A, Fluorescence imaging of tumor; B, Correlation of fluorescence intensity, fluorescence area and tumor weight in transplanted tumor.*

positive cells) were detected. In contrast to wild-type mice and Nude mice, no B- and T-lymphocytes or NK cells were detected in Nude-RJ mice (Figure 1).

Tumor cell engraftment. The ability of Nude-RJ mice to engraft human hematopoietic malignancies was compared with Nude mice. Subcutaneous administration of K562 cells resulted in solid tumor formation in both strains of Nude mice. Subcutaneous solid tumors were larger in Nude-RJ mice than Nude mice (Figure 2A). The mice were sacrificed and subcutaneous tumors were removed and weighed on day 16. Tumors in Nude-RJ mice weighed significantly more than those in Nude mice (Nude-RJ: 1.54±0.64 g, Nude: 0.39±0.27 g, n=6 each, $p<0.01$) (Figure 2B, C).

Fluorescence detection of subcutaneous tumors. mCherry (red fluorescence; Figure 3A) expressing cells, a cholangiocarcinoma cell line, M213-mCherry, was

established with lipofection. The florescence of subcutaneously transplanted M213-mCherry was successfully detected with a Maestro *in vivo* fluorescence imaging system. The *in vivo* detected fluorescence intensity and area of fluorescence of M213-mCherry was compared to the weight of the tumors. Statistical analysis revealed that the fluorescence intensity, and the weight and size of the tumors, correlated (Figure 3B). These results indicated that the fluorescence intensity correlated with tumor development, which can be easily followed up in a time-dependent manner in live animals.

Discussion

In the present study, we developed and characterized Nude mice with complete loss of lymphocytes and NK cells with a Balb/c background (Nude-RJ mice). Lack of B- and T- lymphocytes and NK cells improves the efficiency of xeno-transplantation, and lack of hair allows visualization of subcutaneously transplanted tumors using an *in vivo* fluorescence imaging system. Since imaging studies using bioluminescent and fluorescent probes are becoming an increasing important research tool (13), Nude-RJ mice will become valuable in human oncology and immunology research.

Nude mice have been used as the recipients of human tumor xeno-transplantation, since Nude mice have two characteristics; lack of a thymus and hair. The athymic phenotype induces loss of mature T-cells and T-cell-dependent immune responses. The lack of a hair phenotype enables easy measurement of subcutaneous tumors and is suitable for fluorescence detection of tumors. However, only 25-35% of human tumors obtained from patients have been successfully transplanted into nude mice (2). These findings are thought to be related to elevated NK cell activity in nude mice. So, nude mice with additional immunodeficiency have been established to improve the take rate of human tumors in Nude mice. Lasat mice (asplenic athymic mice), NIH type-2 nude mice, CBA/N nude mice with X-linked partial B cell deficiencies, nude-beige mice and NOD/Scid nude mice were established; however, since NK activity is reduced but retained by these mice, the take rate of human tumors is not obviously improved (14). Recently, genetically modified mice have enabled us to establish a complete loss of NK cells, resulting in severe immunodeficient mice, such as NOD/Scid common γ-deficient or Jak3-deficient mice (4, 6, 7) and Balb/c Rag-2 common γ- or Jak3 double-deficient mice (5, 8). These mice are optimized for xeno-transplantation of human primary and tumor cells and are frequently used in human stem cell and tumor cell studies (15). Nude-RJ mice are the first report of a hairless phenotype with complete loss of NK cells.

It is known that the mouse strain background is critical in xeno-transplantation of human cells into immunodeficient mice; that is, the non-obese diabetic (NOD) strain is most efficient, BALB/c is moderate, and C57BL/6 is inefficient for human cell engraftment (8). Recently, it was shown that NOD-specific polymorphism of the signal regulatory protein-α (Sirpa) allows NOD Sirpa to bind human CD47, and the resultant "don't eat me" signaling by this binding prevents rejection of a human graft (16). Balb/c mice also have a Balb/c-specific Sirpa polymorphism although the CD47-binding capacity is weaker than NOD Sirpa. Since immunodeficient mice with a NOD background are prone to develop thymic lymphomas (17, 18), are generally difficult to breed and have a short lifespan (19), a Balb/c background is an alternative recipient of human cell transplantation (5). In this study, we established Nude-RJ mice with a Balb/c background based on Balb/c Rag-2/Jak3 double-deficient mice from these findings.

In conclusion, we established Balb/c Nude Rag-2/Jak3 double-deficient (Nude-RJ) mice, and showed that Nude-RJ mice are optimal for human tumor engraftment and non-invasive *in vivo* fluorescent imaging.

Acknowledgements

We thank I. Suzu and S. Fujikawa for providing technical assistance, and K. Tokunaga and Y. Endo for secretarial assistance. This work was supported in part by a Health and Labour Science Research Grant from the Ministry of Health, Labour and Welfare of Japan (H25-AIDS-I-002), Adaptable and Seamless Technology Transfer Program (A-STEP) from Japan Science and Technology Agency (No. AS2311293E), and Grants-in-Aid for Science Research (Nos. 21107522 and 21591209) from the Ministry of Education, Science, Sports, and Culture of Japan.

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Received April 4, 2014
Revised June 11, 2014
Accepted June 12, 2014