Brief Report

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A novel EGFP-expressing nude mice with complete loss of lymphocytes and NK cells to study tumor-host interactions

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Summary **Enhanced green fluorescent protein (EGFP) expressing Balb/c nude mice strain with Rag-2 and Jak3 double mutants (Nude-R/J-EGFP mice) was established to improve the take rate of human tumors and to distinguish tumor and host cells. EGFP was ubiquitously expressed in all organs including the brain, lung, liver, heart, kidney, spleen, and gastrointestinal tract in Nude-R/J-EGFP mice. The mice showed complete loss of T lymphocytes, B lymphocytes, and NK cells, indicating a higher take rate of human tumor xenograft. M213-mCherry, an mCherry expressing the cholangiocarcinoma cell line, was successfully detected and tumor vessels derived from the host were clearly identified with fluorescence imager. Thus, dual-color fluorescence imaging visualizes the tumor-host interaction by non-invasive *in vivo* fluorescent imaging in Nude-R/J-EGFP mice. These finding suggests that Nude-R/J-EGFP mice are becoming a powerful tool to investigate human tumor-host interactions.**

Keywords: Enhanced green fluorescence protein (EGFP), nude mice, NK cells, fluorescence imaging, xenotransplantation

1. Introduction

Human cancer xenograft models of immunodeficient mice are widely used in oncology research (*1*). Athymic nude mice, Scid mice, and NOD/Scid mice have been used for this purpose. Since nude mice lack T cells but retain functional B cells and NK cells, they show limited growth of human tumors and tumor cell lines (*2,3*). However, they are still commonly used because their lack of fur facilitates tumor implantation and assessment (*4,5*). In particular, recent advances of *in vivo* fluorescent technologies enable us to detect fluorescence-expressing tumor cells inside mice without fur. In addition, several fluorescence protein-expressing transgenic mice have been established to distinguish tumor cells from host cells (*6-9*). Thus, fluorescence protein-expressing severe immunodeficient mice without fur are optimized for *in vivo* imaging.

Recent approaches have involved the use of severe immunodeficient mice with NK defective genetically modified mice (*10-13*), which markedly improved the efficiency of xenotransplantation. We have previously generated Rag-2/Jak3 double-deficient mice with a Balb/c genetic background (Balb/c R/J mice) (*14*). These mice showed a lack of mature T and B lymphocytes and NK cells, and showed high efficiency of human hematopoietic stem cell (HSC) and peripheral blood mononuclear cell (PBMC) transplantation, and human tumor xenograft (*15*). Based on these findings, we established an enhanced green fluorescent protein (EGFP) -expressing Balb/c nude mice strain with Rag-2 and Jak3 double mutants (Nude-R/J-EGFP mice) and evaluated them for use in fluorescence bio-imaging.

2. Materials and Methods

2.1. *Mice*

Transgenic C57/BL6-EGFP mice were obtained from Prof. Masaru Okabe (Osaka University, Osaka, Japan). C57/BL6-EGFP mice express EGFP under the control of chicken β-actin promoter and cytomegalovirus enhancer (*16*). Balb/c-EGFP mice were established by crossing C57/BL6-EGFP mice with the Balb/c strain

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for 10 generations. Balb/c-EGFP Rag-2$^{-/-}$Jak3$^{-/-}$ mice were then established by crossing Balb/c Rag-2$^{-/-}$Jak3$^{-/-}$ mice (14) and Balb/c-EGFP mice. Finally, Balb/c-EGFP nude Rag-2$^{-/-}$Jak3$^{-/-}$ mice (referred to as Nude-R/J-EGFP mice) were established by crossing Balb/c-EGFP Rag-2$^{-/-}$Jak3$^{-/-}$ mice and Balb/c nude mice, and were housed and monitored in our animal research facility according to institutional guidelines. The mice were maintained by mating nu/nu males with nu/+ females as nu/nu females cannot feed infants (*17*). The nude (*18*), Rag-2 (*19*) and Jak3 (*20*) mutations were genotyped using a previously described PCR method using genomic DNA extracted from tail tissue. EGFP mice were detected with Ultra violet lamp. All experimental procedures and protocols were approved by the Institutional Animal Care and Use Committee of Kumamoto University.

2.2. *Cell lines*

The human cholangiocarcinoma cell line, KKU-M213, was cultured in Dulbecco's modified Eagle's medium (DMEM; Wako Pure Chemical, Osaka, Japan) supplemented with 10% (v/v) heat-inactivated fetal bovine serum (JRH Bioscience, Lenexa, KS, USA), 100 u/mL penicillin and 100 μg/mL streptomycin (*21*). mCherry-transfected KKU-M213 (M213-mCherry) was established with 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 medium containing neomycin (G418; Carbiochem, Darmstadt, Germany), followed by limiting dilution to isolate stable clones.

2.3. *Flow cytometry*

Mouse spleen cells were stained with DX5-APC (pan NK marker), mCD122 (IL-2Rβ)-PE, mCD19-PE, and mCD3-Pacific Blue (eBiosciences, San Diego, CA, USA), and analyzed using LSR II (BD Biosciences, San Diego, CA, USA) to detect murine lymphocytes (*14*). Data were analyzed with FlowJo (Tree Star, San Carlos, CA, USA).

2.4. *Xenograft mouse model*

Eight-ten-week-old Nude-R/J-EGFP mice were subcutaneously inoculated with M213-mCherry (6 × 10^6 cells) suspended in 100 μL phosphate-buffered saline (PBS) in both flanks. On day 16, xenotransplanted mice were euthanized and imaged with an *in vivo* imaging system.

2.5. *Image acquisition*

We confirmed that organs and cells obtained from nude-R/J-EGFP mice could be visualized fluorescently. In brief, after euthanizing Nude-R/J-EGFP mice, internal organs were placed on a tray and imaged using an Maestro *in vivo* fluorescence imaging system (Cambridge Research & Instrumentation, MA, USA). For M213-mCherry inoculated mice, euthanized nude-R/J-EGFP mice were placed on a tray and imaged using a Nuance multispectral imaging system (Cambridge Research & Instrumentation).

3. Results and Discussion

In the present study, we developed and characterized nude mice with ubiquitously expressed EGFP and complete loss of lymphocytes and NK cells on a Balb/c background (Nude-R/J-EGFP mice). The generated Nude-R/J-EGFP mice survived and bred well under specific pathogen-free conditions. Green fluorescence expression can be readily detected by the naked eye under fluorescent light in nude-R/J-EGFP and clearly detected in Nude-R/J-EGFP using a hand-held UV lamp (Figure 1A). Almost all internal organs showed green fluorescence under the imaging instrument (Figure 1B). The expression of EGFP in spleen cells was confirmed with flow cytometry (Figure 2A). To confirm the predicted immunophenotype of Nude-R/J-EGFP 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). In contrast to wild-type mice, no B (CD19 positive) and T (CD3 positive) lymphocytes or NK cells (DX-5 and CD122 double-positive cells) were detected in Nude-R/J-EGFP mice as expected (*14*) (Figure 2B).

The fluorescence of Nude-R/J-EGFP mice (green) and subcutaneously transplanted M213-mCherry

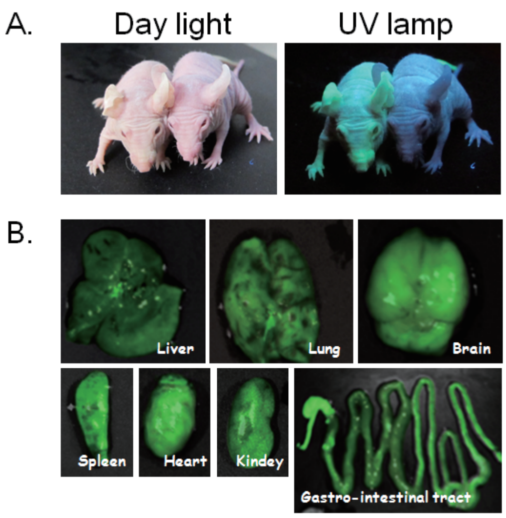

Figure 1. EGFP expression of the Nude-R/J-EGFP mice. (A) Nude-R/J-EGFP mice visualized in daylight or using a handheld UV lamp (left: Nude-R/J-EGFP mice, right: Nude mice). **(B)** Internal organs showing strong EGFP expression in the heart, lung, kidney, liver, brain, spleen, and gastrointestinal tract using a fluorescence imaging system.

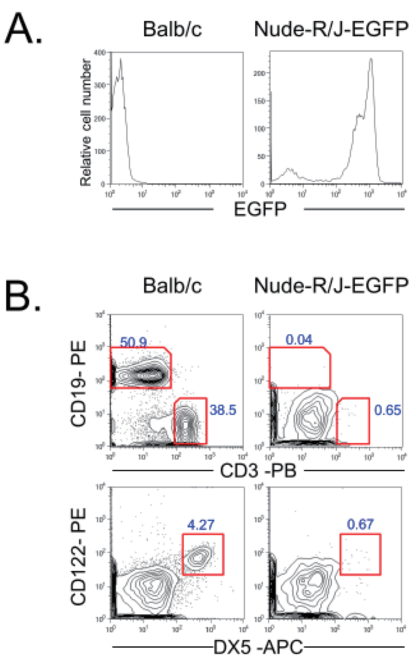

Figure 2. Flowcytometric analysis of EGFP and lineage markers of the Nude-R/J-EGFP mice spleen cells. (A) Strong expression of EGFP in spleen cells of Nude-R/J-EGFP mice. **(B)** Lack of mature lymphocytes and NK cells in Nude-R/J-EGFP mice. Spleen cells from Balb/c wild-type mice and Nude-R/J-EGFP mice were stained with DX5-APC (pan NK marker) and mCD122 (IL-2Rβ)-PE, or mCD19-PE and mCD3-Pacific Blue, and analyzed with flow cytometry. No T and B lymphocytes or NK cells were observed in the spleen of Nude-R/J-EGFP mice.

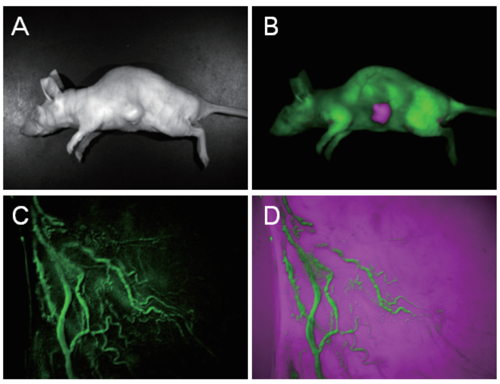

Figure 3. Visualization of mCherry expressing tumor in Nude-R/J-EGFP mice. (A and **B)** Direct fluorescence of M231-mCherry tumor grown in the subcutaneous region of Nude-R/J-EGFP mice and visualized using a non-invasive fluorescence imaging system. **(C)** Direct green fluorescence of tumor vessels in the M213-mCherry tumor visualized using a multispectral imaging system (Nuance). **(D)** Merged with tumor red fluorescence.

(red) was successfully detected with a Maestro *in vivo* fluorescence imaging system (Figure 3). The tumor vessels inside M213-mCherry were further detected with a Nuance multispectral imaging system.

Nude mice are used as recipients of human tumor xenotransplantation, since the lack of a hair phenotype enables easy measurement of subcutaneous tumors and makes them suitable for fluorescence detection of tumors. However, only 25-35% of human tumors obtained from patients have been successfully transplanted into nude mice, because nude mice lack mature T cells, but retain B cells and NK cells. To overcome this weakness, several attempts have been made to develop more immunodeficient mice such as beige-nude, CBA/N nude and hairless scid mice (*22,23*). However, significant NK activity remains in these mice. Recent advances in developmental engineering have enabled to develop immunodeficient mice with complete loss of NK cells, such as NOD/Scid/commonγc$^{-/-}$ (NOG and NSG) mice (*24*), NOD/Scid/Jak3$^{-/-}$ mice (*13*), Balb/c Rag-2$^{-/-}$commonγc$^{-/-}$ mice (*11*), Balb/c Rag$^{-/-}$/-Jak3$^{-/-}$ mice (*14*), which markedly improved the efficiency of xenotransplantation. In addition, several immunodeficient mice expressing fluorescence protein have been developed to optimize *in vivo* imaging (*7,9*). These mice are very useful to distinguish host cells and transplanted human tumor cells (*25*). However, the level of immunodeficiency is not sufficient for transplantation of human cells in nude based mice (*7*). NOD/Scid based mice are sufficient for human cell transplantation; however, their fur prevented precise assessment of tumor size and detection of fluorescence subcutaneously and within the body (*9*). Newly generated Nude-R/J-EGFP mice have favorable attributes for *in vivo* bio-imaging, *i.e.* high immunodeficiency with NK deficiency, being hairless, and expressing EGFP, indicating that Nude-R/J-EGFP mice are optimized for human cancer xenotransplantation and detection using a Nuance multispectral imaging system.

In summary, we established an EGFP-expressing Balb/c nude mice strain with Rag-2 and Jak3 double mutants (Nude-R/J-EGFP mice) and showed that Nude-R/J-EGFP mice are optimal for human tumor engraftment and non-invasive *in vivo* fluorescent imaging.

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