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# A Novel Enhanced Green Fluorescent Protein-Expressing NOG Mouse for Analyzing the Microenvironment of Xenograft Tissues

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**Abstract:** The interaction between transplanted cells and host tissues is important for the growth and maintenance of transplanted cells. To analyze the mechanisms of these interactions, a systemic fluorescent protein-expressing mouse is a useful recipient. In this study, we generated a novel NOG strain, which strongly expresses enhanced green fluorescent protein (EGFP; PgkEGFP-NOG), especially in the liver, kidney, gastrointestinal tract, and testis. Because the host tissues expressed EGFP, xenotransplanted human cancer cells were clearly identified as EGFP-negative colonies in PgkEGFP-NOG mice. Immunohistochemical analysis revealed that EGFP-expressing stromal tissues formed a complicated tumor microenvironment within xenograft tissues. Moreover, a similar microenvironment was observed in human iPS cell-derived teratomas. Collectively, these results indicated that a suitable microenvironment is essential for the growth and maintenance of xenotransplanted cells and that PgkEGFP-NOG mice represent a useful animal model for analyzing the mechanisms of microenvironment formation.

**Key words:** NOG mouse, teratoma, tumor microenvironment, xenotransplantation

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## Introduction

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Cell transplantation analysis is performed for safety testing (tumorigenicity), *in vivo* functional characterization of engrafted cells, and establishment of a “humanized” mouse model [5, 10, 16, 17]. Growth and progression of transplanted cells are dependent on the formation of a suitable microenvironment [1–4, 14, 21]. For example, host stromal tissue components, such as vessels and fibroblasts, interact with engraftment derivatives and

provide oxygen and other essential nutrients. To elucidate the mechanisms that form such microenvironments, various fluorescent protein-expressing murine models have been established [20, 22, 23] by mating systemically fluorescent protein-expressing mice with nude mice, which lack T cell production because of thymic deficiency, but retain production of B cells and other immunocompetent cells. The survivability of transplanted cells is dependent on the immune status of the recipient. It was previously demonstrated that nude mice showed

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a little resistance to xenotransplantation [10]. Another group reported the nonobese diabetic/severe combined immunodeficient (NOD/Scid) transgenic mouse expressing enhanced green fluorescent protein (EGFP) [11]. NOD/Scid mice lack B and T lymphocytes and have low natural killer (NK) cells and hemolytic complement activity, defects in myeloid development, and poor antigen-presenting-cell function. The NOD/Scid mouse demonstrates immunodeficiency that is severer than that in the nude mouse. However, it was reported that lymphoma developed in the NOD/Scid mouse with high incidence [7].

We previously established a nonobese diabetic/severe combined immunodeficiency interleukin-2 receptor  $\gamma^{\text{null}}$ (NOD/Shi-*scid* *Il2rg<sup>null</sup>*) (NOG) mouse that incorporated the *Il2rg<sup>null</sup>* mutation from C57BL/6-*Il2rg<sup>tm1Sug</sup>* mice by backcross mating with NOD/Shi-*scid* mice [6]. NOG mice have no lymphocytes or NK cells and have impaired dendritic cells and macrophage function [9, 13]. Additionally, we previously reported an EGFP-expressing NOG mouse line (NOG-EGFP mouse) [18]. Since the NOG-EGFP mouse demonstrates severe immunodeficiency and systemic EGFP expression, it is considered the most appropriate recipient mouse for analyzing the microenvironment of xenograft tissues [15].

The NOG-EGFP mouse demonstrated strong EGFP expression especially in the skin, muscle, and pancreas. However, EGFP expression in the hepatic parenchymal cells was not observed (Fig. S1). This suggests that the NOG-EGFP mouse is not suitable as a recipient for liver metastatic model. In the present study, we established a novel EGFP-expressing NOG mouse that expressed EGFP under the control of the phosphoglycerate kinase promoter (PgkEGFP-NOG mouse). The PgkEGFP-NOG mouse retains equivalent immunodeficiency to the NOG mouse, and whole-body immunohistochemical analysis revealed that PgkEGFP-NOG mice demonstrated strong EGFP expression especially in the liver. Xenograft colonies in the PgkEGFP-NOG mice were clearly identified as EGFP-negative colonies, and the tumor microenvironment in xenograft colonies was composed of EGFP-expressing fibroblasts and vessels. Moreover, a similar microenvironment was observed in human induced pluripotent stem (iPS) cell-derived teratomas. These results demonstrated that PgkEGFP-NOG mice present a useful host model for analysis of the microenvironment of xenograft tissues.

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## Materials and Methods

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### DNA construction

The *EGFP* gene expression unit was constructed as follows. The promoter region (621 bp) of the murine phosphoglycerate kinase 1 gene was amplified by polymerase chain reaction (PCR) at an annealing temperature of 60°C with the primers mPGK-F-*Ase*I (5'-AAAATTAATACCGGGTAGGGGAGGCGCT-3') and mPGK-R-*Bam*HI (5'-TTTGGATCCACGCGCTTCTACAAGGCGCT-3') and then cloned into the pEGFP-N1 plasmid (Clontech Laboratories, Inc., Mountain View, CA, USA) at the *Ase*I-*Bam*HI restriction site (pmPgkEGFP). A vector-free 1.6-kb *EGFP* expression fragment was prepared by cleavage of the pmPgkEGFP plasmid DNA at unique *Ase*I and *Ssp*I restriction sites.

### Generation of the PgkEGFP-NOG mouse

The PgkEGFP-NOG construct was microinjected into fertilized NOD/Shi strain mouse eggs using standard methods. Transgenic offspring were identified by PCR (annealing temperature 60°C) using GFP1 forward and reverse primers (5'-CTGGTCGAGCTGGACGGC-GACG-3' and 5'-CACGAACTCCAGCAGGAC-CATG-3', respectively). Genomic DNA extracted from tail tissue was amplified in a 20  $\mu$ l reaction volume under the following conditions: 94°C for 2 min; 30 cycles at 94°C for 30 s, 60°C for 30 s, and 72°C for 30 s; and a final extension step at 72°C for 3 min. Transgene DNA showed an amplified product band of 630 bp on agarose gel electrophoresis. For cloning of the transgene/transgene junction, PCR amplification was conducted using PgkEGFP-NOG mouse DNA and the GFP1 forward primer and PGKR1 reverse primer, 5'-AGAAAGC-GAAGGAGCAAAGCT-3'. Nucleotide sequences of the transgene/transgene junction were determined using an ABI PRISM 3130  $\times$  1 Genetic Analyzer (Life Technologies Corporation, Carlsbad, CA, USA) and ABI PRISM BigDye Terminator Cycle Sequencing Ready Reaction Kits (Life Technologies Corporation). Transgenic females were mated with NOG males to confer the *scid* and *Il2rg<sup>null</sup>* mutations to the offspring. The *Il2rg<sup>null</sup>* mutations were genotyped using a previously described PCR method [6]. The *scid* mutations were genotyped using Cycling Probe Technology (Cycleave<sup>®</sup> PCR Reaction Mix, Takara Bio Inc., Shiga, Japan). Information for the primers and probes is provided in the supplementary table. Reaction mixture composition and cycling conditions were in

accordance with the instruction manual. Real-time PCR was performed using an Applied Biosystems 7500 Fast Real-Time PCR System, and data were collected using the SDS 2.6 application software (ABI). EGFP expression in the P<sub>gk</sub>EGFP-NOG mouse was then confirmed with the IVIS Spectrum CT and Living Image software (Perkin Elmer Inc., Waltham, MA, USA). For flow cytometric analysis of peripheral blood, hemolysis was performed as previously described [6]. Samples were analyzed using a FACSCanto analyzer (BD). Data were recorded with the BD FACSDiva Software (BD) and analyzed using the FlowJo software (Tree Star). The murine line used in the present study was assigned the genetic designation NOG-Tg (*Pgk-EGFP*) 16/ShiJic (formally, NOD.Cg-*Prkdc*<sup>scid</sup>*Il2rg*<sup>tm1Sug</sup> Tg (*Pgk-EGFP*) 16/ShiJic, abridged name: P<sub>gk</sub>EGFP-NOG). We also generated BALB/cA.Cg-Tg (*Pgk-EGFP*) 16/Jic (abridged name, P<sub>gk</sub>EGFP-BALB) and C57BL/6J.Cg-Tg (*Pgk-EGFP*) 16/Jic (abridged name, P<sub>gk</sub>EGFP-C57BL/6) by crossing the inbred P<sub>gk</sub>EGFP-NOG strain with the BALB/cA or C57BL/6J strain, respectively, using the speed congenic method [18]. This study was performed in accordance with the guidelines of our institution and was approved by the Animal Experimentation Committee of the Central Institute for Experimental Animals.

#### Cell culture

The human colorectal cancer cell line HCT 116 was obtained from the American Type Culture Collection (Manassas, VA, USA). HCT 116 cells were maintained in Dulbecco's Modified Eagle Medium (DMEM; Invitrogen Corporation, Carlsbad, CA, USA) supplemented with 10% fetal bovine serum (Invitrogen), 2 mM L-glutamine (L-Gln; Nacalai Tesque, Inc., Kyoto, Japan), 50 units/ml of penicillin, and 50  $\mu$ g/ml of streptomycin (P-S; Nacalai Tesque, Inc.).

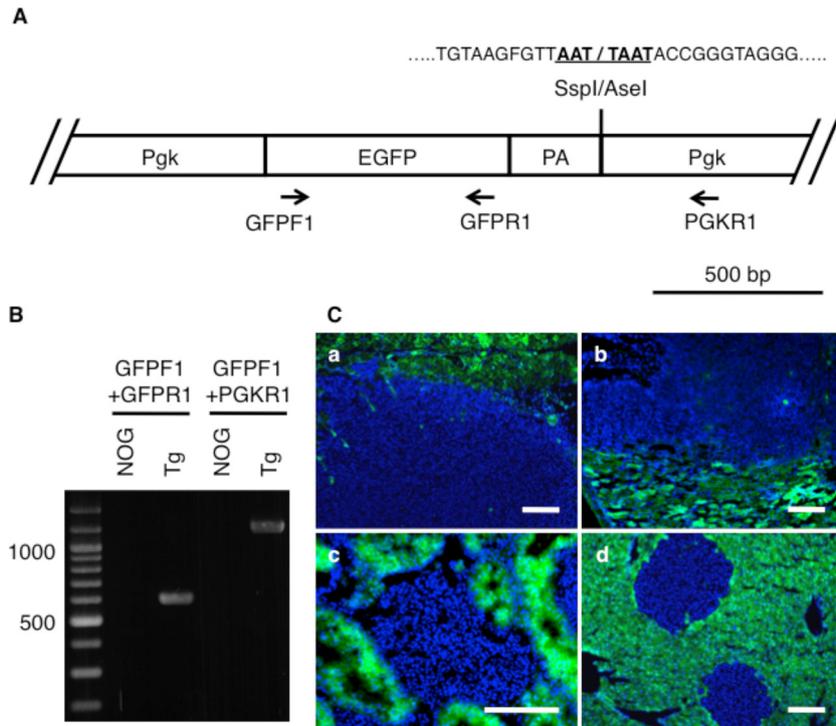
Human iPS cell line 201B7 was provided by the RIKEN BRC (Ibaraki, Japan) through the National Bio-Resource Project of the Ministry of Education, Culture, Sports, Science and Technology (MEXT), Japan [19]. Human iPS cells were maintained with mitomycin C-treated mouse embryonic fibroblast feeder cells in DMEM:Nutrient Mixture F-12 (Invitrogen) supplemented with 20% knockout serum replacement (KSR; Invitrogen), L-Gln, P-S, 100  $\mu$ M nonessential amino acids (Invitrogen), 100  $\mu$ M  $\beta$ -mercaptoethanol (Sigma-Aldrich, St. Louis, MO, USA), and 5 ng/ml of basic fibroblast growth factor (Wako Pure Chemical Industries,

Ltd., Osaka, Japan).

#### Transplantation

Confluent HCT 116 cells were dissociated using 0.25% trypsin- ethylenediaminetetraacetic acid (EDTA; Invitrogen). For subcutaneous transplantation,  $1 \times 10^5$  HCT 116 cells were suspended in 100  $\mu$ l of Hanks' Balanced Salt Solution (HBSS, Invitrogen) and subcutaneously injected. For transplantation under the kidney capsule, the kidney was exteriorized through a dorsal-horizontal incision, and a syringe with a 29-G needle with a flattened tip was introduced into the kidney at a site away from the transplanted region. The kidney was penetrated, the tip of the needle was held just beneath the kidney capsule, and  $1 \times 10^4$  HCT 116 cells in 10  $\mu$ l of HBSS were injected. For transplantation into the testis,  $1 \times 10^4$  HCT 116 cells in 10  $\mu$ l of HBSS were injected into the testis using a syringe with a 29-G needle. In the case of intrasplenic (isp) injection,  $1 \times 10^4$  HCT 116 cells in 50  $\mu$ l HBSS were injected isp using a Hamilton syringe with a 26-G needle, as previously described [8]. Recovered grafts were embedded in optimal cutting temperature compound (Sakura Finetechnical Co., Ltd., Nagoya, Japan). Fresh frozen sections were immediately fixed with 4% paraformaldehyde (PFA) or 100% ethanol. For the observation of EGFP fluorescence, PFA-fixed samples were embedded in ImmunoSelect Antifading Mounting Medium (Dianova, Inc., Pinole, CA, USA) and then analyzed by fluorescent microscopy (Carl Zeiss Microscopy GmbH, Jena, Germany).

To assess teratoma formation, confluent human iPS cells were treated with phosphate-buffered saline (Nacalai Tesque, Inc.) supplemented with 20% KSR, 0.25% trypsin (Invitrogen), 1 mg/ml of collagenase IV (Invitrogen) and 10 mM CaCl<sub>2</sub> (Nacalai Tesque, Inc.) for 5 min. Cell clusters were gently detached using a cell scraper and collected in a 15-ml conical tube. A part of the cell suspension was treated with 0.25% trypsin-EDTA and used for cell counting. Next,  $1 \times 10^7$  human iPS cells were suspended in 0.2 ml of HBSS and then subcutaneously injected into the mice. Eight weeks post-transplantation, the teratomas were recovered and fixed with 10 nM Mildform formaldehyde solution (Wako Pure Chemical Industries, Ltd.). Paraffin-embedded tissues were then sliced and stained with hematoxylin and eosin (HE).



**Fig. 1.** Establishment of the PkgEGFP-NOG mouse. **A.** A schematic representation of the inserted transgenes. The GFFP1+GFFP1 primer set was used for genotyping PCR, and the GFFP1+PGKR1 primer set was used to sequence the tandem transgene junction. The nucleotide sequence of the tandem transgene junction was described. **B.** The PCR products of the primer sets GFFP1+GFFP1 (630 bp) and GFFP1+PGKR1 (1288 bp). NOG mice were used as negative controls (Non). Tg indicates the PkgEGFP-NOG offspring. **C.** HCT 116 cells were transplanted into subcutaneous (a), kidney (b), testis (c), and liver tissues (d) of the PkgEGFP-NOG mice. Xenotransplanted organs were recovered 2 weeks after transplantation, and fresh frozen sections were analyzed by fluorescence microscopy. The green fluorescence indicates EGFP, and blue indicates nuclear staining (DAPI). Xenografts in the PkgEGFP-NOG mice were clearly identified as EGFP-negative colonies. \*Bar=200  $\mu$ m.

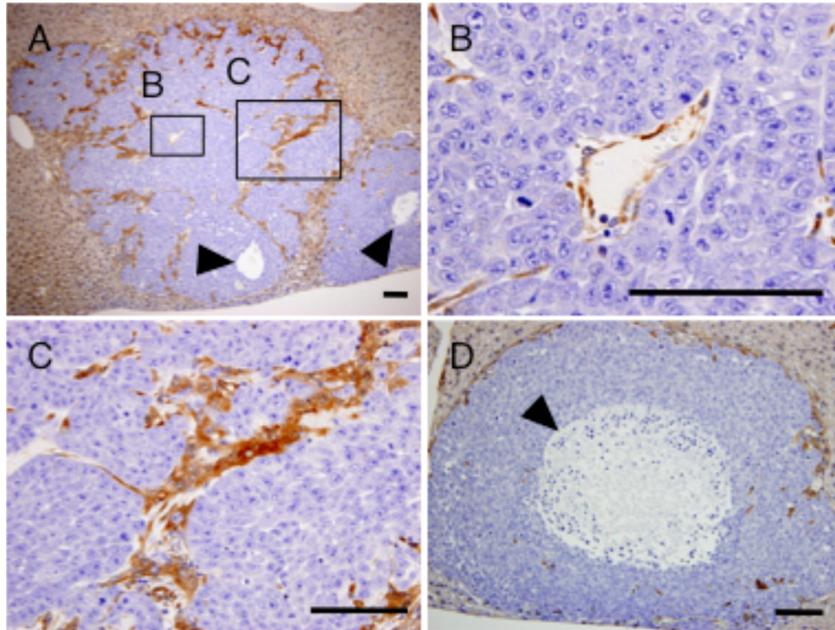
### Immunohistochemical analysis

The following antibodies were used for immunohistochemical analysis: mouse anti-human leukocyte antigen (Hokudo Co., Ltd., Hokkaido, Japan), rabbit anti-vimentin (Nichirei Bioscience, Tokyo, Japan), and anti-GFP (Abcam Inc., Cambridge, MA, USA). The antibodies for mouse immunoglobulins were visualized using amino acid polymer/peroxidase complex-labeled antibodies (Histofine Simple Stain Mouse MAX PO (M); Nichirei Bioscience) and diaminobenzidine (Dojindo Laboratories, Kumamoto, Japan) substrate (0.2 mg/ml 3,3'-diaminobenzidine tetrahydrochloride, 0.05 M Tris-HCl (pH 7.6), and 0.005% H<sub>2</sub>O<sub>2</sub>). Sections were counterstained with hematoxylin.

### Results

#### Establishment of the PkgEGFP-NOG mouse line

The PkgEGFP transgene was microinjected into fertilized NOD/Shi strain mouse eggs and confirmed by genomic PCR, and the sequence of the tandem transgene junction was defined (Figs. 1 A and B). Transgenic offspring were mated with NOG mice, and insertion of the *scid* and *Il2rg<sup>null</sup>* mutations was confirmed as previously described [6]. Next, we confirmed EGFP expression in the PkgEGFP-NOG mice (Figs. S1–S3). Although EGFP expression in the cardiac muscle, skeletal muscle and pancreatic exocrine cells was faint (Figs. S1 and S3), EGFP expression was systemically observed (including in the liver parenchymal and ductal epithelium cells). Furthermore, no sexual dimorphism was observed (data



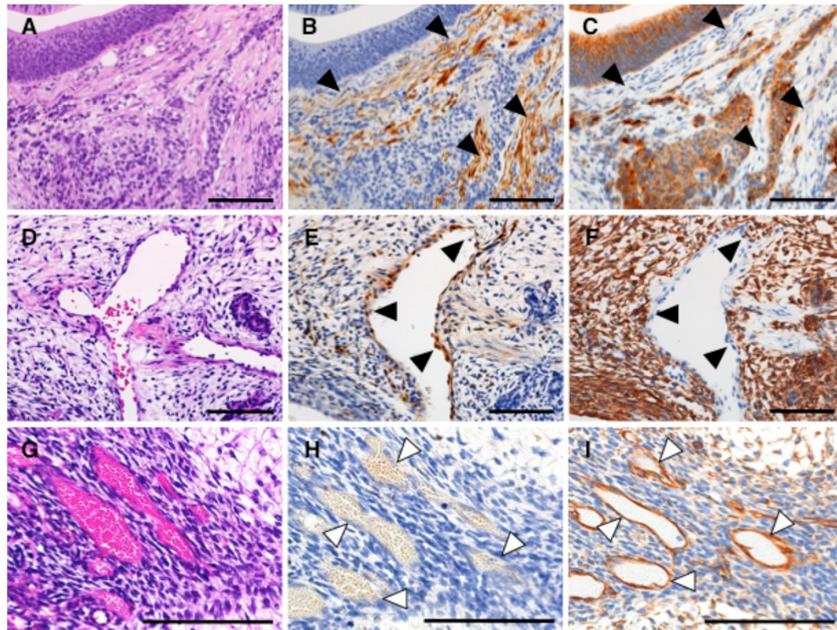
**Fig. 2.** Tumor microenvironment in the xenograft colonies composed of EGFP-expressing stromal tissue components. HCT 116 cells were intrasplenically transplanted into the livers of PgkEGFP-NOG mice. Xenotransplanted livers were recovered 3 weeks after transplantation, and EGFP-expressing tissues in the xenografts were subjected to immunohistochemical analysis. In the recovered livers, the xenografts were identified as EGFP-negative colonies (A). EGFP-expressing vessels (B) and fibroblasts (C) formed the tumor microenvironment in the xenograft colonies. On the other hand, EGFP-expressing stromal tissues were not observed in the necrotic areas (A and D, arrowheads). \*Bar=100  $\mu$ m.

not shown). Since the host tissues expressed EGFP, engraftment derivatives that were transplanted in the PgkEGFP-NOG mice appeared to be EGFP-negative. We then transplanted the human colon cancer cell line, HCT 116, into the PgkEGFP-NOG mice. HCT 116 cells were transplanted into subcutaneous, kidney capsule, testis, and liver tissues of the PgkEGFP-NOG mice, and each transplanted organ was recovered 2 weeks posttransplantation. EGFP fluorescence analysis revealed that the HCT 116-derived xenografts could be clearly distinguished from host tissues, which displayed EGFP fluorescence (Fig. 1 C). Immunohistochemical analysis of the xenografts demonstrated that EGFP-negative tissues were positively counterstained with human leukocyte antigen (HLA; Fig. S4). Collectively, these results indicated that the xenografts in the PgkEGFP-NOG mice were clearly identified by the absence of EGFP expression.

#### *Tumor microenvironment analysis in PgkEGFP-NOG mice*

To investigate the tumor microenvironment, we per-

formed *in situ* transplantation of the HCT 116 cells into PgkEGFP-NOG mice. The xenotransplanted livers were recovered 3 weeks after transplantation and subjected to immunohistochemical analysis. EGFP-expressing stromal tissue components (i.e., vessels and fibroblasts) were readily identified in the xenograft tissues and formed a complicated tumor microenvironment (Figs. 2 A–C). We also observed necrotic areas in the xenografts (Figs. 2 A and D). In accordance with a previous report, EGFP-expressing vessels and fibroblasts were not observed in the necrotic areas [22]. Collectively, these results indicated that the interaction between the xenograft and host tissues is important for tumor growth and maintenance. Next, we examined teratoma formation in the subcutaneously injected PgkEGFP-NOG mice. It is well known that human iPS cells can differentiate into stromal tissue components. However, EGFP-expressing vascular cells and fibroblasts were clearly distinguishable from xenotransplanted derivatives (Figs. 3 B and E). The microenvironment was composed of EGFP-expressing tissues and human iPS cell derivatives, which stained posi-



**Fig. 3.** The microenvironment in human iPS cell-derived teratomas. The human iPS cells were subcutaneously transplanted into PgkEGFP-NOG mice. Formed teratomas were recovered 8 weeks after transplantation. We stained the paraffin sections of the teratomas with HE stain (A, D, and G), and serial sections were counterstained with GFP (B, E, H), HLA (C), or human vimentin (F and I). The microenvironment is composed of EGFP-expressing fibroblasts (B and C; black arrowheads) or vascular cells (E and F; black arrowheads), and iPS cell derivatives, which were positively stained using human-specific markers. Vimentin-stained human vessels were not stained with EGFP (H and I; white arrowheads). \*Bar=200  $\mu$ m.

tively for HLA and vimentin (Figs. 3 C and F). On the other hand, vimentin-positive human vascular cells were not EGFP-positive (Figs. 3 H and I). Collectively, these results indicated that human iPS cell-derived teratomas contained components of host stromal tissues that composed the complicated microenvironment.

### Discussion

In the present study, we established a PgkEGFP-NOG mouse that displayed systemic EGFP expression and equivalent immunodeficiency to the NOG mouse. Previously, we reported another EGFP-expressing NOG mouse line, which was established by the congenic method using a marker-assisted selection protocol (NOG-EGFP mouse) [18]. We summarized the systemic EGFP expression patterns of PgkEGFP-NOG and NOG-EGFP mice in Figs. S1, S2, and S3. Quantification of EGFP fluorescence revealed that the NOG-EGFP mouse line showed enhanced EGFP expression, especially in the heart, skeletal muscle, and pancreas. On the

other hand, EGFP expression in the liver and ductal epithelium of PgkEGFP-NOG mice was stronger than that of NOG-EGFP mouse. One of the differences between PgkEGFP-NOG and NOG-EGFP mice was the transgene driver. In the NOG-EGFP mice, EGFP is expressed under the control of the cytomegalovirus early enhancer element and chicken beta-actin (CAG) promoter [12]. Moreover, transgene expression might be affected by the genomic environment. These factors reflect the differences in transgene expression between PgkEGFP-NOG and NOG-EGFP mice.

The use of PgkEGFP-NOG mice makes it easy to investigate formation of the microenvironment in the recipient liver. Transplantation of human cancer cells into the liver of PgkEGFP-NOG mice demonstrated that the tumor microenvironments in the xenograft tissues were composed of host fibroblasts and vessels (Fig. 2). In addition, the tumor microenvironment was not observed in the necrotic areas of the xenograft colonies. A previous study demonstrated that angiogenesis is induced from transplanted tumor cells, which is essential for

tumor growth [21]. Our results indicate that formation of a suitable microenvironment is essential for xenotransplanted tumor cell growth and maintenance.

Teratoma formation is regarded as a landmark of the pluripotency of human iPS cells. Since the teratoma contains three germ layer derivatives, it is difficult to distinguish between host tissues and iPS cell derivatives by morphological characterization. However, the origin of iPS cell derivatives can be easily identified in P<sub>gk</sub>EGFP-NOG mice (Fig. 3). Moreover, teratoma formation in the P<sub>gk</sub>EGFP-NOG mouse demonstrated that EGFP-expressing stromal tissue components were the primary components of the microenvironment in the teratomas (Fig. 3). Our results indicated that formation of a suitable microenvironment is induced simultaneously with teratoma formation.

Taken together, our established P<sub>gk</sub>EGFP-NOG mouse demonstrated severe immunodeficiency and systemic EGFP expression. Transplanted cell derivatives in the P<sub>gk</sub>EGFP-NOG mice were readily identified by the absence of EGFP expression, suggesting that the P<sub>gk</sub>EGFP-NOG mouse presents a useful tool to analyze the interactions between xenotransplanted cells and host stromal tissues.

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