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*Establishing EGFP Congenic Mice in a NOD/Shi-*scid *IL2Rg^{null} (NOG) Genetic Background Using a Marker-Assisted Selection Protocol (MASP)*

Hiroshi SUEMIZU, Chie YAGIHASHI, Tomoko MIZUSHIMA, Tomoyuki OGURA, Tomoo ETOH, Kenji KAWAI, and Mamoru ITO

Central Institute for Experimental Animals, 1430 Nogawa, Miyamae, Kawasaki 216-0001, Japan

Abstract: *Severely immunocompromised NOD/Shi-*scid *IL2Rg^{null} (NOG) mice, which show higher engraftment efficiency, are useful as recipients in xenotransplantation studies. We generated a NOG-enhanced green fluorescent protein (EGFP) transgenic (Tg) mouse (NOG-EGFP) that was introduced the EGFP transgene from the C57BL/6-EGFP Tg mouse using the speed congenic method with a marker-assisted selection protocol (MASP). With this method, the selection of the male with the closest NOG strain type was repeated four times. When human cord blood CD34$^+$ cells were transplanted into NOD/Shi-*scid*, NOG, and NOG-EGFP mice (N_6), the engraftment efficiency of the NOG-EGFP mice was significantly higher than that of the NOD/Shi-*scid *mice and was similar to that of the NOG mice. These results suggest that the NOG-EGFP mice, which were generated using the congenic method with MASP, acquired the immunological properties of the NOG mice.*

Key words: *microsatellite marker, NOG mouse, speed congenic*

Previously established techniques of introducing additional genetic changes into transgenic or knockout mice include transgenic methods involving the direct microinjection of fertilized eggs and congenic strategies using extant genetically modified mice. The congenic strategy is the classical method and is easiest to perform, but, backcrossing must be performed for at least seven generations to replace over 99% of the genetic background (99.2% is theoretically replaced), which is extremely time-consuming. One of the fastest ways to introduce additional genetic modification is the marker-assisted selection protocol (MASP) known as the "speed congenic" method. In MASP, the male mouse showing the most complete replacement with the targeted genetic background is selected as the "best" male and is used in the next backcross [11–13]. NOG (formally, NOD.Cg-*Prkdc^{scid} Il2rg^{tm1Sug}*/ShiJic) mice, which were developed by introducing IL2Rg^{null} mutation from C57BL/6-*Il2rg^{tm1Sug}* mice with backcross mating to NOD/Shi-*scid* mice [3], have no lymphocytes (neither T nor B) or natural killer (NK) cells, and have impaired dendritic cell function [4, 7]. Therefore, NOG mice can be used to develop "humanized mice", which possess high levels of human-derived cells or tissues. Because it is difficult to label human cells (e.g. hematopoietic stem cells or neural stem cells) with visible markers, we

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Address corresponding: H. Suemizu, Central Institute for Experimental Animals, 1430 Nogawa, Miyamae, Kawasaki 216-0001, Japan

attempted to introduce the enhanced green fluorescent protein (EGFP) gene into the NOG mouse as a visible recipient marker, which if successful would make it easier to differentiate donor cells from recipient cells in studies of transplantation and regenerative medicine.

This study was performed in accordance with institutional guidelines and was approved by the Animal Experimentation Committee of the Central Institute for Experimental Animals. NOG mice expressing the EGFP gene can be obtained reliably by backcrossing with an animal in which the phenotype of the modified gene is already expressed in the donor. Therefore, we mated an inbred line of C57BL/6-*Tg(Act-EGFP)C14-Y01-FM1310sb* (C57BL/6-TgEGFP) mice [6, 8] with a NOG mice to make a NOG-EGFP (NOD.Cg-*Prkdc*^{scid} *Il2rg*^{tm1Sug} *Tg(Act-EGFP)C14-Y01-FM1310sb*/ShiJic) mice. Tail clips of the mice were obtained and digested with proteinase K using standard methods [9]. Genomic DNA was extracted using the MagExtractor System MFX-9600 Magnia R Plus (Toyobo, Osaka, Japan) according to the manufacturer's instructions. The EGFP transgene was genotyped by PCR with the forward primer GFP-F1 (5'-CTGGTCGAGCTGGACGGCGACG-3') and reverse primer GFP-R1 (5'-CACGAACTCCAGCAGGACCATG-3'). The *scid* and IL2Rg^{null} mutations were genotyped using a previously described PCR method [2, 3]. Three to six microsatellite markers were selected from each chromosome, including the X-chromosome. We selected 87 microsatellite markers to evaluate the mouse genetic backgrounds of the C57BL/6, NOG, and 129S6/SvEv strains because the Tg EGFP mice originated in C57BL/6, and the IL2Rg^{null} mutants were generated with mouse ES cells (CCE) derived from the 129S6/SvEv strain. To evaluate the genetic background in more detail, an additional 17 microsatellite markers were arranged on chromosome 9. General information about the primers, including the size of the PCR product, is listed in Table 1. First, 10–100 ng of genomic DNA was suspended in a total volume of 12.5 μl PCR buffer containing 20 mM Tris-HCl (pH 8.4), 50 mM KCl, 1.5 mM $MgCl_2$, 0.1–0.3 μM fluorescence-labeled primers, 0.2 mM dNTP, and 1.0 unit of Platinum GenoType *Tsp* DNA polymerase (Invitrogen, Carlsbad, CA, USA). Using a Bio-Rad iCycler (Bio-Rad Laboratories, Hercules, CA, USA), the thermal cycling conditions consisted of 1 cycle at 95°C for 2 min followed by: 10 cycles at 94°C for 45 s, 60°C for 1 min, and 72°C for 1 min; then, 17 cycles at 89°C for 1 min, 60°C for 1 min, and 72°C for 1 min; with a final extension of 10 min at 72°C. Following PCR, 2 μl of product, 0.6 μl of GS500-LIZ size standard (Applied Biosystems, Foster City, CA, USA), and 24.4 μl of Hi-Di formamide (Applied Biosystems) were mixed and denatured at 95°C for 2 min, cooled on ice, and then loaded directly on an ABI 3130xl Genetic Analyzer (Applied Biosystems). The electrophoresis data were processed using GeneMapper 4.0 software (Applied Biosystems). When non-labeled primer pairs were used, the PCR products were electrophoresed on 3% NuSieve 3:1 agarose (Lonza Walkersville, Inc., Walkersville, MD, USA) gel/20 mM Tris-acetate, 2.5 mM EDTA (0.5 × TAE) and then stained with ethidium bromide (0.25 μg/ml). Statistical analyses were performed with StatView 5.0 and Prism 5 software.

To introduce the EGFP transgene into NOG mice, the first generation hybrid (F_1) was obtained by mating a male C57BL/6-TgEGFP mouse as the donor with a recipient NOG female mouse. The F_1 hybrid received a uniform genome (except for the sex chromosome) from the donor and recipient, and the replacement rate (described as "% Recipient genome" in Table 2) for both the expected and observed values was 50% (Table 2). In the next stage, a randomly selected male F_1 mouse with the EGFP transgene was again mated with female NOG recipient mice to obtain the N_2 generation. Marker-assisted selection was started at the N_2 generation to select the "best" male congenic mouse. Using the 61 informative markers, the male with the closest NOG strain type was selected as the parent for the N_3 generation. Using this method, the selection of males with the closest NOG strain type was repeated four times. The observed "% Recipient genome" of the male closest to the NOG background was 81.1, 95.9, 98.4, and 99.2% in the N_2, N_3, N_4, and N_5 generations, respectively. The higher values of the observed "% Recipient genome" in generation N_3 and N_4 were statistically significant (P=0.018 and 0.067, respectively, Fisher's exact probability test). Wakeland *et al*. described the rationale of speed congenics, and stated that the advantage of screening with low-density markers (25 cM marker spacing) was realized in the N_3 and N_4 generations [12]. Com-

Table 1. MASP primer pairs and PCR product sizes in the NOG, C57BL/6, and 129S6/SvEv strains

Marker	Position (cM) [b]	NOG	C57BL/6	129S6/SvEv (CCE ES)	Dye	Marker	Position (cM) [b]	NOG	C57BL/6	129S6/SvEv (CCE ES)	Dye
D1Mit67 [a]	9	125	133	125	NED	*D9Mit182*	55	115	99	104	NL [c]
D1Mit303	34.8	112	122	112	NED	*D9Mit53*	57	198	206	198	FAM
D1Mit132 [a]	43.1	160	141	160	NED	*D9Mit184*	60	125	132	127	FAM
D1Mit91	64	146	146	138	PET	*D9Mit20*	61	95	104	114	FAM
D1Mit102 [a]	73	121	110	125	FAM	*D9Mit214*	62	135	137	108	FAM
D1Mit459 [a]	102	116	120	116	PET	*D9Mit215*	63	116	116	122	NED
D2Mit1 [a]	1	111	115	113	VIC	*D9Mit281*	68	106	111	106	FAM
D2Mit312	1	120	120	111	PET	*D9Mit120*	69	126	146	146	NL [c]
D2Mit182 [a]	38.3	149	153	149	PET	*D9Mit52* [a]	72	173	171	171	VIC
D2Mit311 [a]	83.1	110	121	115	VIC	*D10Mit2* *[a]	16	133	126	133	NED
D2Mit346 [a]	91.8	107	101	118	PET	*D10Mit31* [a]	36	146	148	151	NED
D3Mit149 *[a]	2.4	147	138	138	PET	*D10Mit266* [a]	62	82	90	84	PET
D3Mit62	4.6	117	117	110	NED	*D11Mit21* [a]	20	147	158	147	VIC
D3Mit25 [a]	29.5	127	129	123	VIC	*D11Mit67* [a]	57	133	131	122	VIC
D3Mit85 [a]	72.9	226	218	224	VIC	*D11Mit48* [a]	77	123	129	123	NED
D3Mit89 [a]	86.1	214	220	214	FAM	*D11Mit184*	78	ND	149	160	NED
D4Mit227 [a]	3.2	182	180	172	VIC	*D12Mit109* [a]	19	124	116	124	VIC
D4Mit52 [a]	54.9	110	119	112	PET	*D12Mit156* [a]	34	184	176	171	FAM
D4Mit190	79	ND	138	130	FAM	*D12Mit30*	46	103	103	113	PET
D4Mit256 [a]	82.7	128	132	128	FAM	*D12Mit133* [a]	56	96	111	111	FAM
D5Mit146 [a]	1	125	120	115	PET	*D13Mit132* [a]	4	157	150	157	NED
D5Mit1	5	135	135	129	VIC	*D13Mit13* [a]	35	145	151	145	PET
D5Mit58 [a]	41	116	114	123	NED	*D13Mit51* [a]	59	139	137	130	FAM
D5Mit367 [a]	65	96	102	92	FAM	*D14Mit1* [a]	3	101	104	98	PET
D5Mit97 [a]	74	118	124	118	NED	*D14Mit233* [a]	19.5	182	194	179	VIC
D6Mit86 [a]	0.5	121	132	121	NED	*D14Mit225* [a]	42.5	94	111	102	FAM
D6Mit284 [a]	37.5	129	138	129	FAM	*D15Mit12*	4.7	143	143	151	FAM
D6Mit304 [a]	75	105	115	107	PET	*D15Mit13* [a]	6.7	138	133	116	NED
D7Mit267 [a]	11	174	187	199	VIC	*D15Mit85*	16.4	194	194	199	FAM
D7Mit193	24.5	149	149	159	VIC	*D15Mit171* [a]	54.5	141	132	141	FAM
D7Mit350 [a]	41	138	116	122	FAM	*D15Mit42* [a]	59.2	178	182	159	VIC
D7Mit100	53.5	201	201	193	VIC	*D16Mit129* *[a]	3.4	161	180	167	PET
D7Mit189 [a]	72.4	114	129	114	PET	*D16Mit139* [a]	43.1	150	144	170	VIC
D8Mit155 *[a]	1	156	161	108	NED	*D16Mit48*	43.3	155	155	159	VIC
D8Mit217	6	169	169	179	FAM	*D16Mit106* [a]	71.5	132	142	132	PET
D8Mit191 [a]	21	122	133	122	VIC	*D17Mit163* [a]	3	126	130	124	VIC
D8Mit88 [a]	58	127	114	114	PET	*D17Mit138*	24.2	ND	138	129	FAM
D8Mit93	72	169	169	163	NED	*D17Mit53* [a]	38.5	122	128	122	VIC
D9Mit250	5	123	123	132	VIC	*D17Mit93* [a]	44.5	141	154	141	NED
D9Mit83 [a]	6	129	134	134	PET	*D18Mit19*	2	150	150	133	FAM
D9Mit97 [a]	29	162	151	159	VIC	*D18Mit12* [a]	17	122	110	110	FAM
D9Mit102	31	144	140	144	FAM	*D18Mit91* [a]	29	135	137	137	PET
D9Mit207	33	160	148	155	NL [c]	*D18Mit40*	37	135	137	137	FAM
D9Mit105	35	116	145	145	NL [c]	*D18Mit187* [a]	47	106	110	110	FAM
D9Mit259	38	122	112	115	NL [c]	*D18Mit25*	57	117	117	109	NED
D9Mit107	40	119	120	104	FAM	*D19Mit78* [a]	5	130	126	128	FAM
D9Mit8	42	185	178	169	VIC	*D19Mit14* [a]	15	150	153	146	PET
D9Mit124	42	128	124	124	FAM	*D19Mit103* [a]	52	117	115	123	NED
D9Mit236	43	125	143	125	NL [c]	*DXMit55* [a]	1.4	121	129	127	NED
D9Mit11	48	112	74	100	NL [c]	*DXMit25* [a]	27.8	158	168	158	VIC
D9Mit275	50	115	110	110	FAM	*DXMit130*	55	161	161	136	VIC
D9Mit35	52	112	124	124	NL [c]	*DXMit121* [a]	67	130	147	147	VIC

[a]: Informative microsatellite markers for distinguishing between the NOG and C57BL/6 strains; [b]: according to the Mouse Genome Database (MGD; http://www.informatics.jax.org); *: Primers were changed from the original sequence on the NCBI UniSTS database to redesigned sequences in order to change the size of the PCR product as follows; *D3Mit149*: F 5'-TTCCATACAAACAAAAGCAACG-3', R 5'-CTATATAGCTGTAAATGTAAAGTGTATGTC-3', *D8Mit155*: F 5'-TTGGACAGGGAAAATTCTGC-3', R 5'-GAAAATGTGACACCATTTGAGGAC-3', *D10Mit2*: F 5'-GTTCATTTGAGGCACAAGCA-3', R 5'-TTTGAGCTGCTCACAACCC-3', and *D16Mit129*: F 5'-ATGAGCAGTCTGCAGACCTT-3', R 5'-GAGACTGAGAAAGGGGATGC-3'; [c]: non-labeled primer pairs; ND: not detected.

Table 2. Comparison of the methods used to generate NOG-EGFP mice

Generation number [a] (Number examined [b])	% Recipient genome (R/total) [c]			
	Expected	Observed		
		MAX	MIN	AVG ± SD
P	0	–	–	–
F_1	50 (61/122)	50 (61/122)	–	–
N_2 (13)	75 (91.5/122)	81.1 (99/122)	66.4 (81/122)	73.1 ± 4.1
N_3 (15)	87.5 (106.8/122)	95.9 (117/122) *	88.5 (108/122)	91.3 ± 2.4
N_4 (31)	93.8 (114.4/122)	98.4 (120/122) **	95.9 (117/122)	96.9 ± 0.8
N_5 (4)	96.9 (118.2/122)	99.2 (121/122)	98.4 (120/122)	98.8 ± 0.5

[a] The generation count begins at strain P (parental) defined as 100% original background, 0% recipient background. The F_1 generation contains the offspring from the intercross between P (100% original) × pure recipient NOG strains (100% recipient). F_1 animals have 50% of the recipient genome. [b] The numbers of transgenic male mice whose genetic backgrounds were examined. [c] R: the number of homozygotes for the allele of the recipient strain; total: total number of alleles. **P*=0.015 and ***P*=0.095 (Fisher's exact probability test).

puter simulations of MASP-based congenic strain construction strategies using high-density (10 cM apart) and low-density (25 cM apart) marker spacing, and screening a mean of 40 and 16 progeny per generation revealed that screening 16 progeny per generation with a low-density marker was the most cost-effective strategy. Our MASP strategy is similar to the computer-simulated most cost-effective strategy because 61 informative microsatellite markers were arranged throughout the mouse genome spaced at an average distance of 26.5 cM (low density), and we screened 13, 15, and 31 progeny at N_2, N_3, and N_4, respectively. However, our MASP strategy included some gaps of over 30 cM, with a largest gap of 51.7 cM. One major problem is that a larger gap might not be able to detect a double crossing-over occurring in meiosis. Hameister *et al*. examined the frequency of double crossing-over in a 55.4-cM region between chromosome 15A2 and 15F2-3 in 151 mice and found only one animal with a double crossing-over [2]. In reality, double crossing-over does not occur at a high frequency, even in a larger gap extending over 50 cM. Therefore, detecting double crossing-over using adjacent markers might be possible. Another problem is the inability to detect a small segment of the donor genome. Because screening all gaps over 30 cM is unrealistic, we screened one large gap (43 cM) extending between *D9Mit97* and *D9Mit52* in more detail. We used extremely high-density markers (2.4 cM marker spacing) consisting of 17 informative markers in 12 and 14 progeny at N_2 and N_3, respectively. No animal with a double crossing-over was found in 12 animals in the N_2 generation, while a small contaminating segment of the donor genome was detected between markers *D9Mit207* and *D9Mit107* in progeny #E109 of the N_3 generation (Fig. 1). Considering an undetected contaminating donor genome, Wakeland *et al*. recommended performing one or two additional backcrosses when all of the markers become recipient-derived. This additional backcross reduces the level of undetected contamination in these strains to the equivalent of that at N_{10} or N_{11} using the traditional protocol [12]. When transferring a transgene or modified gene into the NOG or C57BL/6 strain from C57BL/6 or NOG and 129S6/SvEv backgrounds, our selected 61 markers serve as genetic quality standards for generating a congenic strain using MASP. The final backcross could be completed because the NOG-EGFP mouse (N_5) met the genetic quality standards after backcrossing four times. However, we persisted with backcrossing during the production of congenic progeny derived from the N_5 generation for xenotransplantation studies. This additional backcross might confer some benefit through the further elimination of residual donor genomes.

To examine the phenotype of the NOG-EGFP mice, the male mouse closest to the NOG strain with EGFP transgene in the N_5 generation was mated with female NOG mice to obtain many NOG-EGFP mice (N_6) for xenotransplantation studies. NOD/Shi-*scid* mice (male, 10 weeks old), NOG mice (female, 7 weeks old), and NOG-EGFP mice (female, 12 weeks old) were irradiated with 2.4 Gy of X-rays 24 h before transplanting 5

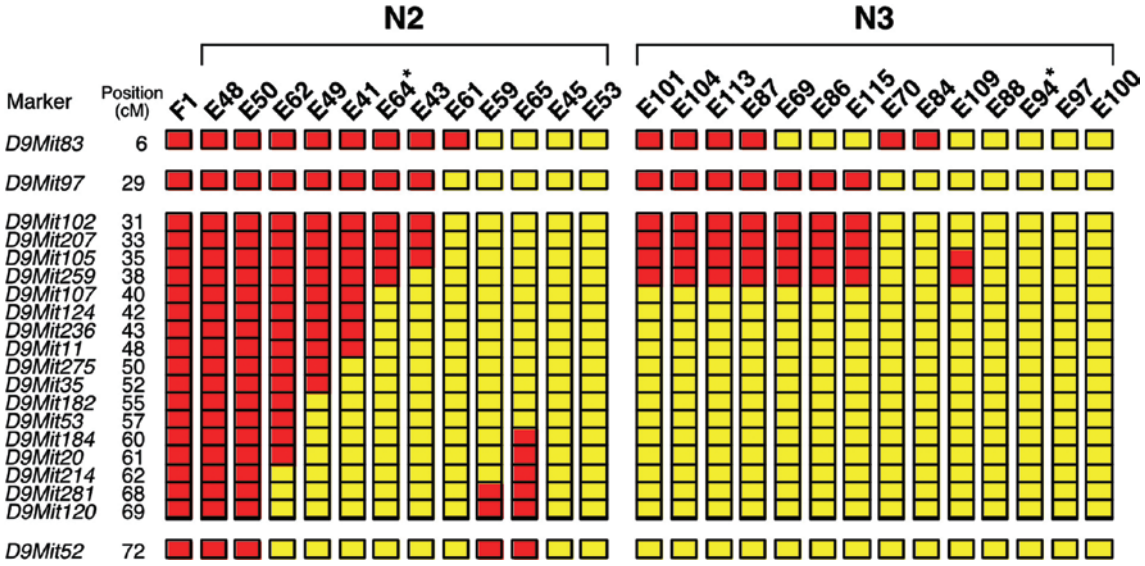

Fig. 1. Detailed screening between *D9Mit97* and *D9Mit52* using extremely high-density markers (2.4 cM marker spacing). The screening is depicted schematically using genotyping data in which each box indicates one informative microsatellite marker primer pair. The yellow boxes represent those PCR products that are identified as NOG homozygotes based on size. The red boxes represent C57BL/6 and NOG heterozygotes. The asterisks indicate the "best" males that were selected as the parents of the next backcross generation.

$\times 10^4$ human $CD34^+$ cells (Lonza Walkersville, Inc.). The engraftment of donor cells was monitored every 4 weeks by detecting the human cells expressing the leukocyte common Ag CD45 (Immunotech, Marseille, France) using a MoFlo flow cytometer (Dako, Glostrup, Denmark) and Summit software. Representative flow cytometric analysis of peripheral blood of mice that underwent transplantation (after 12 weeks) is shown in Fig. 2a. When using NOG-EGFP mice as recipients, we could easily differentiate donor human cells from recipient mouse cells with the fluorescent signals of the EGFP, although the cells were not stained with any cell surface marker for the mouse species. Twelve weeks after transplantation, significantly more growth of human cells was observed in both NOG mice and NOG-EGFP mice compared to NOD/Shi-*scid* mice (*P<0.01; **P<0.001; Fig. 2b), but there was no significant difference between NOG mice and NOG-EGFP mice. These results suggest that the NOG-EGFP mice, which were generated by the congenic method with MASP, acquired the immunological properties of the NOG strain.

In this experiment, we did not use X-chromosome-derived microsatellite markers as informative markers to select a parent for the next generation because the X-chromosome is always derived from the recipient NOG strain in male transgenic mice. However, we checked the X-chromosome with informative markers to distinguish between the NOG and 129S6/SvEv strains (the source of the CCE ES cells) because of the confirmation of an X-chromosome linked $IL2Rg^{null}$ mutation (Chr X, 38 cM) derived from the recipient genome. However, the nearest marker located at 32.1 cM was not informative, and the other three markers on the X-chromosome were shown to be homozygous for recipient-derived alleles.

Nakanishi *et al*. analyzed transgene integration sites in more than 100 EGFP transgenic mouse lines rigorously using fluorescent *in situ* hybridization and determined that the EGFP transgene is located on the D1 region of chromosome 14 in the 131 line (C57BL/6-*Tg (Act-EGFP) C14-Y01-FM1310sb*) [6]. The microsatellite markers that did not become NOG markers in the N_4 generation were the markers *D14Mit233* and *D14Mit225*, both of which are on chromosome 14. Fortunately, we could change the genetic background around the *D14Mit225* region from C57BL/6 to the NOG strain in the N_5 generation, but the genetic background around the *D14Mit233* region remained C57BL/6. *D14Mit233*

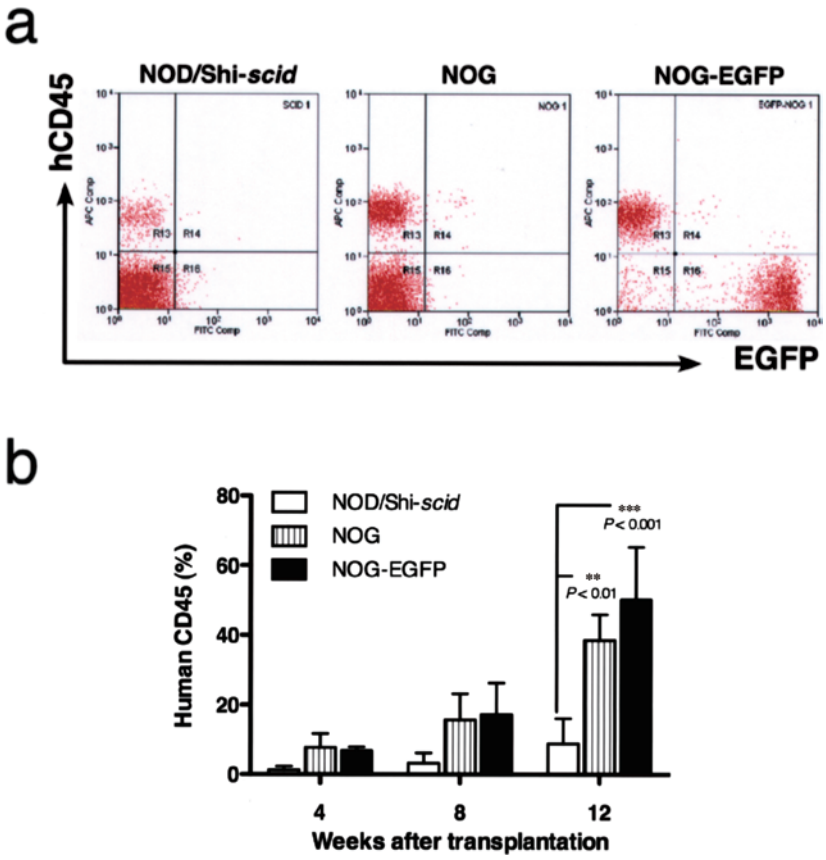

Fig. 2. High engraftment efficiency of human cells in NOG-EGFP mice. (a) Representative flow cytometric data for peripheral blood obtained from NOG-EGFP mice that underwent human cord blood $CD34^+$ cell transplantation (12 weeks after transplantation). (b) Comparison of the engraftment levels of human cells in NOD/Shi-*scid*, NOG, and NOG-EGFP mice. At the indicated times following transplantation of 5×10^4 $CD34^+$ cells, human $CD45^+$ cells in mouse peripheral blood were assayed by flow cytometry (n=3 each). $^{**}P<0.01$, $^{***}P<0.001$.

and *D14Mit225* are located in the B and D1–D3 regions, respectively. These markers are located extremely close to the site of EGFP transgene integration in the C5BL/6-TgEGFP line 131. The *D14Mit233* marker seemed to be closer to the integrated transgene than the *D14Mit225* marker because six recombinants at the *D14Mit225* locus were observed in the 63 male Tg EGFP mice, while no recombinant was observed for the *D14Mit233* marker. The distance from the *D14Mit225* marker to the integrated EGFP transgene can be calculated as roughly 9.5 cM based on the recombination frequency [10]. Based on statistical modeling, the traditional 12 backcross generation (N_{12}) protocol will produce a congenic strain in which more than 99% of the genome is unlinked to the target gene carried in a donor-derived genome segment with an average length of about 20 cM [1]. Therefore, the *D14Mit233* marker genetically linked to the transgene integration site is physically close enough to the transgene that it will not segregate independently during meiosis.

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References

1. Flaherty, L. 1981. pp. 215–221. *In*: The Mouse in Biomedical Research: History, Genetics, and Wild Mice, Vol. 1. (Foster, H.L., Small, J.D., and Fox, J.G., eds.), Academic Press, New York.
2. Hameister, H., Schulz, W.A., Meyer, J., Thoma, S., Adolph, S., Gaa, A., and von Deimling, O. 1992. *Genomics* 14: 417–422.
3. Ito, M., Hiramatsu, H., Kobayashi, K., Suzue, K., Kawahata, M., Hioki, K., Ueyama, Y., Koyanagi, Y., Sugamura, K., Tsuji, K., Heike, T., and Nakahata, T. 2002. *Blood* 100: 3175–3182.
4. Koyanagi, Y., Tanaka, Y., Kira, J., Ito, M., Hioki, K., Misawa, N., Kawano, Y., Yamasaki, K., Tanaka, R., Suzuki, Y., Ueyama, Y., Terada, E., Tanaka, T., Miyasaka, M., Kobayashi, T., Kumazawa, Y., and Yamamoto, N. 1997. *J. Virol*. 71: 2417–2424.
5. Maruyama, C., Suemizu, H., Tamamushi, S., Kimoto, S., Tamaoki, N., and Ohnishi, Y. 2002. *Exp. Anim*. 51: 391–393.
6. Nakanishi, T., Kuroiwa, A., Yamada, S., Isotani, A., Yamashita, A., Tairaka, A., Hayashi, T., Takagi, T., Ikawa, M., Matsuda, Y., and Okabe, M. 2002. *Genomics* 80: 564–574.
7. Ohbo, K., Suda, T., Hashiyama, M., Mantani, A., Ikebe, M., Miyakawa, K., Moriyama, M., Nakamura, M., Katsuki, M., Takahashi, K., Yamamura, K., and Sugamura, K. 1996. *Blood* 87: 956–967.
8. Okabe, M., Ikawa, M., Kominami, K., Nakanishi, T., and Nishimune, Y. 1997. *FEBS Lett*. 407: 313–319.
9. Sambrook, J. and Russell, D.W. 2001. Molecular Cloning, Third Edition: A Laboratory Manual. Cold Spring Harbor Laboratory Press, New York.
10. Sturtevant, A.H. 1913. *J. Exp. Zool*. 14: 43–59.
11. Teppner, I., Aigner, B., Schreiner, E., Müller, M., and Windisch, M. 2004. *Lab. Anim*. 38: 406–412.
12. Wakeland, E., Morel, L., Achey, K., Yui, M., and Longmate, J. 1997. *Immunol. Today* 18: 472–477.
13. Wong, G.T. 2002. *Neuropeptides* 36: 230–236.