detect sheep immune cells were rabbit anti-human CD3 Ab (Dako), anti-human CD79 mAb (Dako), rabbit anti-human lysozyme Ab (Dako), and rabbit anti-human myeloperoxidase (MPO) Ab (Novocastra Laboratories, Newcastle, UK), all of which have been confirmed to react to sheep counterparts [23]. The primary Abs were detected with the Dako EnVision+ System HRP (Dako) and detected with 3,3′-diaminobenzide tetrahydrochloride (Dojindo, Kumamoto, Japan). Nuclei were counterstained with Hematoxylin.

Regarding immunofluorescent staining of frozen sections, tissues were fixed with 4% paraformaldehyde. Primary Abs were anti-human histocompatibility leukocyte antigen (HLA)-A, -B, and -C mAbs (BD Pharmingen), which have been confirmed to react to cynomolgus counterparts, anti-ovine CD4 mAb (Serotec, Oxford, UK), anti-ovine CD8 mAb (Serotec), and rat anti-mouse Foxp3 Ab (eBioscience, San Diego, CA). The sections were incubated with Alexa Flour 488- or 555-conjugated secondary Abs (Invitrogen), nuclei-stained with DAPI (Donjindo), and observed with a confocal laser scanning microscope (Nikon, Tokyo, Japan).

Quantitative PCR

Genomic DNA was extracted from samples with the QIAamp DNA mini kit (Qiagen, Hilden, Germany) and subjected to quantitative DNA-PCR for the cynomolgus-specific β2-microglobulin sequence using the QuantiTect SYBR Green PCR kit (Qiagen) and the ABI Prism 7000 (Applied Biosystems, Foster, CA). Cynomolgus DNA was serially diluted with sheep genomic DNA and used to make the standard amplification curves. The primer set was 5′-GTC TGG ATT TCA TCC ATC TG-3′ and 5′-GGT GAA TTC AGT GTA GTA CAA G-3′[and amplification conditions were 40 cycles of 95°C for 60 sec, 60°C for 60 sec, and 72°C for 60 sec.

Flow cytometry

The expression of MHC class I and Oct-3 in cultured graft cells and cyES cells was analyzed using a FACS Calibur flow cytometer (BD Pharmingen). For major histocompatibility class I (MHC I), cells were incubated with phycoerythrin (PE)-conjugated anti-human HLA-A, -B, and -C mAbs (BD Pharmingen) for 30 min at 4°C. For Oct-3, cells were first fixed using the fixation/permeabilization buffer (eBioscience) for 2 h at 4°C and then incubated with Alexa Fluor 647 (Invitrogen)-conjugated anti-mouse Oct-3 mAb (BD Pharmingen) for 60 min at 4°C. Other cell-surface antigens of fetal sheep peripheral blood leukocytes were also analyzed using the flow cytometer. Fetal sheep peripheral blood (150 μl) were treated with BD Pharm Lyse™ Lysing Buffer (BD Pharmingen) to lyse red blood cells and then incubated with the following conjugated mAbs for 30 min at 4°C: PE-conjugated anti-ovine CD5 mAb (Serotec), Alexa Fluor 647 (Invitrogen)-conjugated anti-ovine CD11b mAb (Serotec), fluorescein isothiocyanate (FITC)-conjugated anti-human CD14 mAb (Serotec), and FITC-conjugated anti-ovine CD45R mAb (Serotec), all of which have been confirmed to react to sheep counterparts. Data acquisition and analysis were performed using CellQuest software (BD Pharmingen). Isotype-matched, fluorescence-conjugated, irrelevant Abs served as negative controls.

Mixed lymphocyte reaction

Mononuclear cells were isolated from heparinized sheep peripheral blood on 55% Percoll (GE Healthcare, Piscataway, NJ) and resuspended in RPMI-1640 medium with 10% fetal bovine serum (FBS). Then, 1×10^5 responder cells and 1×10^5 irradiated (4,000 cGy) stimulator cells were placed in each well of 96-well U-bottomed plates and incubated at 37°C for 5 days. Plates were pulsed with 1 μCi/well of [^{3}H]-thymidine for 24 h and cellular intake of [^{3}H]-thymidine was quantified with a β-scintillation counter (Aloka, Tokyo, Japan). Used as stimulator cells were autologous peripheral blood mononuclear cells (PBMCs), cyES cells, cynomolgus PBMCs, and cultured adherent cells of the grafts that were confirmed to be of cynomolgus origin by karyotyping (SRL, Tokyo, Japan). Cynomolgus PBMCs were isolated on Ficoll-Paque PLUS (GE Healthcare). The mixed lymphocyte reaction (MLR) was assessed with a stimulation index, which was calculated by dividing the mean count per minute of the sample over that of the autologous PBMCs (negative control). Significant differences were examined using the *t*-test.

Xenoantibody detection

Immunoglobulin (Ig) G and IgM xenoantibodies against cyES cells in sheep were determined by flow cytometry. cyES cells (2.5×10^5) were cultured with 10 ml of 1:10 diluted serum taken from the cyES cell-transplanted or naive (control) sheep. In sheep, maternal antibodies do not to pass through the placenta, but they do pass through the milk of the mothers [24]. Serum of newborn sheep was taken before they took first milk to exclude the contamination of maternal antibodies. After secondary staining with PE-conjugated donkey anti-ovine IgG Ab (Abcam, Cambridge, UK) and Alexa Fluor 647 (Invitrogen)-conjugated anti-ovine IgM mAb (Serotec), cells were examined with the FACS Calibur flow cytometer. Data acquisition and analysis were performed using the CellQuest software (BD Pharmingen). Nonviable cells were excluded from analysis by propidium iodide (Sigma, St. Louis, MO) co-staining.

Identification of ovine foxp3

On the basis of the cattle *foxp3* sequence in GenBank (accession nos. DQ322170 and XM582445), the primer set 5′-CCA AGT CAC TGG GCC TGC CCT TGA ACA-3′ and 5′-TTC TCT TCT TGG CTC TGA GAT CAG GGG C-3′ was designed for the ovine *foxp3* complete coding sequence (expected amplicon size, 1,353 bp). Total RNA was extracted from sheep PBMCs using the EZ1 RNA universal tissue kit (Qiagen) and reverse-transcribed using the RNA LA PCR kit (Takara, Shiga, Japan) with an oligo(dT) primer. The resulting cDNA was subjected to PCR with this primer set. The PCR product was sequenced with the ABI Prism 310 (Applied Biosystems). Sequence analysis was performed with the Genetyx-Mac software (Genetyx Corporation, Tokyo, Japan).

Cross-reactivity of Foxp3 Ab

The cloned ovine *foxp3* cDNA was inserted into the plasmid pCMV-IRES-EGFP and introduced into 293T cells. Trans-

fected cells were fixed using the fixation/permeabilization buffer (eBioscience), stained with PE-conjugated rat anti-mouse Foxp3 Ab (eBioscience), and examined for cross reactivity of the Ab to the ovine Foxp3 using the FACS Calibur flow cytometer.

RESULTS

Macroscopic cynomolgus/sheep chimeras

Pregnancy of ewes could be judged at 35 days of gestation and subcutaneous injection of cells into sheep fetuses was technically feasible under ultrasound guidance at 45 days of gestation in our group. We used cyES cells as a transplantation source. Although there are considerable differences in growth and differentiation conditions between mouse and primate ES cells [25,26], human and cynomolgus ES cells have remarkable similarities [21]. Therefore, studies using cyES cells would be desirable as a predictive model for hES cell behavior. Undifferentiated cyES cells were transplanted subcutaneously into sheep fetuses at 43–67 days of gestation. At birth (3 months post-transplant), palpable tumors were found at some of the transplantation sites (Fig. 1A). The overall incidence of tumor formation was 4/15 sheep (6/36 transplantation sites) (Table 1). Notably, no tumor developed when cyES cells were transplanted after 50 days of gestation (Fig. 1B, left).

To examine whether the tumors were derived from transplanted cyES cells, in situ hybridization with a cynomolgus-specific genomic DNA probe was performed. Because a cynomolgus-specific genomic probe had not been available, we first examined whether a probe developed for humans [27] can specifically detect cynomolgus sequences by testing the monkey and sheep liver tissue sections (Fig. 1C, upper). The monkey liver cell nuclei were positive with the probe, whereas signals were not detected in the sheep liver, thus demonstrating that this human probe can be used to distinguish cynomolgus from sheep cells. As shown in Fig. 1C (lower panel), the tumor cells were clearly positive with the probe, indicating their cynomolgus origin, except for blood cells, most of the feeding vessels, and granulation tissues. To exclude the possibility of their fusion with sheep cells, we examined the karyotype of tumor cells (Fig. 1D). On average, 83% of tumor cells in the engrafted sheep (n = 3) had normal cynomolgus 42 chromosomes, a pattern identical to that of the transplanted cyES cells [21]. On the other hand, cultured sheep cells (n = 3) usually consisted of 54 chromosomes. Taken together, the tumors were cyES cell-derived grafts but were fed by the host sheep vessels. The largest graft was $30 \times 28 \times 9$ cm in size and weighed 3.5 kg (sheep no. 5), which implied favorable local microenvironments of fetal sheep for the growth of cyES cells.

Next, we transplanted different numbers of cyES cells into two or four different sites per fetal sheep (Table 1). As a result, the minimal cell number needed to engraft was found to be 1.4×10^6. Among the cyES cell-engrafted sheep, no engraftment was observed at any sites receiving less than 1×10^6 cells (Fig. 1B, right). Thus, the transplanted cell number was also critical for engraftment. A similar result was reported for the allogeneic transplantation of mouse ES cells [28].

The grafts were excised or biopsied from all the engrafted sheep before 1.5 months of age. The grafts contained all three germ layer cells, composing mature tissue structures such as neural epithelia, cartilage, ductal epithelia, and hepatocyte-like cells (Fig. 2A). Immunohistochemistry revealed that the graft cells were all negative for Oct-3, a pluripotent marker of ES cells [29,30]. On the other hand, they were positive for the differentiation markers ectodermal GFAP, NSE, endodermal α-FP, and mesodermal αSMA, vimentin, and desmin (Fig. 2B). Endodermal cells were found much less frequently than ectodermal and mesodermal cells, which might be a reflection of the subcutaneous transplantation sites [31]. Although transplanted cyES cellss were originally negative for MHC class I, many of the cells became positive in the grafts after birth (Fig. 2B).

Histology I: fetal period

The cyES cell-derived grafts in sheep were observed only when the cells were transplanted in utero before 50 days of gestation. It has been believed that early sheep fetuses are immunologically naive and that host immune responses can be circumvented. This concept has received support from the immunological ontogeny in fetal sheep. Morphologically mature lymphocytes first appear in peripheral blood at 32 days of gestation, monocytes at 63 days, and neutrophils at as late as 123 days [32]. IgG and IgM are detected in peripheral blood at 56 and 77 days, respectively, but primary Ab responses remain immature at the onset, and IgG production in response to an antigen challenge occurs only after 87 days [33]. However, a histological examination regarding immune responses after in utero xenogeneic transplantation has not been performed. Here, we transplanted cyESCs expressing GFP (CMK6G, 6×10^6 cells/site) into fetal sheep at either 48 (<50) or 60 (>50) days of gestation, and examined the in vivo fate of transplanted cells and host immune responses at 5 days and 2 weeks after the transplantation.

The transplant sites of delivered fetuses were determined under a fluorescence microscope or by detecting petechiae resulting from the puncture at transplant. When

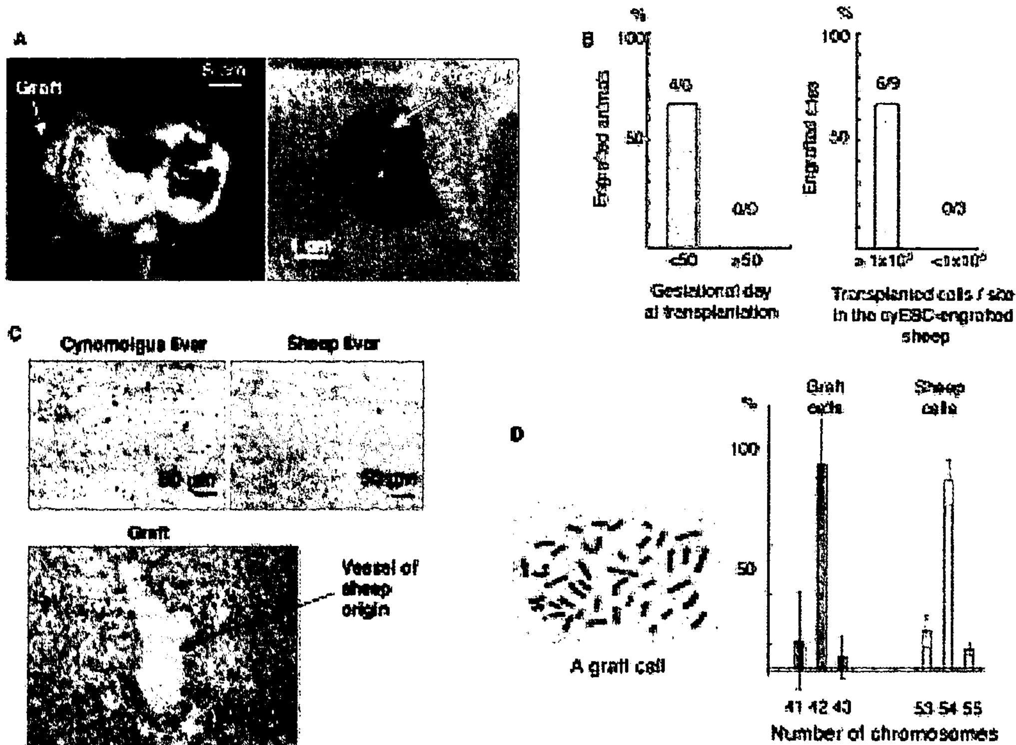

FIG. 1. Macroscopic engraftment after in utero transplantation of cyESCs into fetal sheep. (**A**) Outward appearance and macroscopic view of a tumor are shown. (**B**) The engraftment ratio (macroscopically engrafted number per total transplant number) was compared between the sheep transplanted at <50 days and ≥50 days of gestation (*left*). The engraftment ratio was also compared between $\geq 1 \times 10^6$ and $\leq 1 \times 10^6$ transplanted cells/site in the cyES cell-engrafted sheep (*right*). (**C**) In situ hybridization with the human genomic DNA probe showed that cynomolgus liver cell nuclei were stained positively, whereas sheep liver cell nuclei were not stained, indicating that the probe could be used to distinguish cynomolgus from sheep cells (*upper*). As assessed with the probe, it turned out that the tumors were of cynomolgus origin except for blood cells, some vessels, and granulated areas (*lower*). (**D**) The karyotype of tumor cells was same as that of cyES cells (*left*). The chromosome number was compared between graft and sheep cells (*right*).

cyES cells were transplanted at 48 days of gestation, GFP^+ transplanted cell progeny were detected in ductal structures 5 days later, at which time no immune cell infiltration was observed (Fig. 3A). At 2 weeks post-transplant, GFP^+ cells were found again in ductal structures (Fig. 3B), and had increased in number as suggested by the expansion of areas of GFP-derived fluorescence (Fig. 3C). A considerable number of $CD3^+$ T cells and a small number of $CD79^+$ B cells surrounded the grafts without macrophages (lysozyme-positive) (Fig. 3B). These immune cells were not stained with anti-GFP in the serial sections and thus were of host (sheep) origin. The transplanted cell progeny were still positive for Oct-3 at 2 weeks post-transplant (Fig. 3D). Staining of frozen sections showed that the surrounding T cells were $CD4^+$ and $CD8^-$ (Fig. 3E). Thus, the transplanted cells survived and proliferated, being surrounded by host $CD4^+$ $CD8^-$ T cells, when transplantation was conducted at 48 days of gestation.

When cyES cells were transplanted at 60 days of gestation, the cells were similarly detected in ductal structures 5 days later, at which time immune cell infiltration was not observed (Fig. 3F). At 2 weeks post-transplant, however, the transplantation sites were not stained with anti-GFP (Fig. 3G) and GFP-derived fluorescence was no longer detected (Fig. 3H). Instead, granulation with infiltration by T cells, B cells, and macrophages was observed (Fig. 3G). Thus, the transplanted cells were cleared away in 2 weeks when the transplantation was conducted at 60 (>50) days of gestation.

Histology II: after birth

After birth, the graft sections showed infiltration by host (sheep) T cells, fewer B cells, macrophages, and neutrophils (Fig. 4A). Most T cells were $CD4^+$, but some were $CD8^+$ (Fig. 4B). As time went on, the grafts consisted more and more of host-derived granulated tissue,

TABLE 1. cyES CELL ENGRAFTMENT AT TERM AFTER IN UTERO TRANSPLANTATION IN SHEEP

Animal number	*Transplanted ES cells*	*Gestational day at transplantation*	*Transplanted cell number per site*	*Engraftment*
1	CMK6G	43	2.0×10^7	−
			2.0×10^6	−
2	CMK6G	45	2.5×10^7	+
			5.0×10^6	+
3	CMK6	47	2.6×10^6	+
4	CMK6	47	1.4×10^7	+
			1.4×10^6	+
			1.4×10^5	−
			1.4×10^4	−
5	CMK6	49	7.5×10^6	+
			7.5×10^5	−
6	CMK6	49	7.5×10^6	−
7	CMK6	50	1.1×10^7	−
			1.1×10^6	−
			1.1×10^5	−
			1.1×10^4	−
8	CMK6G	50	6.0×10^6	−
			6.0×10^5	−
			6.0×10^4	−
			6.0×10^3	−
9	CMK6	50	5.0×10^6	−
			5.0×10^5	−
			5.0×10^4	−
			5.0×10^3	−
10	CMK6	53	2.6×10^7	−
11	CMK6	55	5.0×10^7	−
12	CMK6G	63	4.5×10^6	−
			4.5×10^5	−
			4.5×10^4	−
			4.5×10^3	−
13	CMK6	64	9.0×10^6	−
			9.0×10^5	−
			9.0×10^4	−
			9.0×10^3	−
14	CMK6	66	1.4×10^7	−
15	CMK6	67	5.0×10^7	−

for instance at 1.5 versus 6.5 months of age, as shown in Fig. 4C. In one of the engrafted sheep, a quantitative DNA-PCR analysis of the grafts showed that the cynomolgus portion decreased from 78% at 1.5 months to 42% at 6.5 months. Thus, the cynomolgus tissues were replaced by host-derived granulated tissue over months, although they remained engrafted for longer than half a year after birth (9 months post-transplant). The cyES cell-derived grafts after birth were no longer positive for the pluripotent marker Oct-3 (data not shown). Although cultured graft cells were all negative for Oct-3 (Fig. 4D), they could be propagated for more than 6 passages.

Innate immune responses

Although xenograft rejection requires T cells [34], it has been recognized that innate immune responses precede and drive adaptive immune responses in xenograft rejection [35]. Therefore, we examined natural killer (NK) cells (as $CD11b^+CD14^-CD5^-$) and monocytes (as $CD11b^+CD14^+CD5^-$) in the fetal leukocytes at transplantation by flow cytometry. The NK cell fraction was $2.2 \pm 0.1\%$ at 48 days and $1.9 \pm 0.6\%$ at 60 days. The monocyte fraction was $0.1 \pm 0.0\%$ at 48 days, and $0.2 \pm 0.1\%$ at 60 days (Fig. 5A). There were no statistical dif-

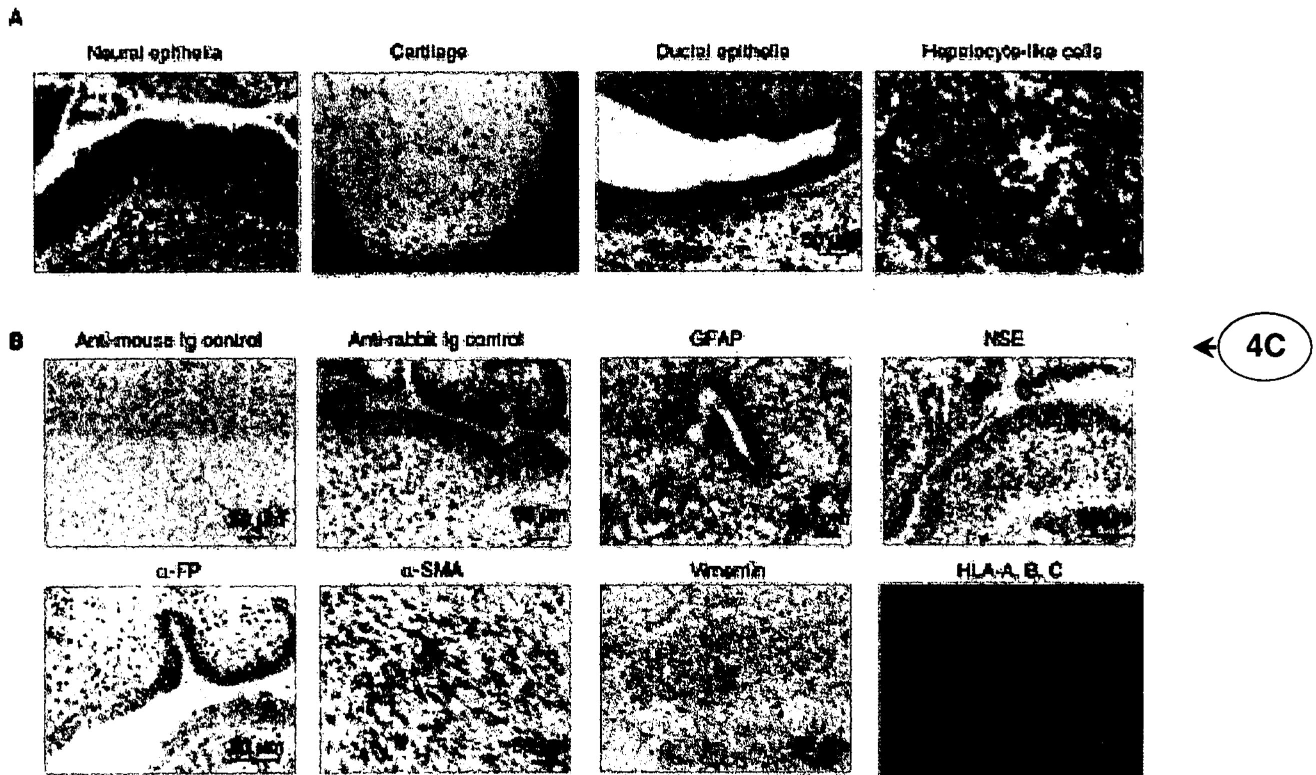

FIG. 2. Cynomolgus grafts in sheep contained mature tissue structures. (A) Hematoxylin & Eosin staining revealed that the grafts contained three germ layer cells with mature tissue structures. (B) Immunohistochemistry revealed that the grafts contained various kinds of mature cells derived from three germ layers. Duct cells were stained positively with anti-GFAP, anti-NSE, and anti-α-FP. Spindle cells in the interstitial areas were stained positively with anti-αSMA and anti-vimentin. Control immunostaining with anti-mouse IgG and anti-rabbit IgG is also shown. Some of the transplanted cyES cell progeny (GFP$^+$, green, *lower right*) still remained negative for HLA-A, B, and C, although most cells became positive (red, *lower right*).

ferences in the percentages of NK cells or monocytes between 48 (<50) days and 60 (>50) days of gestation. The very low prevalence of monocytes was also described in a previous study [32]. Neutrophils did not appear at this gestational age [32]. However, macrophages were infiltrated in the transplanted progeny two weeks later when transplanted at 60 days of gestation (Fig. 3G, lysozyme-positive cells), although they were not infiltrated when transplanted at 48 days of gestation (Fig. 3B, lysozyme-positive cells). Thus, the premature (absent or weak) innate immune responses before 50 days of gestation might be one of the reasons for the survival of xenogeneic ES cells.

Adaptive immune responses

Next, we examined adaptive immune responses. To examine cellular immune responses in the newborn sheep (at 3 months of age), we performed MLRs (n = 3). The reactivity against cyES cells and graft cells was considerably higher in the cyES cell-engrafted sheep than in the nonengrafted (transplanted but not engrafted) and nontransplanted (naive) sheep (Fig. 5B), suggesting that the sheep engrafted with cyES cells were sensitized to the ES cells. The low reactivity to ES and graft cells as compared to cynomolgus PBMCs might be a reflection of low immunogenicity of ES cells and their progeny [34,36]. The low reactivity in the nonengrafted sheep suggests that cyES cells and their progeny were cleared away before the development of conventional T cell memory, which was not established yet at this time of gestation [37,38].

Regarding the humoral immunity (Fig. 5C), considerable levels of IgM against cyES cells were detected at 2 weeks post-transplant when the transplantation was conducted at 60 days of gestation (day 60 + 14). The slight increase in CD45R$^+$ fraction (as B cells) at 60 days of gestation might support the B cell development after 50 days of gestation (Fig. 5A). Anti-cyESC IgM was, however, no longer detectable at birth in the engrafted (n = 4) or nonengrafted sheep (n = 3). Although anti-cyES cell IgG xenoantibodies were not detected at 2 weeks post-transplant regardless of the transplant days (either 48 or 60 days of gestation), they were detected in all of

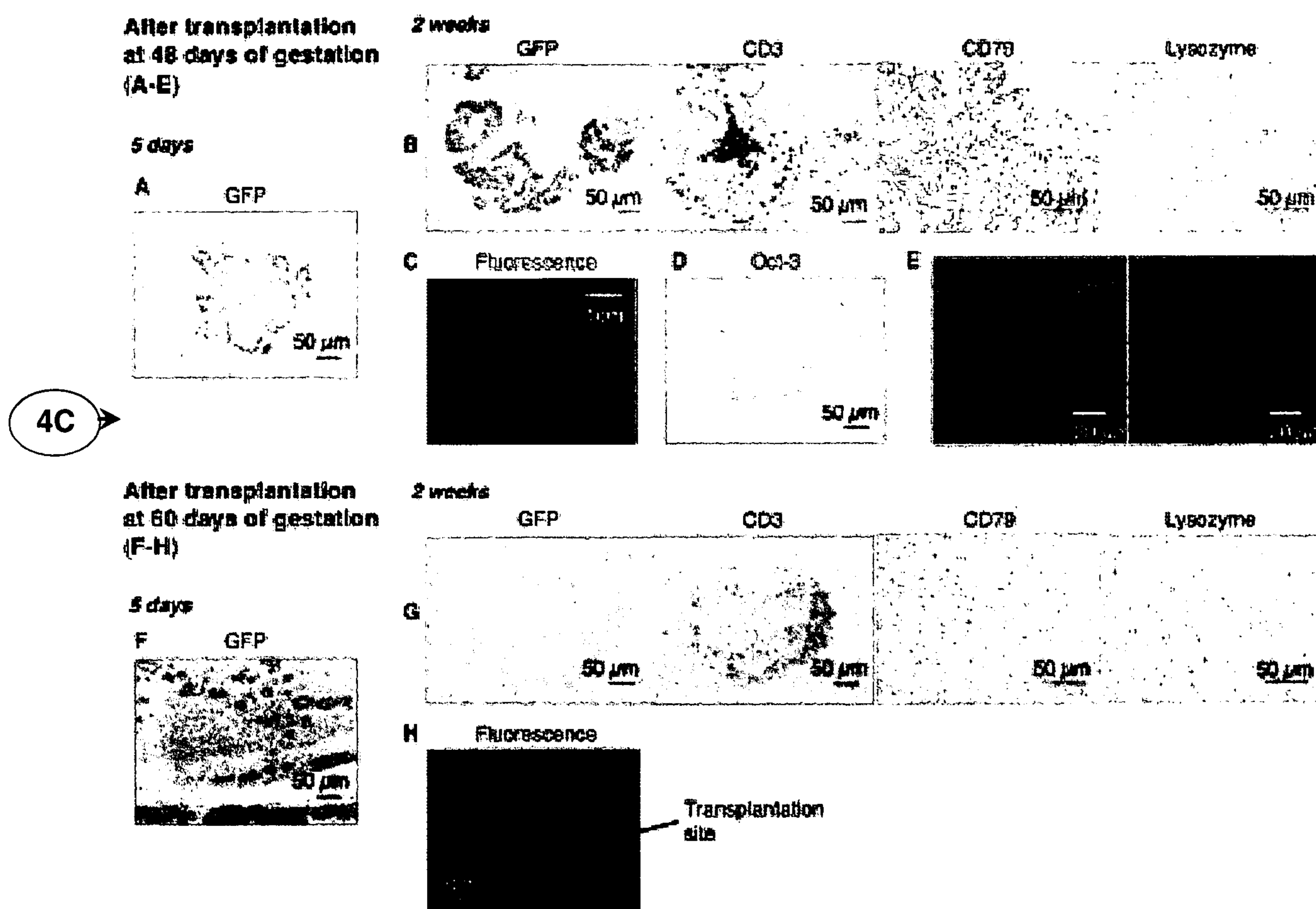

FIG. 3. Cynomolgus grafts in sheep during the fetal period. (**A–E**) When cyES cells expressing GFP were transplanted at 48 days of gestation, GFP^+ transplanted cell progeny were clearly detected both at 5 days (**A**) and at 2 weeks post-transplant (**B** and **C**). (**B**) According to the immunostaining of serial sections, a considerable number of $CD3^+$ T cells and a small number of $CD79^+$ B cells were observed around GFP^+ transplanted cell progeny, but lysozyme-positive macrophages were not observed. (**D**) Transplanted cell progeny at 2 weeks post-transplant were still positive for Oct-3. (**E**) Staining of frozen sections showed that T cells in **B** were $CD4^+$ (red, *left*) and $CD8^-$ (red, *right*). (**F–H**) When cyES cells expressing GFP were transplanted at 60 days of gestation, GFP^+ transplanted cell progeny were observed at 5 days post-transplant (**F**), but they were no longer detected at 2 weeks post-transplant (**G** and **H**). (**G**) According to the immunostaining of serial sections, GFP^- host-derived granulated tissue infiltrated with $CD3^+$ T cells, $CD79^+$ B cells, and lysozyme-positive macrophages was observed, suggesting that the transplanted cells were cleared in 2 weeks when the transplantation was conducted at 60 days of gestation.

the cyES cell-engrafted sheep at birth ($n = 4$). Thus, humoral immune responses against cyES cells also occurred during the pregnancy in the engrafted sheep.

We then tried an additional transplantation into the engrafted sheep to examine the immunological tolerance. cyES cells (1×10^7 cells /site) were transplanted subcutaneously into the engrafted ($n = 2$) and nonengrafted sheep ($n = 3$), which were already tested in the MLR and xenoantibodies detection studies, at more than 6 months after birth, and the sheep were examined 3 months later. No additional engraftment or tumor was observed in any of the sheep, suggesting that immunological tolerance was not achieved. Thus, both cellular and humoral immune responses against cyES cells were generated in the cyES cell-engrafted sheep, despite the sustained macroscopic engraftment of cynomolgus tissues in immunocompetent sheep.

Involvement of regulatory T cells

Because cyES cell-derived grafts could survive long term in xenogeneic sheep despite immune responses, some mechanisms should be involved in the sustained engraftment. Although $CD4^+$ T cells surrounded the transplanted cyES cell progeny at 2 weeks post-transplant, the cyES cell-derived orderly structures were not affected at all (Fig. 3B). Such a circumferential distribution of T cells was reminiscent of 'peri-insulitis'; regu-

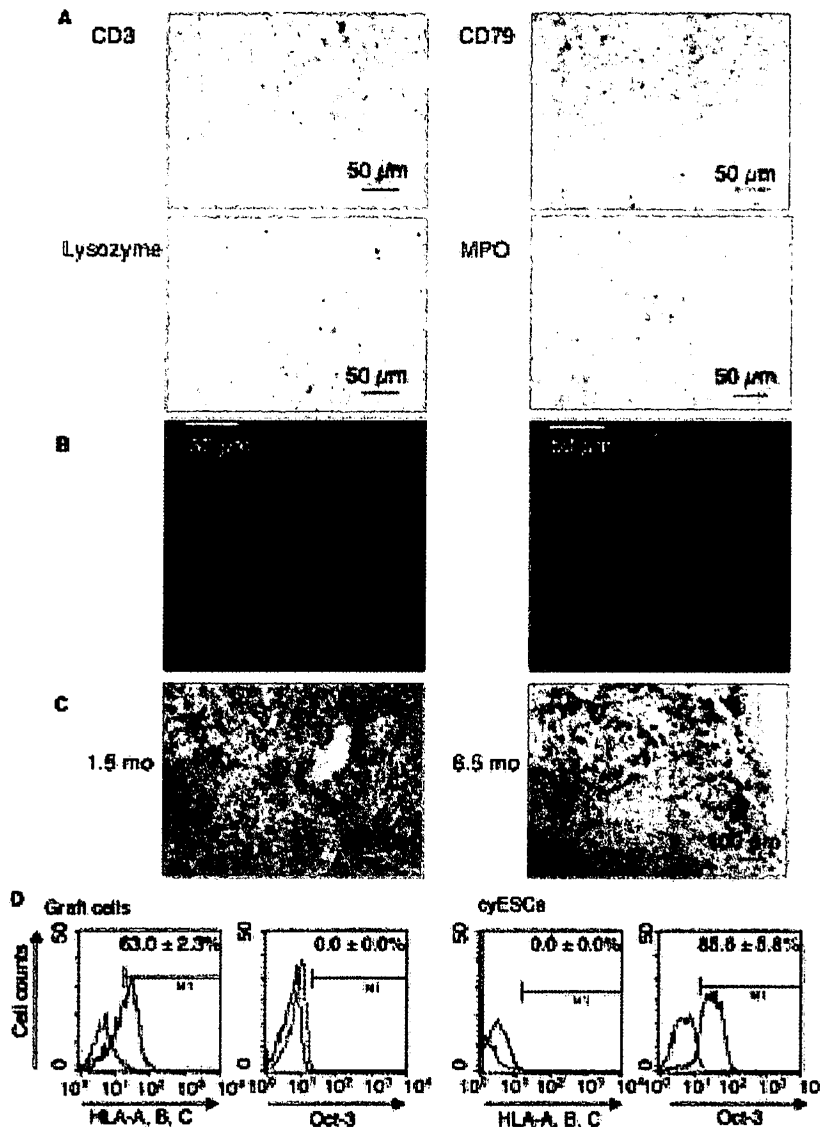

FIG. 4. Cynomolgus grafts in sheep after birth. **(A)** Immunostaining shows infiltration by T cells (positive for CD3), B cells (positive for CD79), macrophages (positive for lysozyme), and neutrophils (positive for MPO) in the cynomolgus-derived grafts in sheep after birth. **(B)** Most of the T cells in the grafts were $CD4^+$ (red, *left*), but some were $CD8^+$ (red, *right*). **(C)** In situ hybridization to detect cynomolgus cells showed that the graft in sheep no. 3 (the longest graft-surviving sheep) at 6.5 months of age (*right*) consisted of more granulated tissues and less cynomolgus components as compared to the graft at 1.5 months (*left*). **(D)** Cultured graft cells were all negative for Oct-3 and 63.0 ± 2.3% of the cells were positive for HLA-A, -B, and -C. On the other hand, cyES cells were all negative for HLA-A, -B, and -n'C and nearly 90% of the cells were positive for Oct-3. Dotted lines show the staining with the isotype-matched, fluorescence-conjugated, irrelevant control Abs.

latory T (T_{reg}) cells were distributed around islets in nonobese diabetic mice in which normoglycemia had been restored [39]. Therefore, we considered that T cells surrounding the cynomolgus tissues in Fig. 3B might be T_{reg} cells, which possibly suppressed immune rejection.

Although sheep T_{reg} cells have not been characterized, the transcription factor Foxp3 is known to be one of the most specific markers of T_{reg} cells that is highly conserved among species [40,41]. Therefore, we first cloned and sequenced the ovine ortholog of *foxp3*. The translated amino acid sequence showed a high homology to other known orthologs of Foxp3 (Fig. 6A, left; human, 90%; mouse, 88%; cattle, 99%). We next examined the cross-reactivity of an anti-mouse Foxp3 Ab to ovine Foxp3. Cells transfected with the cloned ovine *foxp3* were stained positively with the anti-mouse Foxp3 Ab by flow cytometry (Fig. 6A, right). We then stained a fetal sheep spleen at 64 days of gestation with this anti-Foxp3 Ab,

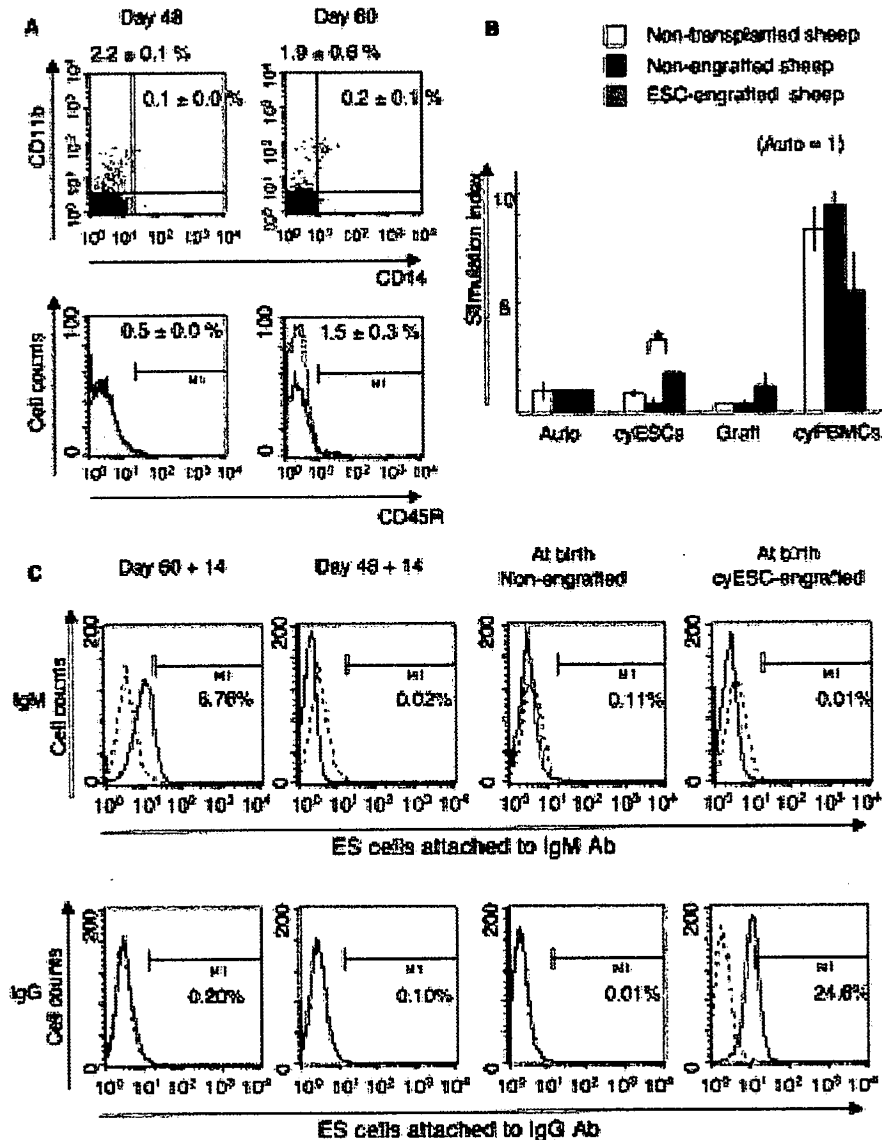

FIG. 5. Host immune responses. (**A**) Cell-surface antigens of fetal sheep peripheral blood leukocytes are compared between 48 days and 60 days of gestation. CD11b and CD14 double staining of the $CD5^-$ cells are shown (*upper*). There were no statistical differences in the percentages of NK cells ($CD11b^+CD14^-CD5^-$) between 48 days and 60 days of gestation. Monocytes ($CD11b^+CD14^+CD5^-$) were scarcely detected at both gestational days. $CD45R^+$ cells (as B cells) are slightly increased at 60 days of gestation (*lower*). Dotted lines show the staining with isotype-matched, fluorescence-conjugated, irrelevant control Abs. (**B**) The MLR against cyES cells and graft cells was higher in the cyES cell-engrafted sheep than in the nonengrafted or nontransplanted sheep. Statistical differences with the *t*-test were indicated (* $p < 0.01$). (**C**) IgM (*upper*) and IgG (*lower*) xenoantibodies against cyES cells were determined by flow cytometry. (Dotted lines) Negative control sera from nontransplanted adult sheep; (solid lines) sample sera. Day 60 + 14; transplanted with cyES cells at 60 days of gestation and examined at 14 days post-transplant. Day 48 + 14; transplanted with cyES cells at 48 days of gestation and examined at 14 days post-transplant.

and found that cells positive for Foxp3 were always positive for CD4 (Fig. 6B). We have also shown that $CD4^+CD25^{high}$ cells of adult sheep PBMCs were mostly $Foxp3^+$ just like human T_{reg} cells (42) (data not shown). These results indicate that this Ab can be used to detect sheep $Foxp3^+$ T cells, namely sheep T_{reg} cells. Using this Ab (Fig. 6C), more than half of the T cells around the grafts at 2 weeks post-transplant were found to be positive for Foxp3. At birth, 10–20% of the T cells in the grafts were positive for Foxp3. These data suggest that T_{reg} cells might be involved in the sustained engraftment of cynomolgus tissues in sheep. To characterize ovine fetal T_{reg} cells further, it would be ideal to isolate T_{reg} cells from the specimen at 2 weeks post-transplant in Fig. 6C

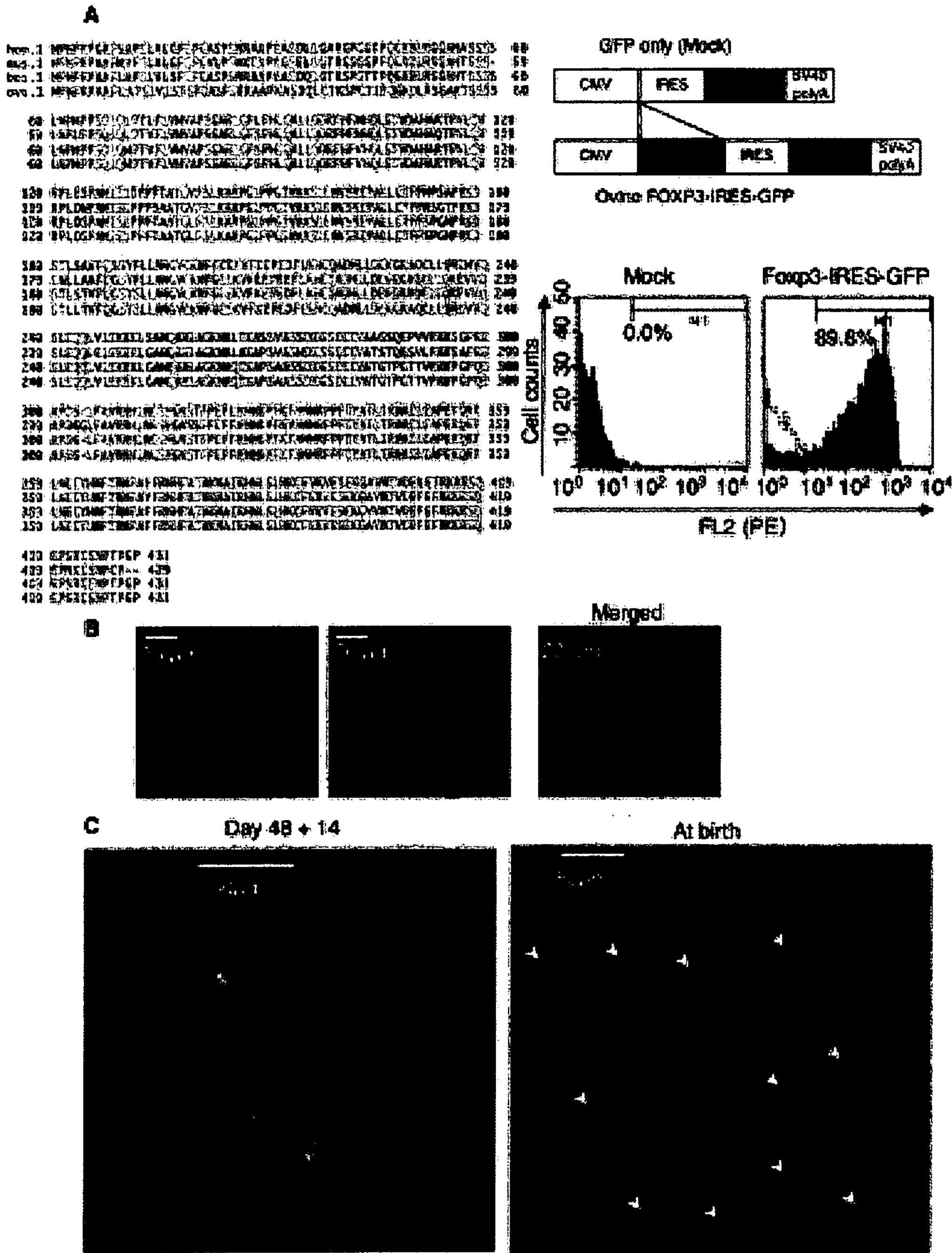

FIG. 6. Detection of Foxp3$^+$ T_{reg} cells. (**A**) Amino acid sequences of Foxp3 in four mammals. Foxp3 of sheep (ovi) is compared with that of human (hom), mouse (mus), and cattle (bos) (GenBank accession numbers; NM014009, NM054039, and DQ322170, respectively). Conserved amino acids are indicated in red (*left*). The ovine *foxp3* cDNA was introduced into 293T cells. The plasmid inserts are shown (*upper right*). Flow cytometric analyses of 293T cells transfected with the mock plasmid and with the plasmid expressing the ovine *foxp3* are shown (*lower right*). Gray areas indicate GFP-expressing cells stained with the PE-conjugated anti-mouse Foxp3 Ab. Dotted lines show GFP-expressing cells stained with PE-conjugated isotype-matched irrelevant Ab. (**B**) Fetal sheep spleen at 64 days of gestation was stained positively with the anti-mouse Foxp3 Ab (red, *left*) and with anti-ovine CD4 (green, *middle*). The merged image revealed that Foxp3$^+$ cells were always CD4$^+$ and they were considered as T_{reg} cells (*right*). (**C**) As assessed with this Ab, when transplanted at 48 days of gestation, more than half of the surrounding T cells at 2 weeks post-transplant (Fig. 3B) were positive for Foxp3 (stained in red, *left*). Ten to 20% of the T cells in the grafts at birth (Fig. 4A) were positive for Foxp3 (stained in red, arrows, *right*).

and demonstrate the suppressive function. However, it was not possible to collect sufficient viable immune cells from the tiny subcutaneous tissues.

DISCUSSION

In the setting of nonprimate-to-primate xenotransplantation, very rapid and vigorous immune rejection occurs because of the interaction between the Gal α1-3Gal epitope abundantly expressed on nonprimate cells and the primate natural anti-Gal α1-3Gal antibody [43–46]. In contrast, primate-to-nonprimate xenotransplantation does not evoke such rejection because primate cells do not express the Galα1-3Gal epitope [47]. Regarding this point, our strategy to generate primate ES cell-derived grafts in sheep has cleared one hurdle of xenotransplantation. In addition, hES cells are less immunogenic even in xenotransplantation settings [34,36]. For instance, when hES cells were transplanted in the leg muscle of immunocompetent mice, no leukocytic infiltration was observed 48 h later, although human mature cells induced rapid granulocytic infiltration within 48 h [36]. Our MLR results showing much lower stimulation evoked by cyES cells or their progeny than by cynomolgus PBMCs might be explained by the less immunogenicity of cyES cells.

In the present study, we transplanted cyES cells into sheep fetuses under several different conditions. Only when transplanted with more than 1×10^6 cyES cells at $<$50 days of gestation did cyES cell progeny show sustained engraftment even after birth. To our knowledge, this is the first report describing the long-term macroscopic engraftment of xenogeneic ES cells after in utero transplantation.

One issue to be discussed is why cynomolgus tissues can engraft for such a long time in sheep. There are several possible explanations for this. First, we showed that the premature innate immunity before 50 days of gestation might be one of the reasons for the survival of the ES cells from early xenorejection. Second, the adaptive immune system during the early fetal period is so premature that even xenogeneic cynomolgus cells introduced in this period can be recognized as a sort of "self." However, both cellular and humoral immune responses against cyES cells were detected in the cyES cell-engrafted sheep, and additional engraftment was not successful in the animals. Therefore, despite their sustained engraftment, the cynomolgus tissues in sheep are recognized as foreign. Third, mixed hematopoietic chimerism (existence of both donor and recipient hematopoiesis) would induce donor-specific T cell tolerance even across a xenogeneic barrier [48–50]. In the mouse allogeneic setting, the transplantation of ES cells is shown to generate such mixed hematopoietic chimerism [51]. Similarly, transplanted cyES cells possibly generated mixed hematopoietic chimerism in sheep, serving to induce cynomolgus-specific tolerance. However, no cynomolgus cells were detectable in the peripheral blood of the cyES cell-engrafted sheep (n = 4) as assessed by a sensitive PCR analysis; that is, there was no mixed hematopoietic chimerism in the sheep (data not shown). Thus, the sustained engraftment of cynomolgus cells in sheep was not attributable to mixed hematopoietic chimerism.

Finally, T_{reg} cells would serve to induce transplant tolerance [52]. When transplanted at $<$50 days of gestation, $CD4^+$ T cells were found mobilized around transplanted cyES cell progeny, many of which were $Foxp3^+$ T_{reg} cells. For allografts to survive, T_{reg} cells had to promote tolerance in mice [53,54]. In a xenogeneic setting, host T_{reg} cells were shown to suppress immune responses to donor antigens in athymic mice that were grafted with neonatal porcine thymus [55]. In the human fetus, preterm cord blood is known to contain a high proportion of T_{reg} cells that declines with gestational age to the level in adult peripheral blood [56]. Fetal $CD4^+$ $CD25^{high}$ T_{reg} cells were reported to play an important role in the suppression of immature fetal T cell responses during early development, which might suppress the auto-reactive T cells or alloreactivity to maternal antigens [57]. Therefore, it is possible that an adequate number of T_{reg} cells were mobilized in the early fetal period and contributed to the engraftment of cynomolgus tissues in sheep.

In this study, however, additional engraftment after birth was not successful. Although further investigation of the fetal immune system is necessary, one plausible possibility is that with the maturation of immune system, the immune responses against xenogeneic cynomolgus tissues might eventually exceed the ability of T_{reg} cells to suppress the immune responses. To provide a sufficient supply of therapeutic cells or tissues by this in vivo differentiation method, further interventions for successful additional transplantation would be necessary. One might be to somehow enhance the ability of T_{reg} cells to suppress the immune responses after birth. Another one might be the induction of immunological tolerance or unresponsiveness through mixed hematopoietic chimerism by in utero co-transplantation of congenic hematopoietic stem cells or those derived from the ES cells. Given that xenograft rejection requires T cells [34], administration of immunosuppressive drugs to the fetal and cyES cell-engrafted sheep might be of help, although it should be considered that T_{reg} cells may also be suppressed.

In conclusion, when a certain quantity of cyES cells are transplanted before 50 days of gestation, $Foxp3^+$ T_{reg} cells are mobilized and cyES cell-derived mature cells are able to survive long term in sheep, although immunological tolerance is not achieved. This finding suggests a possibility of generating sheep with human grafts after in utero transplantation of hES cells, although ma-

jor challenges remain, especially with respect to the in vivo regulation of hES cell differentiation to functional cells. Because the differentiation of ES cells in vivo is influenced by the microenvironment at transplantation sites [31,58], transplantation into specific sites might be of help in regulating the differentiation. Genetic manipulation of ES cells should be of help, for instance transduction with *hoxb4* for in vivo hematopoiesis [59]. Differentiation of ES cells to certain precursor cells in vitro prior to transplant might be also helpful [60]. Because in vivo ES cell-derived grafts after birth were no longer at all positive for the pluripotent marker Oct-3, they are free of undifferentiated ES cells, implying their potential utility for clinical cell preparations [61,62]. In view of clinical application, however, there is concern about potential risk of horizontal infection between species [63]. Although closed housing and breeding of carefully selected specific pathogen-free sheep herd could possibly control infections, the risk of unknown pathogens cannot be eliminated [64]. Therefore, further study and constant vigilance are inevitable.

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Address reprint requests to:
Dr. Yutaka Hanazono
Professor, Division of Regenerative Medicine
Center for Molecular Medicine
Jichi Medical University
3311-1 Yakushiji, Shimotsuke
Tochigi 329-0498, Japan.

E-mail: hanazono@jichi.ac.jp

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YUJIRO TANAKA

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In vivo repopulation of cytoplasmically gene transferred hematopoietic cells by temperature-sensitive mutant of recombinant Sendai viral vector ☆

Kumi Yoshida [a], Yoshikazu Yonemitsu [a,*,1], Sakura Tanaka [a], Shuro Yoshida [b], Satoko Shibata [a], Haruhiko Kondo [a], Shinji Okano [a], Fumihiko Ishikawa [b,c], Koichi Akashi [d], Makoto Inoue [e], Mamoru Hasegawa [e], Katsuo Sueishi [a]

[a] *Division of Pathophysiological and Experimental Pathology, Department of Pathology, Graduate School of Medical Sciences, Kyushu University, 3-1-1 Maidashi, Higashi-ku, Fukuoka 812-8582, Japan*

[b] *Department of Medicine and Biosystemic Science, Graduate School of Medical Sciences, Kyushu University, 3-1-1 Maidashi, Higashi-ku, Fukuoka 812-8582, Japan*

[c] *RIKEN, Research Center for Allergy and Immunology, Yokohama Institute, 1-7-22 Suehiro-cho, Tsurumi-Ku, Yokohama, Kanagawa 230-0045, Japan*

[d] *Center for Cellular and Molecular Medicine, Kyushu University Hospital, 3-1-1 Maidashi, Higashi-ku, Fukuoka 812-8582, Japan*

[e] *DNAVEC Corporation, 1-25-11 Kannondai, Tsukuba-shi, Ibaraki 305-0856, Japan*

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Abstract

Recent clinical studies revealed 'proof of concept' of gene therapy targeting hematopoietic stem cells (HSCs) to treat hematopoietic disorders. However, vector integration-related adverse events of retroviral vectors have slowed progress in this field. As an initial step to overcoming this hurdle, we examined the potential of an improved cytoplasmic RNA vector, temperature-sensitive mutant non-transmissible recombinant Sendai virus (ts-rSeV/dF), for gene transfer to murine HSCs and progenitors. Both conventional vector and ts-rSeV/dF-GFP showed efficient gene transfer to T-lymphocyte-depleted syngeneic bone marrow cells (BMCs) (>85%), but only BMCs treated with ts-rSeV/dF-GFP but not with conventional vector efficiently repopulated in the recipient mice, associated with multilineage differentiation *in vitro* and *in vivo*. To our knowledge, this is the first demonstration of the *in vivo* reconstruction of hematopoietic series by cytoplasmically gene transferred BMCs, that warrants further investigation to realize this strategy in clinical settings.
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Keywords: Sendai viral vector; Hematopoietic stem cells; Cytoplasmic gene therapy; Severe immunodeficiency syndrome; Bone marrow transplantation

Gene therapy targeting hematopoietic cells is a promising strategy for treating inherited hematopoietic disorders. Hematopoietic stem cells (HSCs) in particular are an ideal target, because they can reconstitute the entire hematopoietic system in a recipient during a lifetime. Recent clinical success in clinical gene therapy for X-linked SCID using a retroviral vector expressing the common γ-chain revealed the 'proof of concept' of the strategy and has given encouragement to physicians, patients, and scientists, but a serious adverse event, T-cell leukemia, which is related to the insertion of a pro-viral genome into the host chromosome

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* Corresponding author.
E-mail addresses: yonemitu@med.kyushu-u.ac.jp, yonemitu@faculty.chiba-u.jp (Y. Yonemitsu), sueishi@patholl.med.kyushu-u.ac.jp (K. Sueishi).

[1] Present address: Department of Gene Therapy, Chiba University Graduate School of Medicine, 1-8-1 Inohana, Chuo-ku, Chiba 260-8670, Japan. Fax: +81 43 226 2973.

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mediated by retroviral vector, is an obstacle to developing the gene therapy strategies in this field [1–4].

We recently developed a novel viral vector for efficient gene transfer, recombinant Sendai virus (rSeV), and demonstrated highly efficient gene transfer to various organs *in vivo*, including airway epithelial cells, vascular tissue, skeletal muscle, and joint synovium [5–8] by using replication-competent additive-type rSeV; these findings are also consistent with a clinically available fusion gene-deleted non-transmissible rSeV (rSeV/dF) [9]. Since rSeVs uses a cytoplasmic transcription system, it can mediate gene transfer to a cytoplasmic location without the DNA phase [10] and therefore theoretically avoid a vector integration-related adverse event. In addition, there are technical advantages in the use of rSeVs for gene transfer to HSCs. First, vector particles can be easily concentrated to high titers, unlike retroviral vectors. Second, and most importantly, the modalities of target cell processing and viral transduction are technically non-demanding and feasible in clinical situations that require transduction into large numbers of target cells. Using replication-competent additive-type rSeV, we previously demonstrated a highly efficient gene transfer to HSCs derived from human cord blood preserving their functions to differentiate into the entire hematopoiesis series *in vitro* [11]; however, repopulation of HSCs has been unsuccessful when these cells were treated with additive-type rSeV as well as rSeV/dF (unpublished data). Therefore, we have again turned our focus to improving the rSeV system in view of reduced immunogenicity and cytotoxicity.

Recently, we newly developed temperature-sensitive mutant non-transmissible rSeV (rSeV/dFP^{ts}M^{ts}HN^{ts}L^{ts}, abbreviated as ts-rSeV/dF) [12,13], as an improved version of rSeV/dF. This new vector loses the expression of envelope-related genes M and HN at 37 °C [12,13], resulting in dramatically reduced cytotoxicity as well as innate immune responses in the murine lung [14].

In this study, therefore, we examined the potential of SeV as a cytoplasmic transcription system for gene transfer to HSCs and hematopoietic progenitors.

Materials and methods

Animals. Female 7-week-old C57BL/6 mice of Charles River grade were obtained from KBT Orientals Co., Ltd. (Tosu, Saga, Japan) and kept under specific pathogen-free and humane conditions. The GFP-TG mice (C57BL/6-TgN(act-EGFP) OsbC14-Y01-FM131) [15] were a gift from Prof. Okabe, Osaka University, Osaka, Japan. These mice were used for all experiments except the CTL assay. All animal experiments were carried out according to the protocols approved by the Institutional Committee for Animal Experiments and by the Institutional Committee for Recombinant DNA and Infectious Pathogen Experiments, Kyushu University. The experiments were carried out in accordance with recommendations for the proper care and use of laboratory animals and according to The Law (No. 105) and Notification (No. 6) of the Japanese Government.

rSeV vectors. rSeV/dF-GFP and ts-rSeV/dF-GFP were constructed as previously described [9,12,13]. A series of F-deficient SeV vectors were prepared by using recombinant LLC-MK$_2$cells carrying the F gene (LLC-MK$_2$/F7). An adenovirus vector, AxCANCre, expressing Cre recombinase was used for the induction of F protein in LLC-MK$_2$/F7 cells (referred to as LLC-MK$_2$/F7/A). Recombinant vaccinia virus vTF7-3 carrying T7 RNA polymerase was inactivated with psoralen and long-wave UV irradiation, and then used for the ribonucleoprotein complex recovery. The viral vectors were further amplified by several rounds of propagation. The titers of the recovered viral vectors were expressed as cell infectious units [9].

Gene transfer to BM cells and BMT. BMCs from donor mice were flushed under sterile conditions with RPMI 1640 with 10% fetal calf serum from the medullary cavities of tibiae and femurs using a 23-gauge needle. Red blood cells were depleted using Lysing buffer (0.38% NH_4Cl, Tris–HCl, pH 7.65), and mature T-cells were depleted with anti-Thy1.2 monoclonal antibody (Sigma, St. Louis) plus selected rabbit complement (Cedalene, Ontario, Canada). Gene transfer was carried out by simply adding MOI = 10 of vector solution to the media at 37 °C for 1 h, and 2×10^7 (C57BL/6) BMCs were given by tail vein injection. For bone marrow ablation, the recipient mice were treated by whole-body γ-irradation at lethal dose (10 Gy) 4–5 h before BMT. For blood cell count, PBCs were obtained via the tail vein, diluted, and counted by an automatic cytometer (CelltacαMEK-6158 NIHON KOHDEN, Tokyo, Japan).

Flow cytometry analyses. At appropriate time points, PBCs or tissue samples were obtained and subjected to flow cytometry analysis. For immune cell subset analysis, cells from each organ were stained with CD3-APC/DX5-PE (for pan-T-cells), CD3-APC/CD4-PE (CD4/T-cells), CD3-APC/CD8-PE (CD8/T-cells), CD11b-PE/CD11c-APC (monocyte/macrophages), and B220-APC/IgM-PE (B-cells) (all antibodies were from BD Pharmingen, CA) by FACSCalibur (Becton Dickinson, CA). For *in vitro* analysis of each progenitor population, whole BMCs, 48 h after ts-rSeV/dF-GFP exposure, were stained with biotin-conjugated anti-lineage (lineage panel; BD pharmingen), anti-Sca-1-APC, anti-c-kit-PE antibodies and streptavidin for 30 min. Propidium iodide-positive dead cells were excluded. Data were evaluated using Cellquest™ (Becton Dickinson) or Flowjo (Tree Star Inc., CA) software.

Enrichment of HSCs and colony assay. Enrichment of HSC via FACS Aria (Becton Dickinson) was previously described [16,17]. Briefly, mononuclear cells were obtained from BMCs supplemented with Lympholyte-M (Cedalene), and lineage$^+$ cells were removed with sheep anti-rat IgG conjugated magnetic beads (Dynal, A.S.), and with anti-c-kit-APC antibody and anti-APC microbeads (Dynal, A.S.). The remaining cells were stained with biotin-conjugated anti-lineage-cocktail (BD Pharmingen), streptavidin-perCP-Cy5, anti-c-Kit-APC, and anti-Sca-1-PE, and were sorted in duplicate. For the colony assays, an enriched HSC population was cultured in MethoCult™ Media (M3434; StemCell Technologies, Vancouver, BC, Canada), and exposed to ts-rSeV/dF-GFP for 1 h. After washing, 1000 cells were cultured in 1 ml Methocult media per dish (35 mm) for 10–12 days. CFU-Mix colonies were observed and analysed by BZ-8000 and BZ-Analyzer (KYENECE, Osaka, Japan).

Statistical analysis. All data are expressed as the mean ± SD, and were analyzed by one-way ANOVA with Fisher's adjustment, with the exception of the data on animal survival. Survival was plotted using Kaplan–Meier curves, and statistical relevance was determined using a log-rank comparison. $P < 0.05$ was considered significant.

Results

Efficient engraftment and repopulation of cytoplasmically gene transferred murine bone marrow cells by ts-rSeV/dF in C57BL/6 syngeneic recipient mice

We first evaluated the gene transfer efficiency of recombinant ts-rSeV/dF-GFP compared to that with rSeV/dF-GFP mediated gene transfer to murine (7-week-old female C57BL/6) BMCs depleted with erythrocyte and mature T-cells. We here used whole BMCs instead of an enriched stem cell fraction to avoid the loss of experimental animals

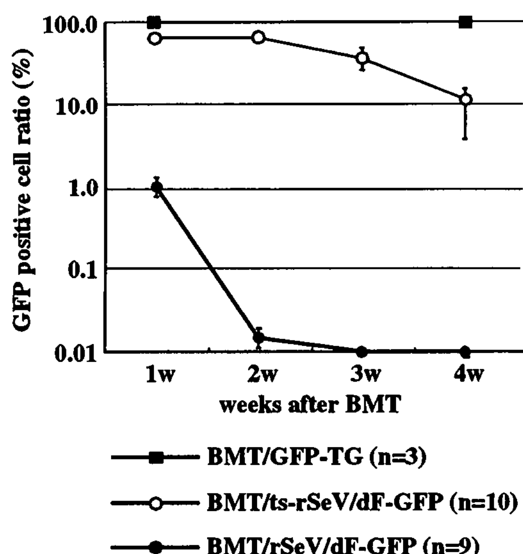
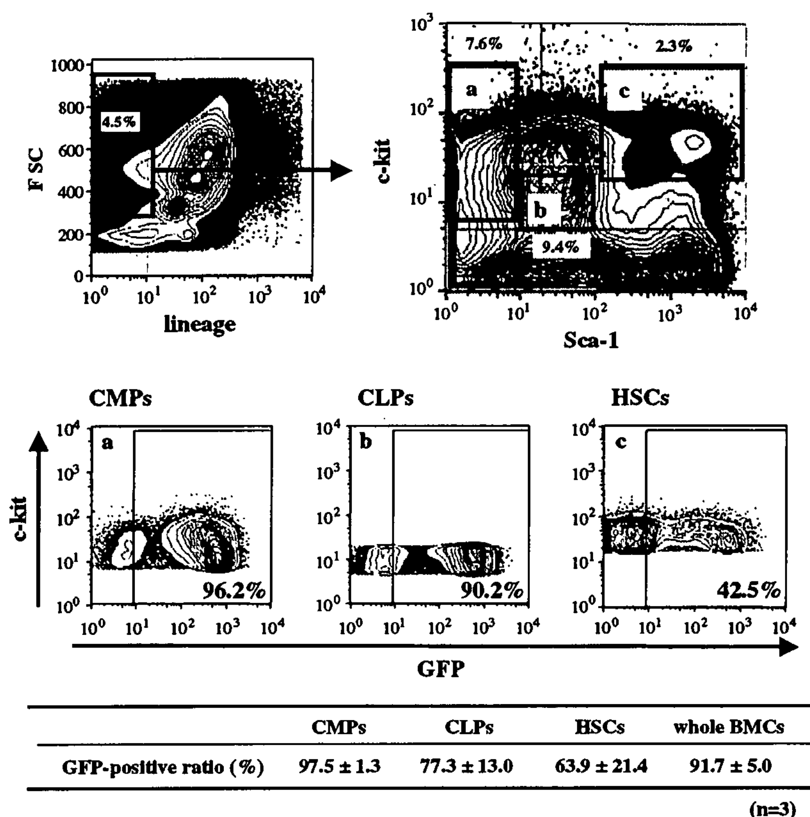


Fig. 1. Time course of GFP gene transferred cell ratio in peripheral blood cells (PBCs) after bone marrow transplantation (BMT). After bone marrow transplantation of 2×10^7 BMCs treated with ts-rSeV/dF-GFP (BMT/ts-rSeV/dF-GFP, $n = 10$) or conventional rSeV/dF-GFP (BMT/rSeV/dF-GFP, $n = 9$), the PBCs were collected via the tail vein of C57BL/6 recipient mice at each time point, and GFP expression was determined by FACS analysis. BMCs from GFP-transgenic mice ($n = 3$) were used as a positive control.

from unsuccessful reconstruction after lethal irradiation. BMCs were isolated from medullary cavities of tibiae and femurs. Erythrocytes were depleted in lysing buffer (0.38% NH_4Cl in Tris–HCl, pH 7.65) and mature T-lymphocytes were removed by anti-Thy1.2 antibody (mouse ascitis IgM monoclonal antibody) with rabbit complement; the removal of more than 99.9% of T-cells was constantly done and checked by FACS analysis. These BMCs were incubated with vectors at a multiplicity of infections 10 (MOI = 10) for only 1 h at 37 °C, and GFP expression was determined 48 h later, as described previously [15]. Both vectors constantly showed high gene transfer efficiency (>85%) to murine BMCs in repeated experiments (data not shown; similar data are shown in Fig. 2A).

Next, we engrafted these BMCs (2×10^7 cells/200 μl/head, 1 h after virus exposure) to lethally irradiated (γ-X ray 10 Gy) female C57BL/6 mice via the tail vein, and then the GFP expression in the cells from peripheral blood (PBCs) was monitored every week. BMCs from β-actin promoter-driven GFP transgenic mice (C57BL/6-TgN(act-EGFP)OsbC14-Y01-FM131) [15] were also used as a positive control of successful bone marrow transplantation (BMT).

As shown in Fig. 1, all recipients receiving BMCs from GFP transgenic mice (GFP-TG) demonstrated nearly



	CMPs	CLPs	HSCs	whole BMCs
GFP-positive ratio (%)	97.5 ± 1.3	77.3 ± 13.0	63.9 ± 21.4	91.7 ± 5.0

(n=3)

Fig. 2. *In vitro* gene transfer efficiencies of ts-rSeV/dF-GFP to populations of hematopoietic stem cells (HSC) and common myeloid and lymphoid progenitors (CMPs and CLPs). A typical result of the FACS analyses is given as panels, and the summary of triplicate experiments is demonstrated in the bottom table. Each enriched population was gated by $Lin^-/c\text{-}Kit^+/Sca\text{-}1^-$ for CMPs, $Lin^-/c\text{-}Kit^{low}/Sca\text{-}1^{low}$ for CLPs, and $Lin^-/c\text{-}Kit^{high}/Sca\text{-}1^{high}$ for HSCs. Data are expressed as mean ± SD.

100% GFP-positive cells in PBCs, indicating successful engraftment of, and almost total replacement by, donor cells under this experimental condition. In contrast, PBCs from mice of BMT/rSeV/dF-GFP showed very low repopulation of GFP-positive cells (~1%) at 1 week, and were rapidly eliminated during the experimental course, a representative result of our previous experiments (unpublished data). Importantly, recipient mice with BMT/ts-rSeV/dF-GFP exhibited a relatively high repopulation of PBCs (50–70%) 1 week after BMT; the ratio gradually declined to ~10% at 4 weeks.

To our knowledge, this is the first demonstration of the efficient repopulation of donor cells that were transferred by cytoplasmic RNA vector.

Efficient ts-rSeV/dF-mediated gene transfer to HSCs and progenitor populations

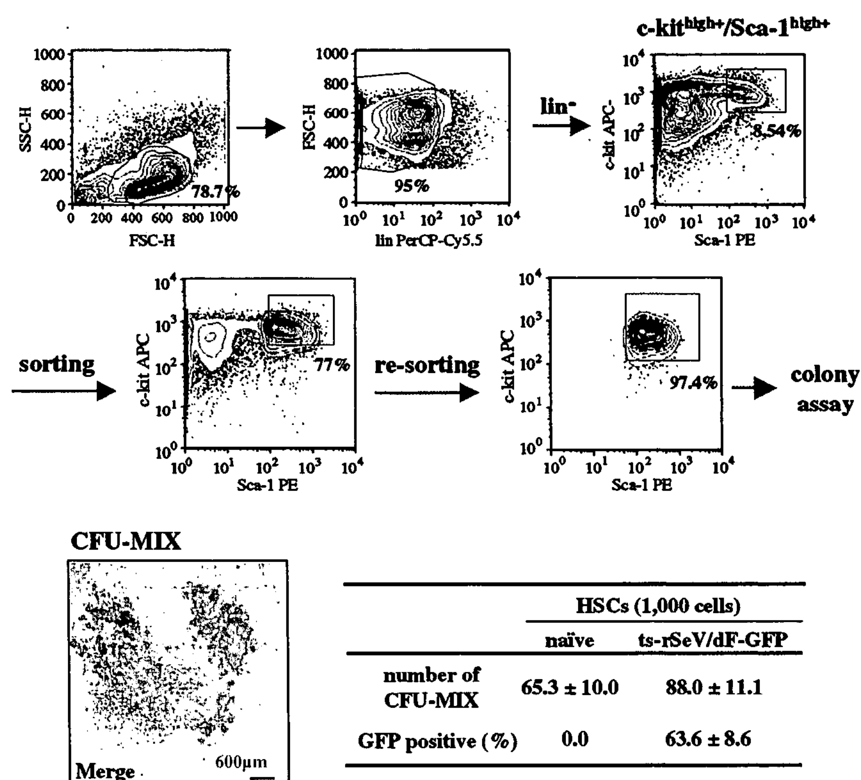
We examined the gene transfer efficiencies of ts-rSeV/dF-GFP to common myeloid progenitors (CMPs: enriched in Lin$^-$Sca-1$^-$c-Kit$^+$) [16], common lymphoid progenitors (CLPs: enriched in Lin$^-$Sca-1^{low}c-Kit^{low}) [17], and HSCs (enriched in Lin$^-$Sca-1^{high}c-Kit^{high}) by flow-cytometric analysis (Fig. 2). Constant high gene transfer, over 90%, was seen in T-cell-depleted BMCs by ts-rSeV/dF-GFP, and relatively high gene transfer also occurred in these populations (mean = 97.5% of CMPs, 77.3% of CLPs, and 63.9% in HSCs, $n = 3$).

Multilineage differentiation of cytoplasmically gene transferred HSCs in vitro

Subsequently, we investigated whether gene transferred HSCs by ts-rSeV/dF-GFP might sufficiently differentiate to various types of colonies by a colony-forming assay. As shown in Fig. 3, 1000 cells of highly enriched HSCs were sorted from BMCs twice and transfected with the GFP gene, and then sparsely cultured for 12 days [16]. Colony-forming units (CFU) were then assessed by fluorescence microscope. We counted the number of multilineage mixed colonies (CFU-Mix) as CFU from single cells with pluripotency. The result in Fig. 3 shows, a reduction in the number of CFU-Mix was not seen in cells treated with ts-rSeV/dF-GFP, suggesting no significant effect of gene transduction to cell differentiation. In addition, the ratio of GFP-positive colonies was almost comparable to gene transfer efficiency to HSCs, suggesting no significant effect on cell growth by ts-rSeV/dF-GFP, at least *in vitro*.

In vivo repopulation and multilineage differentiation of cytoplasmically gene transferred BMCs

Using six animals 5 weeks after BMT with BMC treated ts-rSeV/dF-GFP, we next examined the distribution, repopulation and differentiation of GFP-positive cells in typical lymphoid organs (thymus, spleen, bone marrow)



	HSCs (1,000 cells)	
	naïve	ts-rSeV/dF-GFP
number of CFU-MIX	65.3 ± 10.0	88.0 ± 11.1
GFP positive (%)	0.0	63.6 ± 8.6

Fig. 3. *In vitro* colony-formation assay. Cells were sorted twice (FACS panels) in order to enrich HSC fraction, a typical photomicrograph of GFP-positive CFU-Mix (bottom left), suggesting multilineage differentiation, and the table summary of the quantitative data (bottom right) are given. Data are expressed as mean ± SD.

as well as PBCs by flow cytometry. As shown in Fig. 4A, these six mice showed a representatively high GFP-positive ratio ($55.9 \pm 5.9\%$, $n = 6$) in PBCs, and the thymus, spleen, and bone marrow of these animals also exhibited a mean = 47.7%, 79.3% and 17.1% of GFP-positive cells, respectively, confirming the repopulation of transplanted cells in each lymphoid organ. A simultaneous experiment using the BMT/SeV/dF-GFP of three animals did not show any GFP-positive cells at all 5 weeks after BMT (data not shown).

Subsequently, we investigated the GFP-positive ratio of each cell type in the spleen and bone marrow by FACS analyses. Cells from each organ were determined by antibodies specific for the following surface-markers: NK cells = $CD3^-/DX5^+$, NKT cells = $CD3^+/DX5^+$, pan-T-cells = $CD3^+/DX5^-$, CD4/T-cells = $CD3^+/CD4^+$, CD8/T-cells = $CD3^+/CD8^+$, dendritic cells (DCs) = $CD11b^+/CD11c^+$, monocytes/macrophages (Mφ) = $CD11b^+/CD11c^-$, B-cells = $B220^+/IgM^+$. As shown in Fig. 4B, even though BMCs were used after complete depletion of T-cells, all these subsets contained GFP-positive cells at various ratios including T-cell lineages, suggesting that these GFP-positive cells were from HSCs and progenitors. Interestingly, DCs and Mφs, both from CMPs [16], included a relatively large number of GFP-positive cells in both the spleen and bone marrow, probably reflecting the high gene transfer to CMPs (Fig. 2).

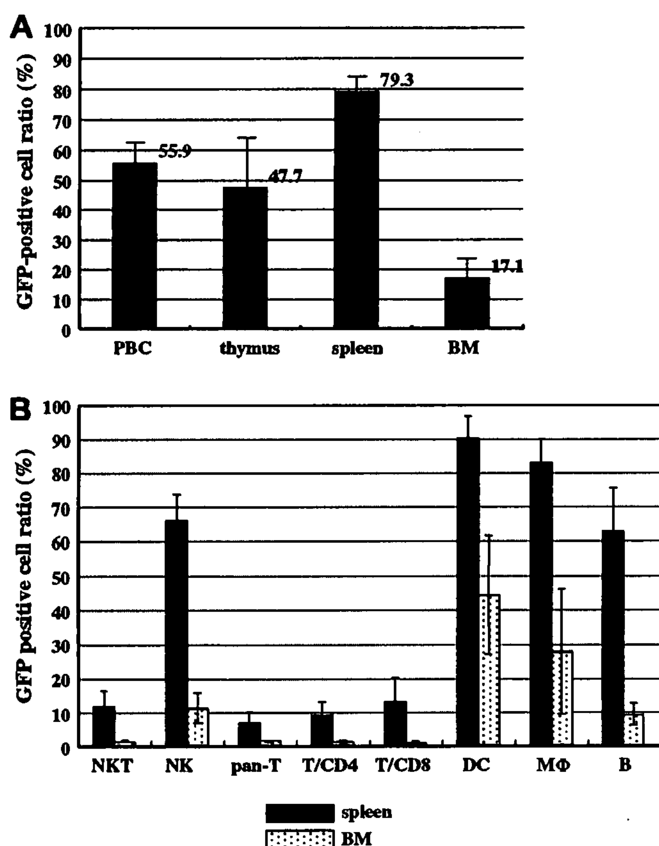


Fig. 4. *In vivo* repopulation and multilineage differentiation of BMCs transfected by ts-rSeV/dF-GFP after BMT. Data are expressed as mean ± SD. (A) Repopulation efficiency of ts-rSeV/dF-GFP treated BMCs in typical lymphoid organs at 4 weeks after BMT *in vivo*. (B) FACS analysis of ration of GFP expression in various cellular subsets in spleen and bone marrow of recipient mice at 5 weeks after BMT. Each cell subset was determined as follows: NK = $CD3^-/DX5^+$, NKT = $CD3^+/DX5^+$, pan-T-lymphocytes = $CD3^+/DX5^-$, CD4/T-cells = $CD3^+/CD4^+$, CD8/T-cells = $CD3^+/CD8^+$, DCs = $CD11b^+/CD11c^+$, Mφ = $CD11b^+/CD11c^-$, and B-lymphocytes = $B220^+/IgM^+$.

These findings, therefore, strongly suggested that BMCs transfected with ts-rSeV/dF-GFP were capable of differentiating the whole hematopoietic series *in vivo*.

Discussion

Physicians and scientists in the gene therapy community have been greatly encouraged by the dramatic outcome of clinical studies to treat X-linked SCID conducted in France [1]. However, this trial has also raised safety concerns about vector integration to the host chromosomes.

Using vectors that express the transgene in cellular cytoplasm is one possible way of addressing safety concerns; in the present study, therefore, we attempted to seek the potential of a cytoplasmic RNA vector, rSeV, as a new modality for gene therapy to treat hematopoietic disorders. To the best of our knowledge, this is the first study to make use of a newly developed version of the cytoplasmic RNA vector, ts-rSeV/dF, to show the high gene transfer efficiency to hematopoietic stem/progenitor populations and their successful repopulation associated with differentiation to multiple lineages of hematopoietic series.

Apparently, an advance seen in this study is that the repopulation of cytoplasmically gene transferred BMCs could be done by using an improved design of rSeV/dF, a temperature-sensitive mutant ts-rSeV/dF, but not of conventional rSeV/dF (and prototype additive rSeV, unpublished data). However, there is no definitive explanation as to why ts-rSeV/dF realized an efficient repopulation. As previously demonstrated, ts-rSeV/dF shows a reduced cytotoxic effect on some types of cells, but this is not a likely explanation for its ability to realize BMC repopulation because our *in vitro* study using murine HSCs, which is shown in Fig. 3, along with a previous study using human HSCs [11], could not demonstrate the apparent cytotoxicity and the disturbance of their differentiation caused by any type of rSeV vector. It is therefore possible that the reduced expression of HN protein due to the insertion of temperature-sensitive mutations enabled BMCs to repopulate in the recipient.

The findings obtained in this study suggested the possible application of 'cytoplasmic gene therapy' for hematopoietic disorders; however, our preliminary study assessing long-term engraftment also revealed the potential limitations of the current construct *in vivo* in view of its clinical application. In other words, more than 40% of recipient mice showing efficient hematopoietic gene transfer were dead in later phase due to severe pancytopenia (Yoshida K and Yonemitsu Y, unpublished data), therefore, more studies and biological information are thus

needed to realize cytoplasmic gene therapy for hematopietic diseases.

In summary, we here demonstrated the successful repopulation and reconstruction of hematopoietic series by cytoplasmically gene transferred BMCs using newly developed ts-rSeV/dF. This study is, to the best of our knowledge, the first report reconstituting whole hematopoietic series using a bone marrow gene transferred by cytoplasmic transcription, but the transient repopulation is an issue that must be resolved. Since 'cytoplasmic gene therapy' is an attractive and challenging strategy for physicians and scientists in this field, further studies are needed to overcome the current problems.

Competing interest statement

Dr. Yonemitsu is a member of the Scientific Advisory Board of DNAVEC Corporation.

Acknowledgments

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