cells were isolated from mice. Cynomolgus ES cells and their differentiated progenitors were used as targets. Spleen cells were mixed with target cells at a ratio of 5:1 to 40:1 and incubated for 4 h at 37°C. The supernatant was examined for the release of lactate dehydrogenase using a Cytotoxicity Detection Kit (Takara, Shiga, Japan). Percent cytotoxicity was calculated as follows: cytotoxicity (%) = (experimental value − low control) × 100/(high control − low control). Low and high controls were obtained after incubating target cells alone or with 1% Triton X-100, respectively. The percent cytotoxicity was indicated as average values from assays performed in triplicate.

RESULTS

There is considerable variation among reports regarding the number of ES cells needed for teratomas to form in mice: from 10–15 clumps (200 cells) per site (18) to 5×10^6 cells per site (30). First, we examined how many nonhuman primate ES cells are necessary to generate teratomas in mice. In all transplantation experiments, we used cynomolgus ES cells expressing GFP (CMK6G) to detect the transplanted cells and track their fate in vivo. As recipient mice, we used two immunodeficient strains: NOD/SCID and NOG mice. NOG mice are more immunodeficient than NOD/SCID mice: NK as well as T and B cells are absent in NOG mice, although NK cells are present in NOD/SCID mice. Cynomolgus ES cells usually grow as clusters. When the cells were transplanted as clumps (not dissociated into single cells) into the limb muscle of NOG and NOD/SCID mice, teratomas developed in all mice that were transplanted with 1×10^5 or more cells, but they were observed only in some of the mice that were transplanted with 1×10^4 and 5×10^3 cells. Teratomas were not observed in any mice transplanted with 1×10^3 cells (Table 1). A similar cell–dose dependency was observed regardless of the mouse strains.

To see whether cellular state affects the tumorigenesis, cynomolgus ES cells as clumps or dissociated cells were transplanted into the lower limb muscle in NOD/SCID mice. As previously reported for human ES cells (18,24), cynomolgus ES cells also exhibit poor plating efficiency. During subculturing, limited dissociation with 0.1% collagenase (to maintain clumps of 10–50 cells) is recommended to enable continued growth (21). As expected, the number of ES cells necessary for teratomas to form in all mice was different: 5×10^4 for clumps and 5×10^5 for single cells (Table 1, Fig. 1). A larger number of ES cells was required for single cells than clumps to form a teratoma.

ES cells have been transplanted into different sites: subcutaneously into the hind leg muscle, testis capsule, abdominal cavity, etc., for teratoma-forming assays. To detremine whether the site affects tumorigenesis, ES cells (as clumps) were transplanted into the lower limb muscle or subcutaneously into the back in NOD/SCID mice. The number of ES cells necessary for teratomas to develop in all mice differed: 5×10^4 for the lower limb muscle and 5×10^5 for the subcutis in the back (Table 1, Fig. 1).

Teratoma-forming assays are often performed to test the safety of ES cell-derived progenitor preparations. To prepare cynomolgus ES cell-derived hematopoietic precursor cells, ES cells were cultured for 6 days to induce the hematopoietic differentiation. On day 6, the expression of CD34, VEGFR2, and Scl was upregulated, but CD45 was not yet expressed (data not shown). Therefore, day 6 cells likely included hematopoietic precursor cells (20,26,28). After the 6-day culture, 85% of cells were still positive for SSAE-4, an undifferentiated marker of both human and nonhuman primate ES cells (24,25) (data not shown). These cells (1×10^6) were transplanted into the lower limb muscle in NOG and NOD/SCID mice. Notably, the incidence of tumorigenesis differed between the two strains. Teratomas devel-

Table 1. Formation of Teratomas in Immunodeficient Mice

	Recipient Mouse Strain			
Transplanted Cell No.	NOD/SCID (Clumps/Muscle)*	NOG (Clumps/Muscle)	NOD/SCID (Dissociated Cells/Muscle)	NOD/SCID (Clumps/Subcutis)
1×10^6	**3/3**	**3/3**	**3/3**	**3/3**
5×10^5	**3/3**		**3/3**	**3/3**
1×10^5	**3/3**	**3/3**	2/3	2/3
5×10^4	**3/3**		1/3	2/3
1×10^4	1/3	2/3	0/3	0/3
5×10^3	0/3		0/3	0/3
1×10^3	0/3	0/3	0/3	0/3

Bold type indicates the formation of teratomas in all mice.
*Indicates cellular state/transplant site.

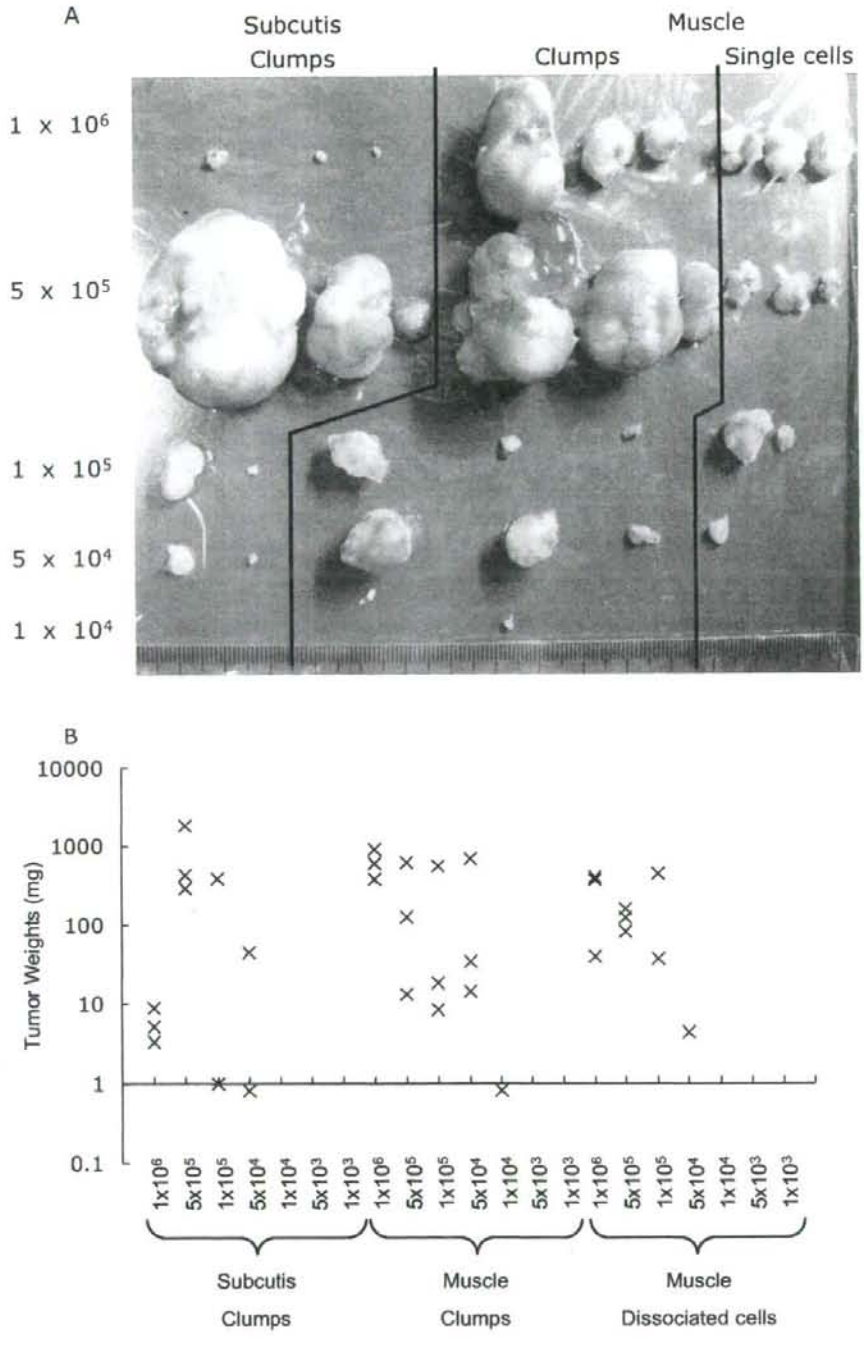

Figure 1. Formation of teratomas dependent on cell number and state. Cynomolgus ES cells (1×10^3 to 1×10^6 per site) were transplanted into NOD/SCID mice. The cells, either as clumps or dissociated single cells, were injected subcutaneously into the back or intramuscularly into the lower limb. Dissected tumors (A) and their weights (B) are shown.

Table 2. Formation of Teratomas Dependent on Recipients

Transplanted Cells	NOD/SCID	NOG	Fetal Sheep*	Fetal Monkeys†	Immunosuppressed Adult Monkeys‡
Cynomolgus ES cells (undifferentiated)	5/5	3/3	4/15	3/3	NT
Cynomolgus ES cell-derived progenitors	2/5	5/5	1/10	3/3	2/2

NT, not tested.
*As published in Tanaka et al. (23).
†As published in Shibata et al. (20).
‡As published in Nara et al. (15). In this experiment, cynomolgus ES cell-derived neural precursor cells were transplanted. In other experiments, cynomolgus ES cell-derived hematopoietic precursor cells were transplanted.

oped in all NOG mice (5/5). However, they developed in only two of five NOD/SCID mice (Table 2), despite that 85% of the transplanted cells (8.5×10^5) were positive for SSEA-4, which should have been enough for teratomas to form in terms of the number of undifferentiated ES cells transplanted (Table 1).

The innate immunity of the recipient mice was then evaluated. It turned out that the cytotoxic activity of spleen cells (putative NK cell activity) in the mice was greater against the ES cell-derived progenitors than undifferentiated ES cells (Fig. 2), suggesting that ES cell-derived progenitor cells are more immunogenic than undifferentiated ES cells.

Histological examination revealed that, in NOD/SCID mice, neutrophils (as Gr-1 positive) were already present around the grafts on day 1 after transplantation, but they had disappeared and were replaced by dendritic cells/active NK cells (as CD45/B220 positive) and macrophages (as BM8 positive) by day 3 (Fig. 3). These immune cells likely eliminated the transplanted cells, presumably more vigorously when ES cell-derived progenitor cells were transplanted (as suggested from Fig. 2), resulting in the failure of teratomas to develop in some NOD/SCID mice as shown in Figure 3 (detected only in two out of five NOD/SCID mice as shown in Table 2).

A similar failure to detect tumorigenesis was also noted in fetal sheep (23) (Table 2). On the other hand, teratomas developed in all monkeys, whether fetal monkeys or immunosuppressed adult monkeys, tested in the

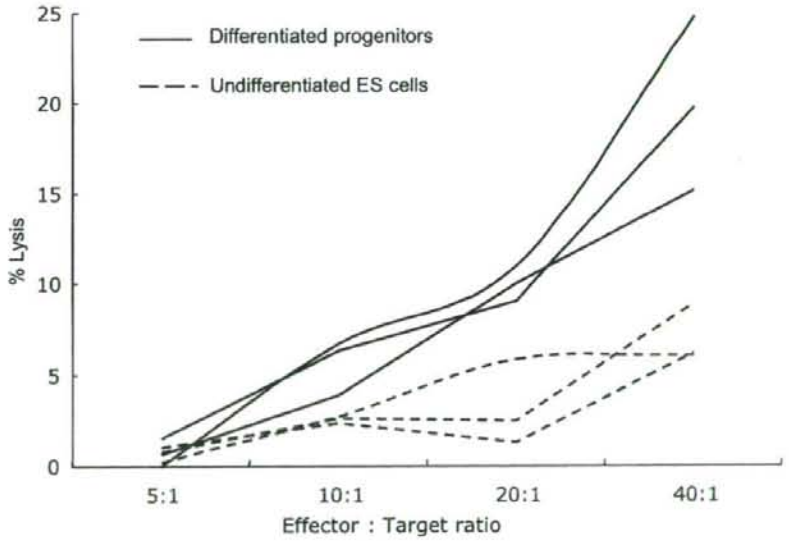

Figure 2. Cytotoxic activity against ES cells and their differentiated progenitors. Effectors: spleen cells of NOD/SCID mice. Targets: cynomolgus ES cells or their differentiated progenitors (hematopoietic precursor cells). The cytotoxic activity of mouse spleen cells (putative NK cell activity) was found to be stronger against the ES cell-derived progenitors than undifferentiated ES cells.

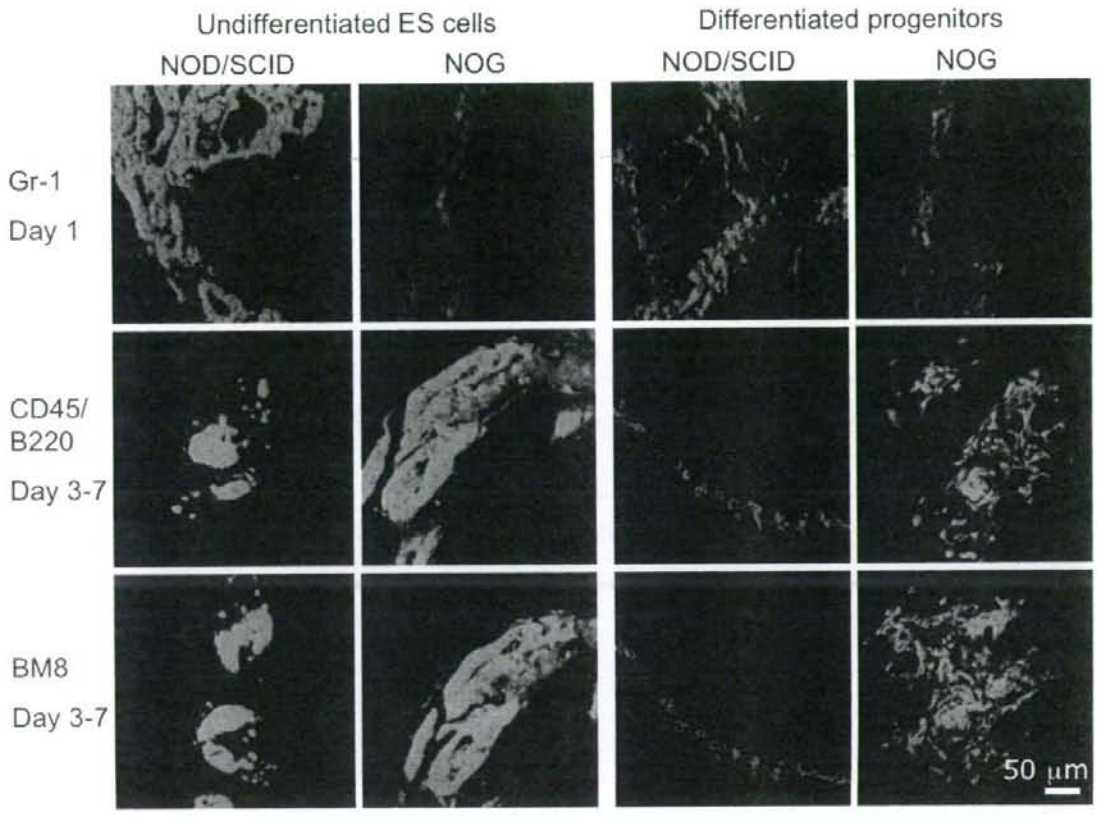

Figure 3. Immune responses to transplanted cells. Cynomolgus ES cells or their differentiated progenitors (1×10^6 cells) were transplanted into the hind leg muscle in NOD/SCID and NOG mice. Gr-1-positive cells (as neutrophils) were present on day 1 (upper panel) but disappeared by day 3. Instead, CD45/B220-positive cells (as plasmacytoid dendritic cells and active NK cells) and BM8-positive cells (as macrophages) were present around the transplanted cells and progeny after day 3, especially in NOD/SCID mice, resulting in the failure of engraftment (middle and lower panels). Green shows GFP-positive transplanted cells and their progeny. Gr-1, CD45/B220, and BM8 were stained in red.

allogeneic settings (15,20) (Table 2), even though the inclusion of the GFP gene in transplanted cells might enhance the immune response.

DISCUSSION

This study showed that the incidence with which teratomas form after cynomolgus ES cells are transplanted into immunodeficient mice is highly variable and depends on several conditions. First, the number of cells transplanted is critical. A similar result was reported for the allogeneic transplantation of mouse ES cells (16). Transplanted ES cells may be liable to apoptosis due to physical stress and environmental changes at transplantation, or they may be eliminated by innate immunity including neutrophils and macrophages present even in immunodeficient mice, as shown in Figure 3. Taking into account the apoptosis and clearance of cells post-transplant, a sufficient number of ES cells (1×10^5 or more) must be transplanted to assess the ability to form teratomas.

Second, teratomas developed much less frequently after the transplantation of dissociated ES cells than of cell clumps; that is, a larger number of ES cells is required for single cells than clumps to form a teratoma. This may be related to the finding that human ES cells are liable to apoptosis upon cellular dissociation (1,17, 18,24). They undergo massive cell death, particularly after complete dissociation, and the cloning efficiency of dissociated human ES cells is generally very low (<1%) (8,29,31). Therefore, cellular state is also important when transplanting ES cells to form teratomas.

Third, the transplant site also affected the formation

of teratomas. One-log larger numbers of ES cells were needed for teratomas to develop after subcutaneous compared to intramuscular transplantation. Connective tissue is sparse in the subcutis compared to muscle, and thus cells transplanted into the subcutis may disperse and so an adequate regional density cannot be retained. Similarly, it has been reported that larger numbers of pancreatic β-cells (9) and tumor cells (11,12) survived after intramuscular than subcutaneous transplantations. In addition, no teratoma was noted at all after intraperitoneal injection, even of 1×10^6 ES cells (data not shown), which should be enough to generate teratomas in the muscle (Table 1). Alternatively, in the muscle, blood flow is abundant and high concentrations of various factors are available for the survival and proliferation of transplanted cells, and thus the muscle may provide conditions more favorable for the tumorigenesis.

Another point is that the host immunity is related to the tumorigenesis. In fact, phagocytes were present around ES cells soon after transplantation in both NOG and NOD/SCID mice. Notably, teratomas developed more frequently in NOG mice (Table 2), in which NK as well as T and B cells were absent, than in NOD/SCID mice, in which NK cells were present. The presence of NK cells in NOD/SCID mice might be attributable to the lower incidence of teratomas. Thus, host innate immunity should be considered when assessing the formation of teratomas.

Finally, regarding the tumorigenesis of ES cell-derived progenitors, the incidence of teratomas posttransplant was very different depending on the recipient mice. We have shown that when ES cell-derived hematopoietic precursor cells (of which 85% were still positive for SSEA-4) were transplanted into NOD/SCID mice, teratomas only developed in some of the animals tested. However, teratomas developed in all NOG mice after the transplantation of these same cells. Furthermore, teratomas developed in all NOG mice even with ES cell-derived progenitor cells of which only 2% were positive for SSEA-4 (data not shown). Therefore, when ES cell-derived progenitors are subjected to an assay of tumorigenesis in vivo using NOD/SCID mice (instead of NOG mice), the risk of tumors developing will likely be underestimated. A predictive assay to detect the tumorigenesis of progenitor cell preparations prior to clinical application is critically important. In this regard, unlike other xenogeneic recipients, either NOD/SCID mice or fetal sheep, NOG mouse recipients closely reflect the results of tumorigenesis in the allogeneic monkey recipients (Table 2), and thus NOG mice should be useful as recipients to predict the tumorigenesis of ES cell-derived progenitor preparations.

We have previously shown that the risk of tumor formation was considerably high after transplantation of monkey ES cell-derived progenitor cells in the allogeneic setting (15,20). In fact, teratomas developed in all monkeys, whether fetal monkeys or immunosuppressed adult monkeys, tested in the allogeneic settings (Table 2). It has been considered that residual pluripotent cells are responsible for the tumor formation posttransplant (4,20). If that is the case, why didn't the residual pleuripotent cells in the ES cell-derived progenitor preparations form teratomas in every NOD/SCID mouse (Table 2)? We and others have shown that immunogenicity increases with the differentiation of ES cells (7) (Fig. 2). The differentiated cell population expressed potent immunogens, which might have functioned as adjuvants. As a result, the residual undifferentiated fraction might be eventually rejected together with the differentiated fraction.

In conclusion, the present study demonstrated that several factors (cell number, cellular state, transplant site, differentiation state, and mouse strain) would affect whether teratomas form after the transplantation of nonhuman primate ES cells and their progenitors into immunodeficient mice. These factors must be taken into consideration when examining the pluripotency of ES cells and, more importantly, when addressing the safety of ES cell-derived progenitor preparations.

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Knockout Serum Replacement (KSR) Has a Suppressive Effect on Sendai Virus-Mediated Transduction of Cynomolgus ES Cells

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Abstract

Sendai virus (SeV) vectors can introduce foreign genes efficiently and stably into primate embryonic stem (ES) cells. For the application of these cells, the control of transgene expression is important. Cynomolgus ES cells transduced with a SeV vector expressing the green fluorescent protein (GFP) gene were propagated in Knockout serum replacement (KSR)–supplemented medium, used widely for the serum-free culture of ES cells, and growth and transgene expression were evaluated. The SeV vector-mediated GFP expression was suppressed in the KSR-supplemented medium, although it was stable in regular fetal bovine serum (FBS)-supplemented medium. Propagation in the KSR-supplemented medium eventually resulted in a complete suppression of GFP expression and eradication of the SeV genome. The inhibitory effect of KSR on the transduction was attributable to the positive selection of untransduced ES cells in addition to the removal of the SeV vector from transduced cells. KSR also reduced the efficiency of the transuction. SeV vector-mediated transgene expression in ES cells was suppressed in the KSR-supplemented medium. Although the suppression is limited in specified cells such as ES cells, these findings will help elucidate how to control transgene expression.

Introduction

SENDAI VIRUS (SeV) is an enveloped virus with a nonsegmented, negative-stranded RNA genome (15,384 nucleotides) and a member of the family *Paramyxoviridae* (Tashiro and Seto, 1999). SeV vectors replicate exclusively in the cytoplasm of transduced cells, and do not go through a DNA phase (Bitzer et al., 2003; Li et al., 2000; Yonemitsu et al., 2000); therefore, there is no concern about the unwanted integration of foreign sequences into chromosomal DNA of the host, which might cause life-threatening diseases as reported previously (Hacein-Bey-Abina et al., 2003; Seggewiss et al., 2006). We previously reported that SeV-mediated gene transfer into primate embryonic stem (ES) cells was efficient and stable (Sasaki et al., 2005).

Because human ES cells have the ability to proliferate indefinitely and differentiate into multiple tissue cells (Hay et al., 2007; Reubinoff et al., 2000; Thomson et al., 1998), they are expected to have clinical applications as well as to serve as models for basic research. Consistent and reliable propagation of undifferentiated ES cells is of particular interest in progenitor cell preparation and drug screening. Fetal bovine serum (FBS) has been the only option for nutritive supplementation of primate ES cell media. Unfortunately, it contains undefined factors, which possibly promote differentiation of ES cells (Bettiol et al., 2007). It has also been reported that the uptake and expression of FBS-derived materials on the cell surface may induce immune responses upon transplantation (Martin et al., 2005). In addition, overall variation in the lots of FBS is significant, and each lot must be screened prior to use. Knockout serum replacement (KSR) is a defined formulation that provides consistent growth conditions for ES cell cultures (Cheng et al., 2004; Goldsborough et al., 1998).

However, we have found that the profiles of SeV-mediated transgene expression in the KSR-supplemented medium are very different from those in the FBS-supplemented medium. Here we show that KSR has an inhibitory

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effect on the transduction of primate ES cells with SeV vectors and discuss the mechanism.

Materials and Methods

Cell culture

Cynomolgus ES cells (CMK6) were maintained on a feeder layer of mitomycin C (Kyowa, Tokyo, Japan)-treated mouse (BALB/c) embryonic fibroblasts as described previously (Suemori et al., 2001). The regular culture medium consisted of Dulbecco's modified Eagle's medium (DMEM)/F12 (Invitrogen, Carlsbad, CA) supplemented with 15% ES cell-qualified FBS (Invitrogen), 0.1 mM 2-mercaptoethanol (Sigma, St. Louis, MO), 2 mM glutamine (Invitrogen), and antibiotics (100 U/mL penicillin and 100 mg/mL streptomycin; Sigma). The serum-free culture medium consisted of DMEM/F12 supplemented with 20% KSR (Invitrogen), 0.1 mM 2-mercaptoethanol, 2 mM glutamine, 0.1 mM nonessential amino acids (Invitrogen), 0.1 mM sodium pyruvate (Invitrogen), and the antibiotics.

Cynomolgus ES cells usually grow as colonies. ES cells were routinely passaged every 3–4 days after dissociation with a combination of digestion with 0.1% collagenase type IV (Invitrogen) and mechanical cutting. ES cells were observed using an Olympus U-LH100HGAPO fluorescence microscope. The colonies were generally oval in shape, and

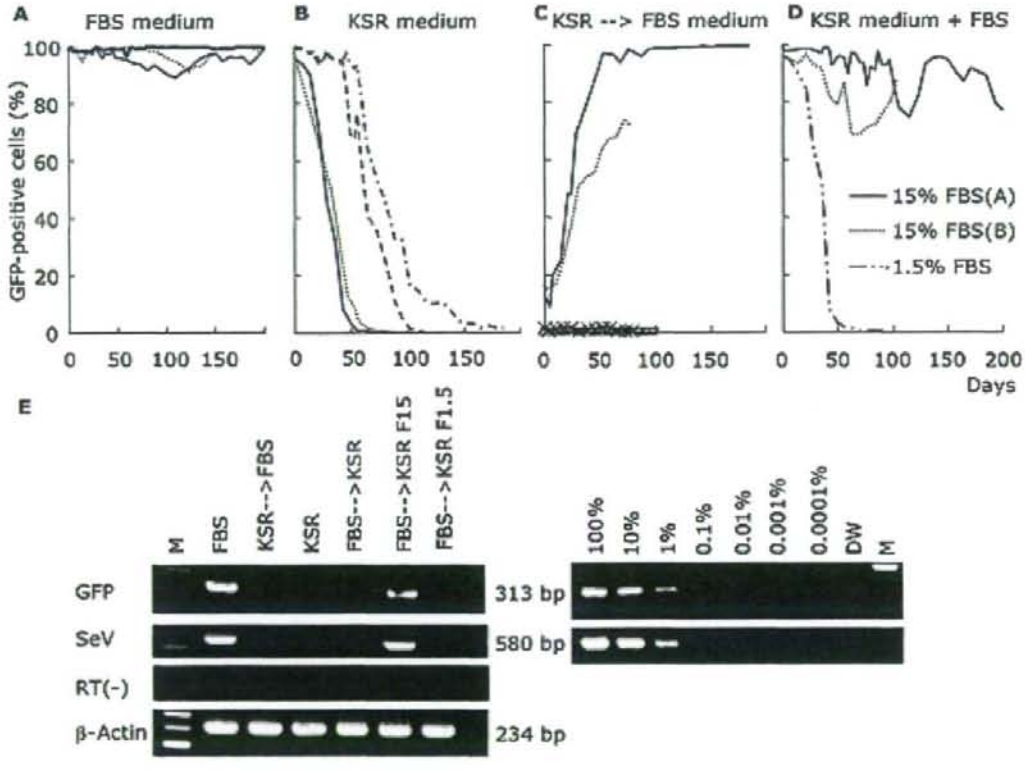

FIG. 1. Inhibitory effect of KSR on SeV transduction. Cynomolgus ES cells transduced with the SeV vector expressing the GFP gene (CMK6/SeV) were propagated in the FBS- (**A**) or KSR-supplemented medium (**B**). The time course profiles of GFP expression were examined by flow cytometry and the results from several independent experiments are shown. (**C**) The cells that had been cultured with KSR were transferred back to the original FBS-supplemented medium. The GFP fluorescence never recovered unless some GFP-positive ES cells (10–20%) were left over at day 0 (at the change of medium). (**D**) CMK6/SeV cells were propagated in the KSR-supplemented medium containing 1.5% or 15% FBS. The inhibitory effect of KSR on the GFP expression was nearly cancelled by the addition of 15% FBS. (**E**) RNA-PCR to detect the SeV genome in transduced cells. The SeV genome became undetectable. The 100% standard consisted of RNA extracted from CMK6/SeV (GFP-positive cells >90%) serially diluted with control (yeast) RNA. The cynomolgus β-actin sequence was used as an internal control, and negative results obtained without reverse transcriptase [designated RT(−)] confirmed that the amplified products were not derived from cellular DNA. M, 100-kb DNA ladder; KSR F15, the KSR-medium containing 15% FBS; KSR F1.5, the KSR-medium containing 1.5% FBS. Arrows (→) indicate the change of medium.

thus the area could be calculated from values of the longest and shortest axes.

Vector

The Fusion (F) gene-defective SeV vector expressing the GFP gene was constructed as previously described (Li et al., 2000). The vector titer was 4.1×10^{10} transducing units (TU)/mL as determined by counting fluorescent cells after the transduction of LLC-MK2 cells, standard control cells for SeV transduction. Gene transfer was conducted by adding the SeV vector solution to culture media at 10 TU per cell. The culture medium during gene transfer was the FBS- or KSR-supplemented medium described above, or Hanks' balanced salt solution (HBSS; Invitrogen) containing 1% BSA (Fraction V; Sigma). Unless otherwise indicated, the cells were washed twice with phosphate-buffered saline (PBS), and fresh medium was added after 24 h of incubation.

Flow cytometry

Expression of GFP and stage-specific embryonic antigen 4 (SSEA-4) was analyzed on a FACScan (Becton Dickinson, Franklin Lakes, NJ) using CellQuest software (Becton Dickinson). For staining SSEA-4, ES cells were incubated with an anti-SSEA-4 antibody (MC-813-70; Chemicon, Temecula, CA), conjugated with Alexa Fluor 647 monoclonal antibody (Invitrogen). Cynomolgus ES cells and cocultured BALB/c feeder cells could be distinguished by using phycoerythin (PE)-conjugated antimouse H-$2K^d$ (SF1-1.1; PharMingen, San Diego, CA), which does not react to cynomolgus cells but does react to BALB/c cells.

RNA-PCR

RNA-PCR for the SeV RNA genomic sequence was carried out as follows. Total RNA was extracted using Quiazol (Qiagen, Hilden, Germany). Reverse transcription was conducted using a RNA LA PCR kit (Takara, Shiga, Japan). The products (250 ng) obtained were then used for the subsequent PCR. Amplification conditions were 30 cycles of 94°C for 1 min, a variable annealing temperature (noted below) for 1 min, and 72°C for 1 min. The amplified products were run on a 2% agarose gel and visualized by ethidium bromide staining. Primer sequences, annealing temperatures, and product sizes were as follows. The SeV vector genome (polymerase gene portion): 5'-AGA GAA CAA GAC TAA GGC TAC C-3' and 5'-ACC TTG ACA ATC CTG ATG TGG-3' (55°C, 580 bp). The GFP gene: 5'-AAG GAC GAC GGC AAC TAC AA-3' and 5'-ACT GGG TGC TCA GGT AGT GG-3' (65°C, 313 bp). The cynomolgus β-actin gene: 5'-CAT TGT CAT GGA CTC TGG CGA CGG-3' and 5'-CAT CTC CTG CTC GAA GTC TAG GGC-3' (60°C, 234 bp).

Results and Discussion

Inhibitory effect of KSR on the transduction

Cynomolgus ES cells (CMK-6) were transduced with the SeV vector expressing the GFP gene. We plucked fluorescent ES-cell colonies under a fluorescence microscope 3 days after transduction and propagated them in the regular FBS-supplemented medium (GFP-positive cells >90%). We defined the transduced, GFP-positive cynomolgus ES cells as CMK6/SeV cells. As we had shown previously (Sasaki et al.,

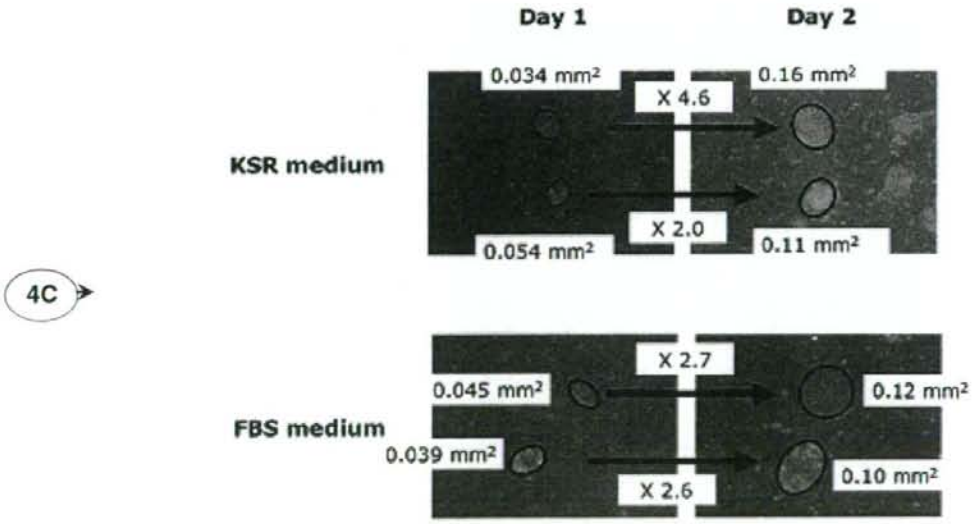

FIG. 2. Positive selection of untransduced ES cells in the KSR-supplemented medium. ES cells were observed with a fluorescence microscope under a bright field and dark fields. The two images were merged into single panels. Typical ES-cell colonies on days 1 and 2 are shown. Values of the area are also indicated. ES cells not expressing the transgene proliferated more rapidly than those expressing the transgene in the KSR-supplemented medium (upper panels). A difference in the growth rate was not observed in the FBS-supplemented medium (lower panels).

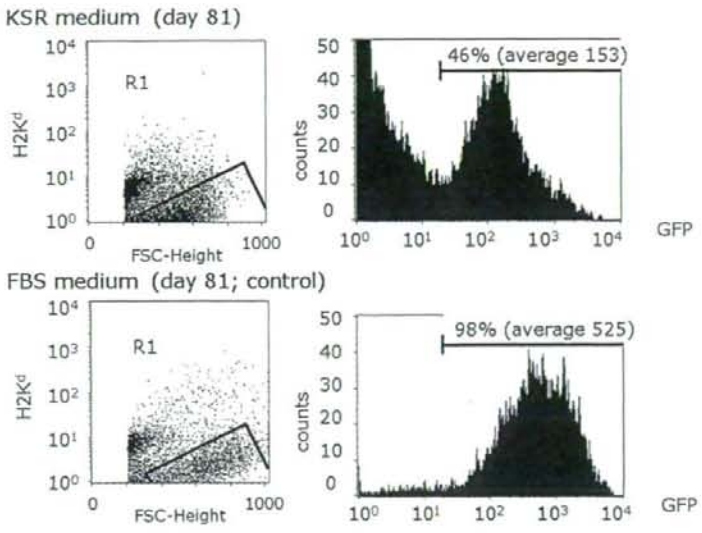

FIG. 3. Removal of the SeV vector from transduced ES cells in the KSR-supplemented medium. Cynomolgus ES cells could be distinguished from cocultured BALB/c feeder cells by using PE-conjugated antimouse H-2K^d, which does not react to cynomolgus cells but does react to BALB/c cells (left panels). Flow cytometric analysis of transduced ES cells after the propagation in the KSR-supplemented medium shows that the mean fluorescence intensity per GFP-positive ES cell decreased compared to the control (right panels).

2005), the expression of GFP in CMK6/SeV cells in the regular FBS-supplemented medium was stable for months (Fig. 1A).

However, when CMK6/SeV cells were propagated in the KSR-supplemented medium, GFP-positive cells decreased in number gradually and eventually no cells fluoresced 2–6 months after transduction (Fig. 1B). The loss of GFP expression was repeatedly observed in several independent experiments. On the other hand, the expression of SSEA-4, an undifferentiated marker for primate ES cells, was stable during the period (data not shown). The cells were then transferred back to the original FBS-supplemented medium, but the fluorescence did not recover unless some GFP-positive ES cells, for instance 10–20%, were still left over at the change of medium (Fig. 1C).

We examined whether the addition of FBS influenced the inhibitory effect by KSR. We propagated CMK6/SeV cells in the KSR-supplemented medium containing 1.5% or 15% FBS. The GFP expression levels again decreased to zero in the former conditions (20% KSR/1.5% FBS); however, the latter conditions (20% KSR/ 15% FBS) resulted in a decrease of less than 30% over the original levels of GFP expression (Fig. 1D). Thus, the inhibitory effect of KSR on the GFP expression was nearly cancelled by the addition of a comparable amount of FBS.

Next, we performed RNA-PCR to examine the SeV genome after the transduction. As shown in Figure 1E, the genome was undetectable on day 77 as assessed by RNA-PCR for both the SeV polymerase and the GFP gene sequences. The limit of detection was 1 transduced cell in 10,000 cells (0.01%). In addition, we have confirmed the loss of vector by a second independent method, immunoblotting, with anti-SeV protein NP (data not shown). On the other hand, KSR did not inhibit the SeV-mediated GFP expression in the rhesus monkey kidney cell line LLC-MK2, a standard control cell line for SeV transduction (data not shown).

A number of hypotheses can be proposed concerning the

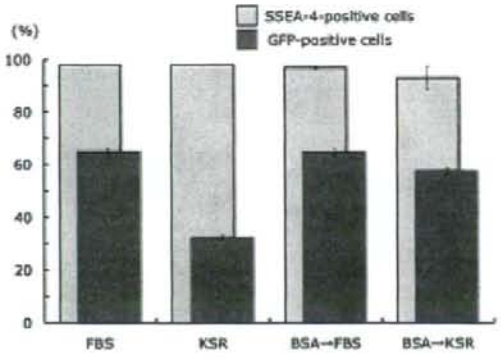

FIG. 4. KSR hampers SeV transduction. The efficiency of transduction with the SeV vector in cynomolgus ES cells decreased by half in the KSR-supplemented medium compared to the FBS-supplemented one. However, the presence of KSR after the transduction (BSA → KSR) did not reduce the efficiency. The transduction efficiency was assessed by flow cytometry for GFP expression at 2 days post-transduction (10 TU per cell). The differentiation status was also assessed by SSEA-4 expression. Arrows (→) indicate the change of culture medium; BSA → FBS, from 1% BSA/HBSS to the FBS-medium; BSA → KSR, from 1% BSA/HBSS to the KSR-medium. The medium was changed 10 h after transduction.

decrease in the SeV vector-mediated expression of GFP in the KSR-supplemented medium. First, ES cells not transduced with the SeV vector might have been selected out in the KSR-supplemented medium ("positive selection of untransduced cells"). To test this hypothesis, we randomly chose 20 ES-cell colonies (10 GFP-positive and 10 GFP-negative) after the exposure to the SeV vector and evaluated colony size by area for 2 days (Fig. 2). In the KSR-supplemented medium, the colonies increased in area by the median 1.9-fold (range, 1.5–4.2) with the expression of GFP and 2.8-fold (range, 1.8–4.6) without the expression (Mann-Whitney's U test, $p = 0.0082$). Thus, ES cells not expressing the SeV-mediated transgene proliferated more rapidly than those expressing the transgene, and cells not expressing the transgene might have been selected out in the KSR-supplemented medium. This hypothesis can explain why KSR does not suppress the SeV-mediated expression of GFP in other cells (e.g., LLC-MK2), considering that KSR does not confer a growth advantage to those cells.

We performed the same experiment in the presence of FBS instead of KSR, but found that the area did not differ between the colonies expressing GFP and those not expressing GFP [median 2.3- (range, 1.6–5.0) and 2.2-fold (range, 1.5–2.8) for 2 days, respectively, $p = 0.76$ by Mann-Whitney's U test). The reduction in growth rate is apparently specific to KSR.

A second hypothesis is that KSR suppresses the replication of the SeV vector in transduced cells ("removal of SeV"). The KSR-supplemented medium might lack constituents that are essential for the replication of SeV vectors in ES cells. In fact, the SeV vector-mediated GFP expression recovered after the addition of FBS (Fig. 1C). Furthermore, the fluorescence intensity of GFP in each GFP-positive ES cells decreased over time in the KSR-supplemented medium as assessed by flow cytometry (Fig. 3) and by fluorescence microscopy (data not shown). These findings cannot be explained by the above-mentioned positive selection hypothesis.

There is another possibility: that KSR has a direct toxic effect on ES cells transduced with SeV vectors, resulting in the predominance of untransduced ES cells in the KSR-supplemented medium. However, this seemed unlikely, given that there was no significant difference in the number of dying or dead cells between the KSR- and FBS-supplemented media (data not shown). Taken together, the inhibitory effect of KSR on the transduction was attributable both to the positive selection of untransduced ES cells and to the removal of the SeV vector from transduced cells. Unfortunately, information on the composition of KSR is not provided by its manufacturer, and we cannot clarify the mechanism by which the vector was lost. Further investigations are awaited.

KSR hampers SeV transduction

Finally, we examined whether KSR influences the efficiency with which cynomolgus ES cells are transduced with the SeV vector. CMK6 cells were exposed to the vector in the FBS- or KSR-supplemented medium. Flow cytometric analysis at 2 days after transduction showed that 65.0 ± 1.37% and 32.3 ± 0.75% of cells fluoresced, respectively (Fig. 4). Thus, the transduction efficacy in the KSR-supplemented medium was approximately half that in the FBS-supplemented one. The transduction did not influence the differentiation status of cells as assessed by SSEA-4 expression (Fig. 4).

The less efficient transduction might be a reflection of a reduced growth rate of transduced cells after the 2-day culture with KSR. To examine this possibility, the cells were transferred to the FBS or KSR medium after a 10-h exposure to the SeV vector in 1% BSA/HBSS. The presence of KSR after the transduction (Fig. 4, BSA → KSR) did not reduce the transduction efficiency compared to the presence of FBS (Fig. 4, BSA → FBS). Thus, the less efficient transduction is due not to a reduced growth rate in the KSR-supplemented medium, but to the transduction conditions: KSR in itself hampers the transduction with SeV vectors.

In conclusion, the present study shows that KSR has an inhibitory effect on transduction with SeV vectors, which was attributable both to the removal of the vector from transduced cells and to the positive selection of untransduced ES cells. Thus, KSR could be used for removal of SeV or the regulation of SeV vector-mediated transgene expression. We are currently investigating methods for regulating transgene expression using KSR or other reagents. However, it should be noted that the KSR-supplemented medium is not suitable for gene transfer into primate ES cells using SeV vectors, given that KSR hampers SeV-mediated transduction. Some other formula not containing KSR would be necessary for serum-free transduction with SeV vectors.

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Author Disclosure Statement

The authors declare that no competing financial interests exist. AU1

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Original Research Report

Sustained Macroscopic Engraftment of Cynomolgus Embryonic Stem Cells In Xenogeneic Large Animals after In Utero Transplantation

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ABSTRACT

Because embryonic stem (ES) cells are able to proliferate indefinitely and differentiate into any type of cell, they have the potential for providing an inexhaustible supply of transplantable cells or tissues. However, methods for the in vitro differentiation of human ES cells are still quite limited. One possible strategy would be to generate differentiated cells in vivo. In view of future clinical application, we investigated the possibility of using xenogeneic large animals for this purpose. We transplanted nonhuman primate cynomolgus ES cells into fetal sheep at 43–67 gestational days (full term 147 days, $n = 15$). After birth, cynomolgus tissues, which were mature teratomas, had been engrafted in sheep when more than 1×10^6 ES cells were transplanted at <50 gestational days. Despite the sustained engraftment, both cellular and humoral immune responses against the ES cells were detected, and additional transplantation was not successful in the animals. At 2 weeks post-transplantation, the ES cell progeny proliferated when transplanted at 48 (<50) gestational days, whereas they were cleared away when transplanted at 60 (>50) gestational days. These results support the rapid development of the xenogeneic immunological barrier in fetal sheep after 50 gestational days. Notably, a large number of Foxp3$^+$ regulatory T cells were present around the ES cell progeny, but macrophages were absent when the transplant was conducted at <50 gestational days, implying that regulatory T cells and premature innate immunity might have contributed to the sustained engraftment. In conclusion, long-term macroscopic engraftment of primate ES cells in sheep is feasible despite the xenogeneic immunological barrier.

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INTRODUCTION

A MAJOR BARRIER TO MOST TISSUE OR CELLULAR transplantation therapies is the shortage of donors. Because embryonic stem (ES) cells are able to proliferate indefinitely and differentiate into any type of cell [1,2], they have potential for providing an inexhaustible supply of transplantable cells or tissues. However, methods for the in vitro differentiation of human (h) ES cells are still quite limited. One possible strategy would be to generate differentiated cells in vivo in animals. In fact, rodent and nonhuman primate allogeneic transplantation models have demonstrated that transplanted ES cells respond to local cues in vivo and showed site-specific differentiation [3,4]. Therefore, the local microenvironment or niche would be important and potentially useful for directed differentiation of ES cells. Given that transplantation experiments using hES cells should be conducted in a xenogeneic setting, there are two major obstacles to this strategy: (1) the xenogeneic immunological barrier [5,6] and (2) the mismatch of microenvironment between the donor and recipient [7].

Although large animals would be clinically relevant as recipients of hES cells for a large supply of therapeutic cells or tissues, there have been very few reports of hES cell-derived engraftment in adult large animals [8]. Generally, such engraftment can be barely achieved by treatment with immunosuppressants. On the other hand, in animal fetuses, it has been considered that the immune system is so premature as not to induce an immune response at the early stages of pregnancy [9,10]. In fact, the in utero transplantation of human hematopoietic stem cells (HSCs) into sheep fetuses before 65 days of gestation (full term, 147 days) led to the generation of human/sheep hematopoietic chimeras (up to 20%). This result also suggests that the fetal sheep microenvironment could provide a proper niche at least for human hematopoietic differentiation [11,12]. Although such chimerism was documented in other animals such as primates, pigs, and mice, the levels of engraftment in sheep were much higher than those in other animals [13,14]. Recently, we and others have shown that cultured primate ES cells also engrafted after in utero transplantation to sheep fetuses, generating microscopic primate/sheep chimeras [15,16]. Furthermore, it has been reported that mouse ES cells engrafted in ischemic hearts of sheep [17] but not in those of baboons [18]. Thus, sheep may be good recipient animals in which stem cells can engraft. In addition, in the fetal period, in vivo microenvironments such as the cytokine milieus are optimized for rapid growth and development of fetuses [19] and this might be favorable for the growth and differentiation of transplanted stem cells. However, primate ES cell-derived macroscopic tissue formation in discordant large animals or systematic studies regarding engraftment and immune responses after in utero transplantation of primate ES cells have not been reported.

In this study, we have transplanted nonhuman primate (cynomolgus macaque) ES cells into fetal sheep and examined whether primate cells can engraft and develop tissues in sheep. In this setting, we have also examined what kind of immune response, if any, is triggered by transplanted primate ES cells.

MATERIALS AND METHODS

Animals

Pregnant Suffolk ewes were purchased from Japan Lamb (Hiroshima, Japan). Fetal sheep at 43–67 days of gestation (full term, 147 days) were used as transplantation recipients. All experiments in this study were performed in accordance with the Jichi Medical University Guide for Laboratory Animals. Experimental procedures were approved by the Animal Care and Use Committee of Jichi Medical University.

Cells

A cynomolgus ES cell (cyES cell) line CMK6 and its subline CMK6G stably expressing green fluorescent protein (GFP) [20] were maintained on a feeder layer of mitomycin C (Kyowa, Tokyo, Japan)-treated mouse (BALB/c, Clea, Tokyo, Japan) embryonic fibroblasts as previously described [21]. Confluent ES cells were dissociated from the feeder layer using 0.1% collagenase type IV (Invitrogen, Carlsbad, CA) for transplantation.

Transplantation and delivery

Before transplantation, ewes were anesthetized with a 0.5–1.0% halothane–oxygen mixture. After a midline laparotomy incision, cells were injected into the fetuses subcutaneously at 1–4 sites/fetus through the uterine wall under ultrasound guidance. The fetuses were delivered at full term or by cesarean section at the indicated days after transplantation.

In situ hybridization and immunohistochemistry

Tissues were fixed with 4% paraformaldehyde and embedded in paraffin. For genomic in situ hybridization, the deparaffinized sections were digested and hybridized with the biotinylated human DNA probe (Dako, Copenhagen, Denmark). The probe was detected with the GenPoint System (Dako) according to the manufacturer's instructions, and nuclei were stained with Hematoxylin. Primary antibodies (Abs) for immunohistochemistry were anti-mouse Oct-3 monoclonal Ab (mAb) (BD Pharmingen, San Diego, CA), rabbit anti-human glial fibrillary acidic protein (GFAP) Ab (Dako), anti-human neuron-specific enolase (NSE) mAb (Dako), anti-human α-smooth muscle actin (α-SMA) mAb (Dako), anti-human desmin mAb (Dako), anti-human vimentin mAb (Dako), rabbit anti-human α-fetoprotein (α-FP) Ab (Dako), and rabbit anti-GFP Ab (Clontech, Palo Alto, CA). The anti-mouse Oct-3 mAb has been confirmed to react to a cynomolgus counterpart [22]. Primary Abs used to

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 visualized 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 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 major histocompatibility complex (MHC) class I and Oct-3 in cultured graft cells and cyES cells was analyzed using a FACS Calibur flow cytometer (BD Pharmingen). For MHC class I, cells were incubated with phycoerythrin (PE)-conjugated anti-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 incubated with 10 ml of 1:10 diluted serum taken from the cyES cell-transplanted or naive (control) sheep. In sheep, maternal antibodies do not 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, 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 × 28 × 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 cells 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 cyES cells 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

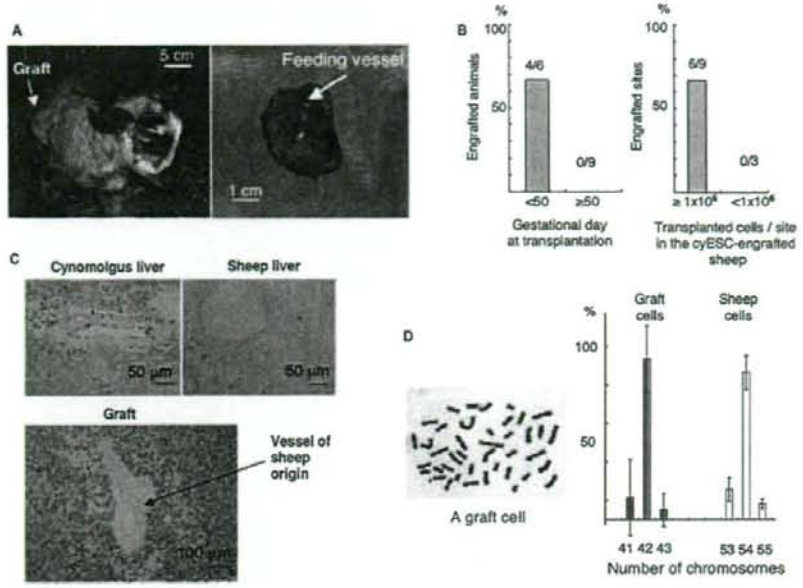

FIG. 1. Macroscopic engraftment after in utero transplantation of cyES cells into fetal sheep. (**A**) Outward appearance and macroscopic view of a tumor are shown. (**B**) The engraftment ratio (macroscopically engrafted number per total transplant number) was compared between the sheep transplanted 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-

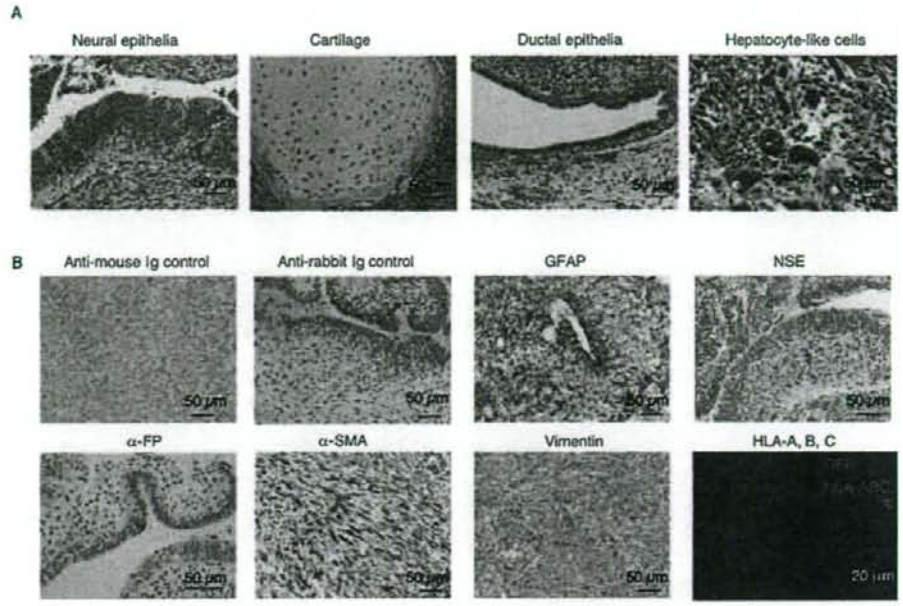

FIG. 2. Cynomolgus grafts in sheep contained mature tissue structures. (**A**) Hematoxylin & Eosin staining revealed that the grafts contained three germ layer cells with mature tissue structures. (**B**) Immunohistochemistry revealed that the grafts contained various kinds of mature cells derived from 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 non-transplanted (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-cyES cell 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

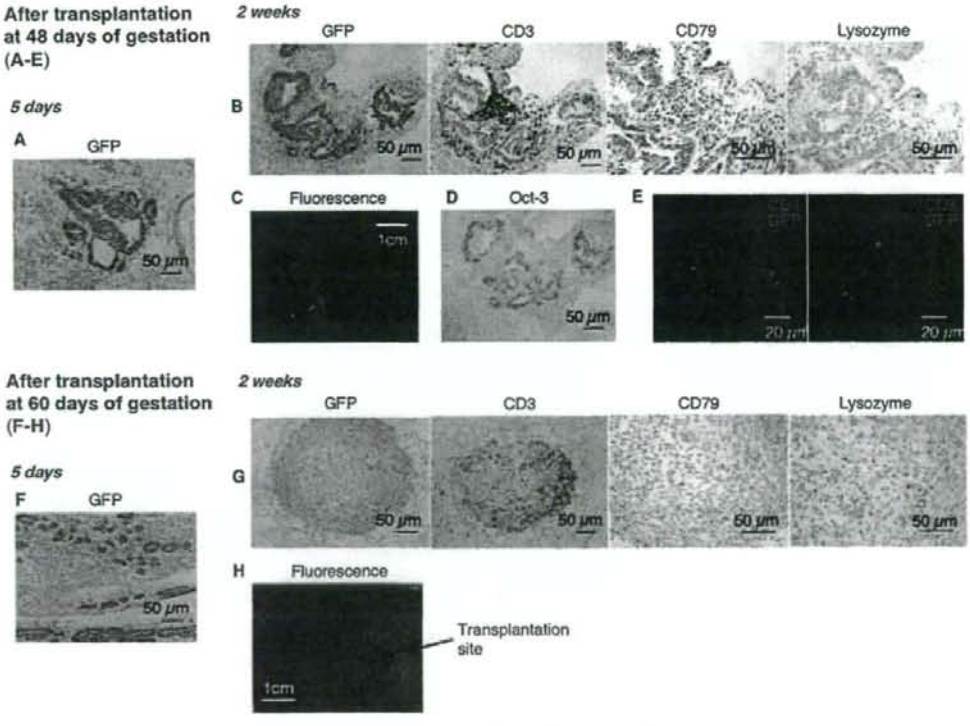

FIG. 3. Cynomolgus grafts in sheep during the fetal period. (**A–E**) When cyES cells expressing GFP were transplanted at 48 days of gestation, GFP^+ transplanted cell progeny were clearly detected both at 5 days (**A**) and at 2 weeks post-transplant (**B** and **C**). (**B**) According to the immunostaining 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.

all of 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-