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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 posttransplantation, 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 45–67 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 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).

F1

T1

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 cellss were originally negative for MHC class I, many of the cells became positive in the grafts after birth (Fig. 2B).

F2

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

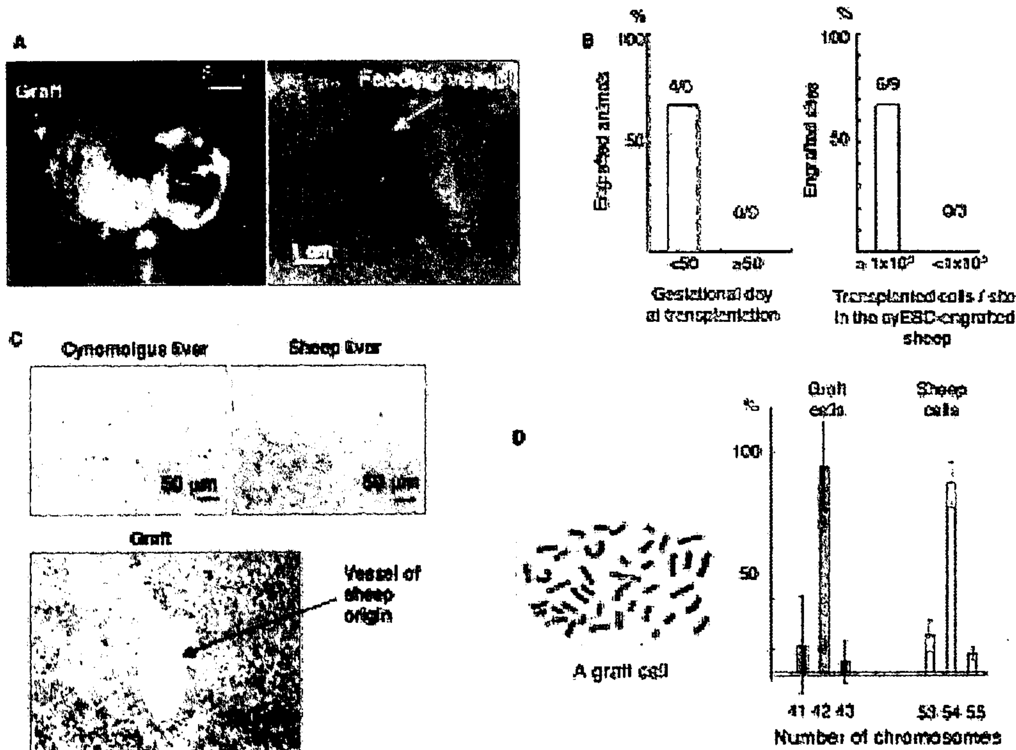

FIG. 1. Macroscopic engraftment after in utero transplantation of cyESCs into fetal sheep. (**A**) Outward appearance and macroscopic view of a tumor are shown. (**B**) The engraftment ratio (macroscopically engrafted number per total transplant number) was compared between the sheep transplanted at <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 ± 0.1% at 48 days and 1.9 ± 0.6% at 60 days. The monocyte fraction was 0.1 ± 0.0% at 48 days, and 0.2 ± 0.1% at 60 days (Fig. 5A). There were no statistical dif-

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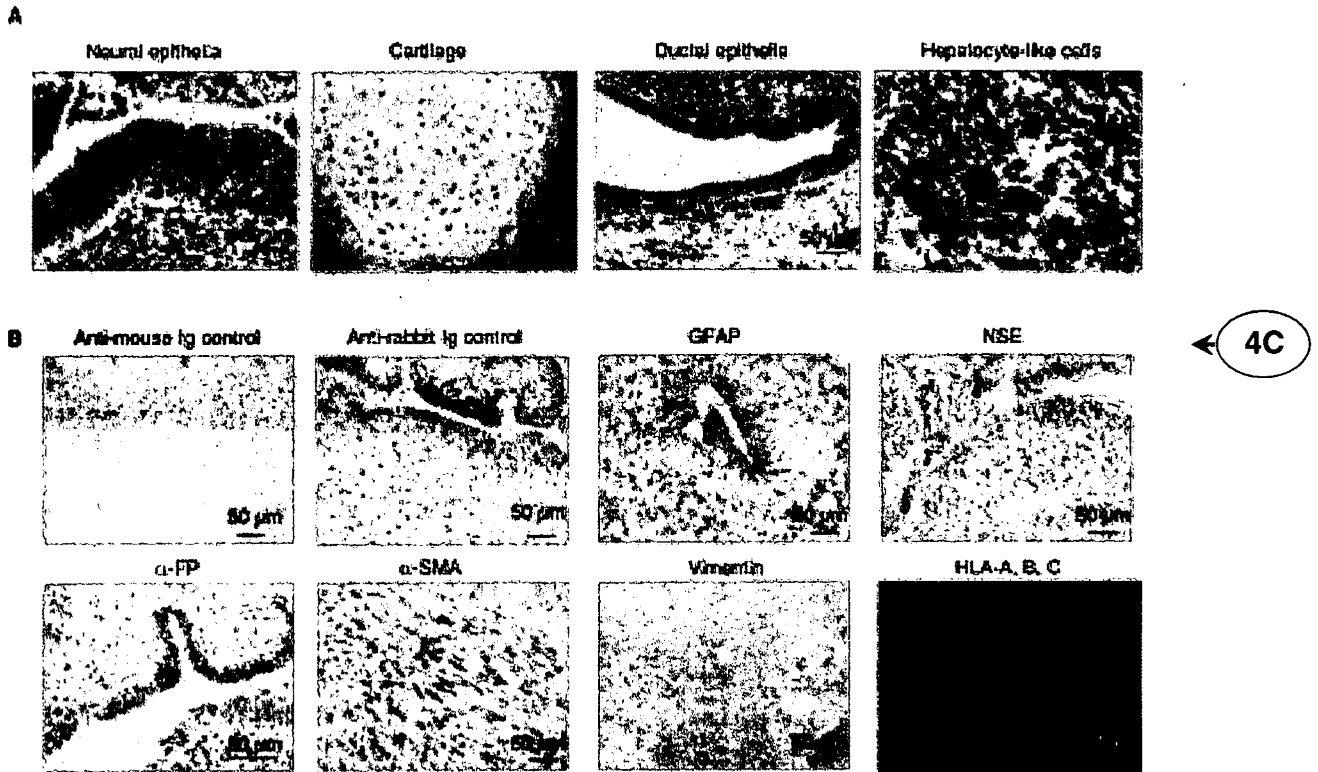

FIG. 2. Cynomolgus grafts in sheep contained mature tissue structures. (**A**) Hematoxylin & Eosin staining revealed that the grafts contained three germ layer cells with mature tissue structures. (**B**) Immunohistochemistry revealed that the grafts contained various kinds of mature cells derived from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

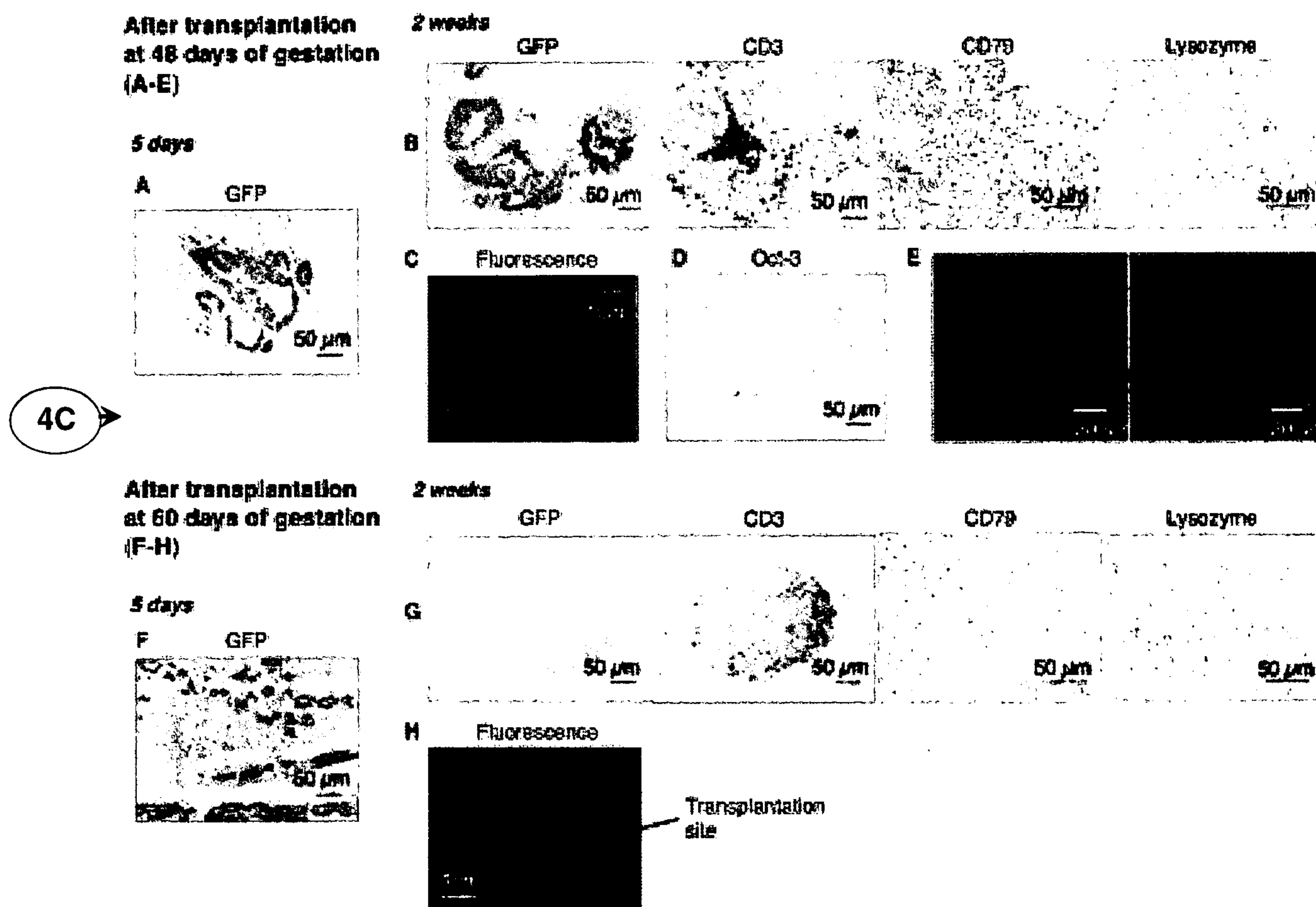

FIG. 3. Cynomolgus grafts in sheep during the fetal period. (**A–E**) When cyES cells expressing GFP were transplanted at 48 days of gestation, GFP$^+$ transplanted cell progeny were clearly detected both at 5 days (**A**) and at 2 weeks post-transplant (**B** and **C**). (**B**) According to the immunostaining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

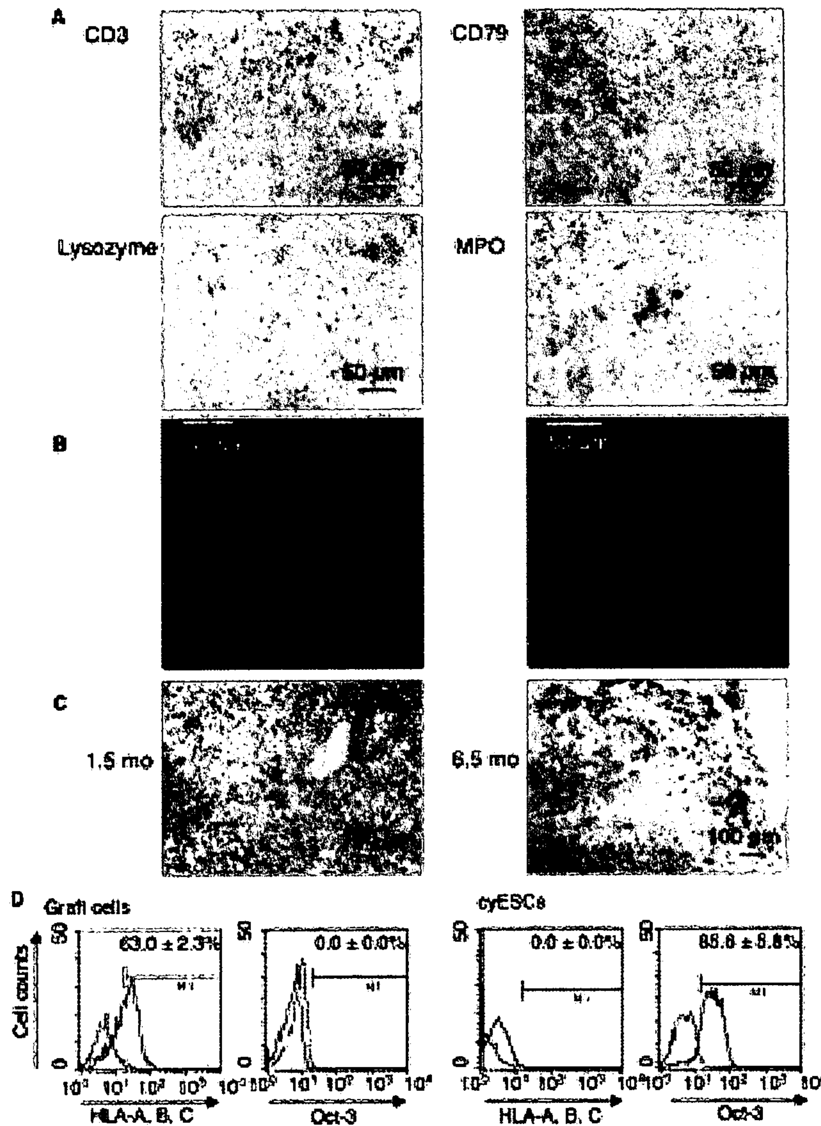

FIG. 4. Cynomolgus grafts in sheep after birth. (**A**) Immunostaining shows infiltration by T cells (positive for CD3), B cells (positive for CD79), macrophages (positive for lysozyme), and neutrophils (positive for MPO) in the cynomolgus-derived grafts in sheep after birth. (**B**) Most of the T cells 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,

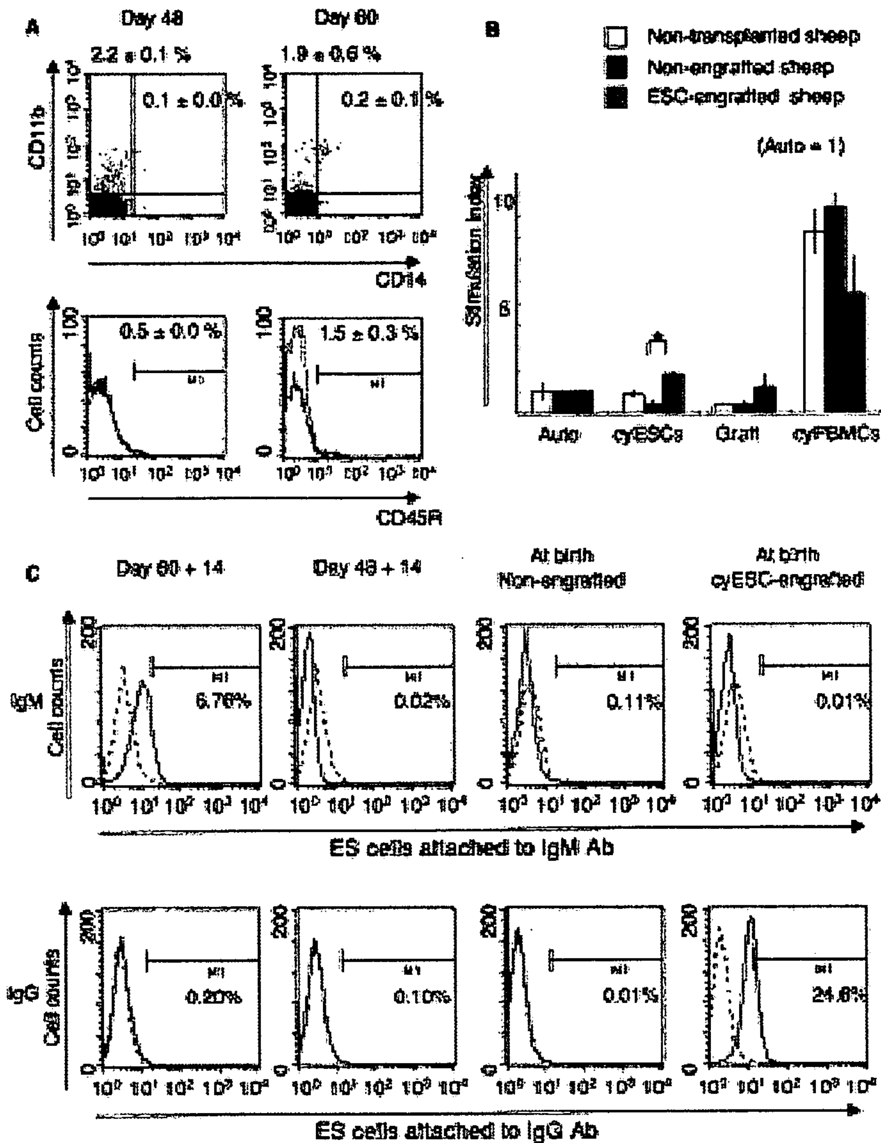

FIG. 5. Host immune responses. (**A**) Cell-surface antigens of fetal sheep peripheral blood leukocytes are compared between 48 days and 60 days of gestation. CD11b and CD14 double staining of the $CD5^-$ cells are shown (*upper*). There were no statistical differences in the percentages of NK cells ($CD11b^+CD14^-CD5^-$) 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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REFERENCES

1. Thomson JA, J Itskovitz-Eldor, SS Shapiro, MA Waknitz, JJ Swiergiel, VS Marshall and JM Jones. (1998). Embryonic stem cell lines derived from human blastocysts. Science 282:1145–1147.
2. Reubinoff BE, MF Pera, CY Fong, A Trounson and A Bongso. (2000). Embryonic stem cell lines from human blastocysts: somatic differentiation in vitro. Nature Biotechnol 18:399–404.
3. Bjorklund LM, R Sanchez-Pernaute, S Chung, T Andersson, IY Chen, KS McNaught, AL Brownell, BG Jenkins, C Wahlestedt, KS Kim and O Isacson. (2002). Embryonic stem cells develop into functional dopaminergic neurons after transplantation in a Parkinson rat model. Proc Natl Acad Sci USA 99:2344–2349.
4. Asano T, N Ageyama, K Takeuchi, M Momoeda, Y Kitano, K Sasaki, Y Ueda, Y Suzuki, Y Kondo, R Torii, M Hasegawa, S Ookawara, K Harii, K Terao, K Ozawa and Y Hanazono. (2003). Engraftment and tumor formation after allogeneic in utero transplantation of primate embryonic stem cells. Transplantation 76:1061–1067.
5. Auchincloss H, Jr. and DH Sachs. (1998). Xenogeneic transplantation. Annu Rev Immunol 16:433–470.
6. Sablinski T, DW Emery, R Monroy, RJ Hawley, Y Xu, P Gianello, T Lorf, T Kozlowski, M Bailin, DK Cooper, AB Cosimi and DH Sachs. (1999). Long-term discordant xenogeneic (porcine-to-primate) bone marrow engraftment in a monkey treated with porcine-specific growth factors. Transplantation 67:972–977.
7. Gritsch HA, RM Glaser, DW Emery, LA Lee, CV Smith, T Sablinski, JS Arn, DH Sachs and M Sykes. (1994). The importance of nonimmune factors in reconstitution by discordant xenogeneic hematopoietic cells. Transplantation 57:906–917.
8. Kehat I, L Khimovich, O Caspi, A Gepstein, R Shofti, G Arbel, I Huber, J Satin, J Itskovitz-Eldor and L Gepstein. (2004). Electromechanical integration of cardiomyocytes derived from human embryonic stem cells. Nature Biotechnol 22:1282–1289.
9. Silverstein AM, RA Prendergast and KL Kraner. (1964). Fetal Response to Antigenic Stimulus. Iv. Rejection of Skin Homografts by the Fetal Lamb. J Exp Med 119:955–964.
10. Flake AW, MR Harrison, NS Adzick and ED Zanjani. (1986). Transplantation of fetal hematopoietic stem cells in utero: the creation of hematopoietic chimeras. Science 233:776–778.
11. Zanjani ED, MG Pallavicini, JL Ascensao, AW Flake, RG Langlois, M Reitsma, FR MacKintosh, D Stutes, MR Harrison and M Tavassoli. (1992). Engraftment and long-term expression of human fetal hemopoietic stem cells in sheep following transplantation in utero. J Clin Invest 89:1178–1188.
12. Civin CI, G Almeida-Porada, MJ Lee, J Olweus, LW Terstappen and ED Zanjani. (1996). Sustained, retransplantable, multilineage engraftment of highly purified adult human bone marrow stem cells in vivo. Blood 88:4102–4109.
13. Flake AW and ED Zanjani. (1999). In utero hematopoietic stem cell transplantation: ontogenic opportunities and biologic barriers. Blood 94:2179–2191.
14. Fujiki Y, K Fukawa, K Kameyama, O Kudo, M Onodera, Y Nakamura, K Yagami, Y Shiina, H Hamada, A Shibuya and H Nakauchi. (2003). Successful multilineage engraftment of human cord blood cells in pigs after in utero transplantation. Transplantation 75:916–922.
15. Narayan AD, JL Chase, RL Lewis, X Tian, DS Kaufman, JA Thomson and ED Zanjani. (2006). Human embryonic stem cell-derived hematopoietic cells are capable of engrafting primary as well as secondary fetal sheep recipients. Blood 107:2180–2183.
16. Sasaki K, Y Nagao, Y Kitano, H Hasegawa, H Shibata, M Takatoku, S Hayashi, K Ozawa and Y Hanazono. (2005). Hematopoietic microchimerism in sheep after in utero transplantation of cultured cynomolgus embryonic stem cells. Transplantation 79:32–37.
17. Menard C, AA Hagege, O Agbulut, M Barro, MC Morichetti, C Brasselet, A Bel, E Messas, A Bissery, P Bruneval, M Desnos, M Puceat and P Menasche. (2005). Transplantation of cardiac-committed mouse embryonic

stem cells to infarcted sheep myocardium: a preclinical study. Lancet 366:1005–1012.

18. Bonnevie L, A Bel, L Sabbah, N Al Attar, P Pradeau, B Weill, F Le Deist, V Bellamy, S Peyrard, C Menard, M Desnos, P Bruneval, P Binder, AA Hagege, M Puceat and P Menasche. (2007). Is Xenotransplantation of Embryonic Stem Cells a Realistic Option? Transplantation 83:333–335.
19. Symonds ME, A Mostyn and T Stephenson. (2001). Cytokines and cytokine receptors in fetal growth and development. Biochem Soc Trans 29:33–37.
20. Takada T, Y Suzuki, Y Kondo, N Kadota, K Kobayashi, S Nito, H Kimura and R Torii. (2002). Monkey embryonic stem cell lines expressing green fluorescent protein. Cell Transplant 11:631–635.
21. Suemori H, T Tada, R Torii, Y Hosoi, K Kobayashi, H Imahie, Y Kondo, A Iritani and N Nakatsuji. (2001). Establishment of embryonic stem cell lines from cynomolgus monkey blastocysts produced by IVF or ICSI. Dev Dyn 222:273–279.
22. Fujimoto Y, K Hasegawa, H Suemori, J Ito and N Nakatsuji. (2006). Molecular cloning and function of Oct-3 isoforms in cynomolgus monkey embryonic stem cells. Stem Cells Dev 15:566–574.
23. Andreoletti O, P Berthon, E Levavasseur, D Marc, F Lantier, E Monks, JM Elsen and F Schelcher. (2002). Phenotyping of protein-prion (PrPsc)-accumulating cells in lymphoid and neural tissues of naturally scrapie-affected sheep by double-labeling immunohistochemistry. J Histochem Cytochem 50:1357–1370.
24. Scanlan CM, ed. (1998). *Introduction to Veterinary Bacteriology*. Iowa State University Press, Ames, Iowa.
25. Kaufman DS and JA Thomson. (2002). Human ES cells—haematopoiesis and transplantation strategies. J Anat 200: 243–248.
26. Hasegawa K, T Fujioka, Y Nakamura, N Nakatsuji and H Suemori. (2006). A method for the selection of human embryonic stem cell sublines with high replating efficiency after single-cell dissociation. Stem Cells 24:2649–2660.
27. Okamura K, K Asahina, H Fujimori, R Ozeki, K Shimizu-Saito, Y Tanaka, K Teramoto, S Arii, K Takase, M Kataoka, Y Soeno, C Tateno, K Yoshizato and H Teraoka. (2006). Generation of hybrid hepatocytes by cell fusion from monkey embryoid body cells in the injured mouse liver. Histochem Cell Biol 125:247–257.
28. Nussbaum J, E Minami, MA Laflamme, JA Virag, CB Ware, A Masino, V Muskheli, L Pabon, H Reinecke and CE Murry. (2007). Transplantation of undifferentiated murine embryonic stem cells in the heart: teratoma formation and immune response. FASEB J 21:1345–1357.
29. Nichols J, B Zevnik, K Anastassiadis, H Niwa, D Klewe-Nebenius, I Chambers, H Scholer and A Smith. (1998). Formation of pluripotent stem cells in the mammalian embryo depends on the POU transcription factor Oct4. Cell 95:379–391.
30. Niwa H, J Miyazaki and AG Smith. (2000). Quantitative expression of Oct-3/4 defines differentiation, dedifferentiation or self-renewal of ES cells. Nature Genet 24:372–376.
31. Cooke MJ, M Stojkovic and SA Przyborski. (2006). Growth of teratomas derived from human pluripotent stem cells is influenced by the graft site. Stem Cells Dev 15: 254–259.
32. Sawyer M, J Moe and BI Osburn. (1978). Ontogeny of immunity and leukocytes in the ovine fetus and elevation of immunoglobulins related to congenital infection. Am J Vet Res 39:643–648.
33. Miyasaka M and B Morris. (1988). The ontogeny of the lymphoid system and immune responsiveness in sheep. Prog Vet Microbiol Immunol 4:21–55.
34. Drukker M, H Katchman, G Katz, S Even-Tov Friedman, E Shezen, E Hornstein, O Mandelboim, Y Reisner and N Benvenisty. (2006). Human embryonic stem cells and their differentiated derivatives are less susceptible to immune rejection than adult cells. Stem Cells 24:221–229.
35. Fox A, J Mountford, A Braakhuis and LC Harrison. (2001). Innate and adaptive immune responses to nonvascular xenografts: evidence that macrophages are direct effectors of xenograft rejection. J Immunol 166:2133–2140.
36. Li L, ML Baroja, A Majumdar, K Chadwick, A Rouleau, L Gallacher, I Ferber, J Lebkowski, T Martin, J Madrenas and M Bhatia. (2004). Human embryonic stem cells possess immune-privileged properties. Stem Cells 22:448–456.
37. Juretic E, A Juretic, B Uzarevic and M Petrovecki. (2001). Alterations in lymphocyte phenotype of infected preterm newborns. Biol Neonate 80:223–227.
38. Thornton CA, JW Upham, ME Wikstrom, BJ Holt, GP White, MJ Sharp, PD Sly and PG Holt. (2004). Functional maturation of $CD4^+CD25^+CTLA4^+CD45RA^+$ T regulatory cells in human neonatal T cell responses to environmental antigens/allergens. J Immunol 173:3084–3092.
39. Chong AS, J Shen, J Tao, D Yin, A Kuznetsov, M Hara and LH Philipson. (2006). Reversal of diabetes in non-obese diabetic mice without spleen cell-derived beta cell regeneration. Science 311:1774–1775.
40. Hori S, T Nomura and S Sakaguchi. (2003). Control of regulatory T cell development by the transcription factor Foxp3. Science 299:1057–1061.
41. Yagi H, T Nomura, K Nakamura, S Yamazaki, T Kitawaki, S Hori, M Maeda, M Onodera, T Uchiyama, S Fujii and S Sakaguchi. (2004). Crucial role of FOXP3 in the development and function of human $CD25^+CD4^+$ regulatory T cells. Int Immunol 16:1643–1656.
42. Condomines M, P Quittet, ZY Lu, L Nadal, P Latry, E Lopez, M Baudard, G Requirand, C Duperray, JF Schved, JF Rossi, K Tarte and B Klein. (2006). Functional regulatory T cells are collected in stem cell autografts by mobilization with high-dose cyclophosphamide and granulocyte colony-stimulating factor. J Immunol 176:6631–6639.
43. Cooper DK, B Gollackner and DH Sachs. (2002). Will the pig solve the transplantation backlog? Annu Rev Med 53:133–147.
44. Sachs DH. (1994). The pig as a potential xenograft donor. Vet Immunol Immunopathol 43:185–191.
45. Minanov OP, S Itescu, FA Neethling, AS Morgenthau, P Kwiatkowski, DK Cooper and RE Michler. (1997). Anti-GaL IgG antibodies in sera of newborn humans and baboons and its significance in pig xenotransplantation. Transplantation 63:182–186.

46. Galili U, SB Shohet, E Kobrin, CL Stults and BA Macher. (1988). Man, apes, and Old World monkeys differ from other mammals in the expression of alpha-galactosyl epitopes on nucleated cells. J Biol Chem 263:17755–17762.
47. Galili U, MR Clark, SB Shohet, J Buehler and BA Macher. (1987). Evolutionary relationship between the natural anti-Gal antibody and the Gal alpha 1-3Gal epitope in primates. Proc Natl Acad Sci USA 84:1369–1373.
48. Abe M, J Qi, M Sykes and YG Yang. (2002). Mixed chimerism induces donor-specific T-cell tolerance across a highly disparate xenogeneic barrier. Blood 99:3823–3829.
49. Lan P, L Wang, B Diouf, H Eguchi, H Su, R Bronson, DH Sachs, M Sykes and YG Yang. (2004). Induction of human T-cell tolerance to porcine xenoantigens through mixed hematopoietic chimerism. Blood 103:3964–3969.
50. Lan P, N Tonomura, A Shimizu, S Wang and YG Yang. (2006). Reconstitution of a functional human immune system in immunodeficient mice through combined human fetal thymus/liver and CD34$^+$ cell transplantation. Blood 108:487–492.
51. Bonde S and N Zavazava. (2006). Immunogenicity and engraftment of mouse embryonic stem cells in allogeneic recipients. Stem Cells 24:2192–2201.
52. Wood KJ and S Sakaguchi. (2003). Regulatory T cells in transplantation tolerance. Nature Rev Immunol 3:199–210.
53. Graca L, SP Cobbold and H Waldmann. (2002). Identification of regulatory T cells in tolerated allografts. J Exp Med 195:1641–1646.
54. Lee MKt, DJ Moore, BP Jarrett, MM Lian, S Deng, X Huang, JW Markmann, M Chiaccio, CF Barker, AJ Caton and JF Markmann. (2004). Promotion of allograft survival by CD4$^+$CD25$^+$ regulatory T cells: evidence for in vivo inhibition of effector cell proliferation. J Immunol 172: 6539–6544.
55. Sun Z, L Zhao, H Wang, L Sun, H Yi and Y Zhao. (2006). Presence of functional mouse regulatory CD4$^+$CD25$^+$T cells in xenogeneic neonatal porcine thymus-grafted athymic mice. Am J Transplant 6:2841–2850.
56. Takahata Y, A Nomura, H Takada, S Ohga, K Furuno, S Hikino, H Nakayama, S Sakaguchi and T Hara. (2004). CD25$^+$CD4$^+$ T cells in human cord blood: an immunoregulatory subset with naive phenotype and specific expression of forkhead box p3 (Foxp3) gene. Exp Hematol 32: 622–629.
57. Michaelsson J, JE Mold, JM McCune and DF Nixon. (2006). Regulation of T cell responses in the developing human fetus. J Immunol 176:5741–5748.
58. Gertow K, S Wolbank, B Rozell, R Sugars, M Andang, CL Parish, MP Imreh, M Wendel and L Ahrlund-Richter. (2004). Organized development from human embryonic stem cells after injection into immunodeficient mice. Stem Cells Dev 13:421–435.
59. Kyba M, RC Perlingeiro and GQ Daley. (2002). HoxB4 confers definitive lymphoid-myeloid engraftment potential on embryonic stem cell and yolk sac hematopoietic progenitors. Cell 109:29–37.
60. Lu SJ, Q Feng, S Caballero, Y Chen, MA Moore, MB Grant and R Lanza. (2007). Generation of functional hemangioblasts from human embryonic stem cells. Nature Methods 4:501–509.
61. Fujikawa T, SH Oh, L Pi, HM Hatch, T Shupe and BE Petersen. (2005). Teratoma formation leads to failure of treatment for type I diabetes using embryonic stem cell-derived insulin-producing cells. Am J Pathol 166:1781–1791.
62. Shibata H, N Ageyama, Y Tanaka, Y Kishi, K Sasaki, S Nakamura, S Muramatsu, S Hayashi, Y Kitano, K Terao and Y Hanazono. (2006). Improved safety of hematopoietic transplantation with monkey embryonic stem cells in the allogeneic setting. Stem Cells 24:1450–1457.
63. Wootton SK, CL Halbert and AD Miller. (2005). Sheep retrovirus structural protein induces lung tumours. Nature 434:904–907.
64. Klymiuk N, M Muller, G Brem and B Aigner. (2003). Characterization of endogenous retroviruses in sheep. J Virol 77:11268–11273.

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Key Words. Cynomolgus monkey • Hematopoiesis • Embryonic stem cell • In utero transplantation • Teratoma • Purging Tumor prevention

ABSTRACT

Cynomolgus monkey embryonic stem cell (cyESC)-derived in vivo hematopoiesis was examined in an allogeneic transplantation model. cyESCs were induced to differentiate into the putative hematopoietic precursors in vitro, and the cells were transplanted into the fetal cynomolgus liver at approximately the end of the first trimester ($n = 3$). Although cyESC-derived hematopoietic colony-forming cells were detected in the newborns (4.1%–4.7%), a teratoma developed in all newborns. The risk of tumor formation was high in this allogeneic transplantation model, given that tumors were hardly observed in immunodeficient mice or fetal sheep that had been xeno-transplanted with the same cyESC derivatives. It turned out that the cyESC-derived donor cells included a residual undifferentiated fraction positive for stage-specific embryonic antigen (SSEA)-4 (38.2% ± 10.3%) despite the rigorous differentiation culture. When an SSEA-4-negative fraction was transplanted ($n = 6$), the teratoma was no longer observed, whereas the cyESC-derived hematopoietic engraftment was unperturbed (2.3%–5.0%). SSEA-4 is therefore a clinically relevant pluripotency marker of primate embryonic stem cells (ESCs). Purging pluripotent cells with this surface marker would be a promising method of producing clinical progenitor cell preparations using human ESCs. STEM CELLS *2006;24:1450–1457*

INTRODUCTION

Human embryonic stem cells (hESCs) hold great potential in the treatment of a variety of diseases and injuries because embryonic stem cells (ESCs) have the ability to proliferate indefinitely in culture and to differentiate into any cell type [1, 2]. Because ESCs are able to form teratomas when transplanted into immunodeficient mice, safety concerns would be raised against the clinical application of hESCs [3, 4]. It will be necessary to test the safety of these cells in animal transplantation models before clinical application. Nonhuman primate transplantation models would be desirable for this purpose; however, there have been only a few reports on these models [5–7]. The successful engraftment of transplanted cells in primates will not be achieved unless the immune rejection of transplanted cells is circumvented (e.g., through immunosuppressive treatment) [6]. The early gestational fetus may be a good recipient with which to circumvent immune rejection because the immune system is premature [8]. In addition, in the animal fetus, transplanted cells would engraft without conditioning of recipients such as irradiation or immunosuppressive treatment [9–12]. We have previously established a system for allogeneic transplantation of cynomolgus ESCs (cyESCs) using preimmune fetal monkeys as recipients [5].

We have also reported a novel method for hematopoietic engraftment from cyESCs in sheep [13]. The method is a combination of three steps: (a) differentiation in vitro to generate the putative hematopoietic precursors [14]; (b) transplantation of the cells in utero [15]; and (c) development into hematopoietic cells in vivo using the hematopoietic microenvironment of the fetal liver [16]. In the present study,

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we have examined the safety as well as the efficacy of hematopoietic engraftment of cells derived from cyESCs in the allogeneic transplantation model.

Materials and Methods

Animals

Pregnant cynomolgus monkeys (16–22 years old) were obtained by mating and were reared at the Tsukuba Primate Research Center in accordance with Rules for Animals Care and Management set forth by the Research Center and Guiding Principles for Animal Experiments Using Nonhuman Primates formulated by the Primate Society of Japan. Experimental procedures were approved by the Animal Welfare and Animal Care Committee of the National Institute of Infectious Diseases. The animals were free of intestinal parasites and were seronegative for herpes virus B, varicella-zoster-like virus, measles virus, and simian immunodeficiency virus.

Cell Preparation

A cyESC line (CMK6G) stably expressing green fluorescent protein (GFP) was established after transfection of the parental cyESC line (CMK6) with the enhanced GFP gene (Clontech, Palo Alto, CA, http://www.clontech.com) [17]. cyESCs were maintained on a feeder layer of mitomycin C (Kyowa, Tokyo, http://www.kyowa.co.jp)-treated mouse (ICR or BALB/c; Clea Japan, Tokyo, http://www.clea-japan.com) embryonic fibroblasts as previously described [18]. The mouse bone marrow stromal cell line OP9 was maintained in α-minimum essential medium (Invitrogen, Carlsbad, CA, http://www.invitrogen.com) supplemented with 20% fetal calf serum (FCS; Invitrogen) [19].

cyESCs were induced to differentiate into the putative hematopoietic precursors as previously described [13]. Briefly, undifferentiated cyESCs were transferred onto mitomycin C-treated confluent OP9 cells and cultured for 6 days in Iscove's modified Dulbecco's medium (Invitrogen) supplemented with 8% FCS, 8% horse serum (Invitrogen), 5×10^{-6} M hydrocortisone (Sigma, St. Louis, http://www.sigmaaldrich.com), and multiple cytokines, including 20 ng/ml recombinant human (rh) bone morphogenetic protein-4 (R&D Systems, Minneapolis, http://www.rndsystems.com), 20 ng/ml rh stem cell factor (Biosource, Camarillo, CA, http://www.biosource.com), 20 ng/ml rh vascular endothelial growth factor (VEGF; R&D Systems), 20 ng/ml rh Flt-3 ligand (PeproTech, Rocky Hill, NJ, http://www.peprotech.com), 20 ng/ml rh interleukin-3 (PeproTech), 10 ng/ml rh interleukin-6 (PeproTech), 20 ng/ml rh granulocyte colony-stimulating factor (PeproTech), and 2 IU/ml rh erythropoietin (Roche, Basel, Switzerland, http://www.roche.com). The cells were resuspended in 0.1% human serum albumin (Sigma)/Hanks' balanced saline solution (Sigma) for transplantation.

Flow Cytometry

Primary antibodies (Abs) used in the present study were anti-human CD34 monoclonal Ab (mAb; BD Pharmingen, San Diego, http://www.bdbiosciences.com/pharmingen), anti-human CD31 mAb (Pharmingen), anti-human CD45 mAb (Pharmingen), anti-human vascular endothelial (VE) cadherin mAb (Pharmingen), rabbit anti-human VEGF receptor (VEGFR)-2 Ab (Santa Cruz Biotechnology, Santa Cruz, CA, http://www.scbt.com), and anti-stage-specific embryonic antigen (SSEA)-4 mAb (Chemicon, Temecula, CA, http://www.chemicon.com). All of them cross-reacted to cynomolgus counterparts as previously demonstrated [18, 20–22]. Secondary Abs were phycoerythrin (PE)-conjugated rabbit anti-mouse immunoglobulins (Ig) Ab (DakoCytomation, Glostrup, Denmark, http://www.dako.com) and Alexa Fluor 647-conjugated goat anti-mouse IgG Ab (Molecular Probes, Eugene, OR, http://probes.invitrogen.com). Cells stained with unlabeled primary Abs were incubated with fluorescence-labeled secondary Abs. Cells were incubated with either primary or secondary Ab for 20–60 minutes at 4°C. Regarding staining with the anti-VEGFR-2 Ab, the cells were incubated with biotin-conjugated goat anti-rabbit IgG Ab (Beckman Coulter, Miami, http://www.beckmancoulter.com), followed by PE-conjugated streptavidin (Beckman Coulter). Fluorescence-labeled cells were analyzed with a FACS Calibur flow cytometer (Becton, Dickinson and Company, Franklin Lakes, NJ, http://www.bd.com). Data analysis was performed using the CellQuest software (Becton, Dickinson and Company). Isotype-matched, irrelevant mAbs (DakoCytomation or Beckman Coulter) served as negative controls. Nonviable cells were excluded from analysis by propidium iodide (Sigma) costaining.

Cell Sorting

Cell sorting was performed to purge SSEA-4^{+} cells from among the cultured cyESCs in vitro. Cells were incubated with the anti-SSEA-4 mAb for 1 hour at 4°C and washed twice with Dulbecco's modified Eagle's medium supplemented with 10% FCS. The cells were then incubated with the PE-conjugated anti-mouse Ig Ab for 1 hour at 4°C and washed twice again. GFP-positive and SSEA-4-negative cells were sorted using an Epics Elite cell sorter (Beckman Coulter). Data acquisition was performed using the Expo2 software (Beckman Coulter).

Transplantation and Delivery

Transplant procedures were previously described [5]. Briefly, animals were anesthetized via an intramuscular administration of ketamine hydrochloride (Ketalar, 10 mg/kg; Sankyo, Tokyo, http://www.sankyo.co.jp) and received 0.5%–1.0% isoflurane by inhalation by means of an endotracheal tube. Cells (0.16–46 $\times 10^6$ cells per fetus; Table 1) were injected into the fetal liver through a 23-gauge needle using an ultrasound-guided technique at approximately the end of the first trimester. The fetuses were delivered by cesarean section at 2–3 months after transplant (gestation 120–157 days, full term 165 days).

Colony Polymerase Chain Reaction

Cynomolgus clonogenic hematopoietic colonies were produced as previously described [20]. After cells were cultured in methylcellulose medium for 10–14 days, well-separated individual colonies were plucked into 50 μl of distilled water and digested with 20 μg/ml proteinase K (Takara, Shiga, Japan, http://www.takara-bio.com) at 55°C for 1 hour, followed by 99°C for 10 minutes. Each sample (5 μl) was used for a nested polymerase chain reaction (PCR) to detect the *GFP* gene sequence. The outer primer set was 5′-AAGGACGACGGCAACTACAA-3′ and 5′-ACTGGGTGCTCAGGTAGTGG-3′, and the inner primer set was 5′-GCATCGACTTCAAGGAGGAC-3′ and 5′-GTTGTGGCGGATCTTGAAGT-3′. Amplification conditions for both the outer and inner PCR were 30 cycles of 95°C for 30 seconds, 65°C for 30 seconds, and 72°C for 30 seconds. The

Table 1. ESC-derived hematopoiesis and tumor formation

Animals	Animal no.	Transplanted cells	Purging SSEA-4+ cells	Cell number per fetus ($\times 10^6$)	Donor-derived CFU in recipients[a] at birth (donor/total colony number)	Tumor formation	Observation period (months)
Monkeys	0031	Undifferentiated	−	3.90	n.d.	+	3
	2311	ESCs	−	0.16	n.d., Dead	+	2
	0321		−	0.21	n.d., Dead	+	2
	0841	Day-6 ESC-	−	10	4.1% (2/49)	+	3
	1551	derived cells	−	46	n.d., Dead	+	2.5
	0021		−	46	4.7% (4/85)	+	3
	0691	Day-6 ESC-	+	0.16	3.2% (2/62)	−	3
	0381	derived cells	+	1.40	5.0% (4/80)	−	3
	0022		+	0.17	2.3% (2/86)	−	3
	0981		+	0.31	4.1% (3/73)	−	3
	0051		+	0.31	n.d., Dead[b]	−	3
	1552		+	0.75	4.4% (2/45)	−	4
Sheep[c]	57	Day-6 ESC-	−	50	1.1% (1/91)	−	18
	55	derived cells	−	50	1.1% (1/91)	−	26
	141		−	78	1.1% (1/91)	−	26
	182		−	14	1.6% (1/63)	−	21

[a]Percentage of donor-derived CFU was calculated by dividing the number of CFU positive for the green fluorescent protein gene sequence by the number of CFU positive for the β-actin gene sequence. Donor-derived CFU were analyzed at delivery.
[b]Death due to ablation of placentae. Other deaths were presumably tumor-related.
[c]As published by Sasaki et al. [13].
Abbreviations: CFU, colony-forming units; ESC, embryonic stem cell; n.d., not done; SSEA, stage-specific embryonic antigen.

outer PCR products were purified using a QIA quick PCR purification kit (Qiagen, Valencia, CA, http://www.qiagen.com). Simultaneous PCR for the β-actin sequence was also performed to ensure DNA amplification of the sample in each colony. The primer set for β-actin was 5′-CATTGTCATGGACTCTGGCGACGG-3′ and 5′-CATCTCCTGCTCGAAGTCTAGGGC-3′. Amplification conditions for β-actin PCR were 40 cycles of 95°C for 30 seconds, 65°C for 30 seconds, and 72°C for 30 seconds. Amplified GFP (131 bp) and β-actin (234 bp) products were resolved on 2% agarose gel (Sigma) and visualized by ethidium bromide (Invitrogen) staining.

RNA PCR

Total RNA was extracted from cells of interest using the EZ1 RNA universal tissue kit (Qiagen). RNA was reverse-transcribed at 50°C for 30 minutes using the RNA LA PCR kit (Takara) with oligo dT primer. The resulting cDNA was then subjected to PCR. Regarding PCR for Oct-4, the primer set was 5′-GGACACCTGGCTTCGGATT-3′ and 5′-TTCGCTTTCTCTTTCGGGC-3′. The PCR conditions were 35 cycles of 95°C for 30 seconds, 67°C for 45 seconds, and 68°C for 1.5 minutes. Regarding PCR for Scl, the primer set was 5′-GGGCGGAAAGCTGTTTGCGATT-3′ and 5′-TCGCTGAGAGGCCTGCAGTT-3′. The PCR conditions were 35 cycles of 95°C for 30 seconds, 63°C for 1 minute, and 72°C for 1 minute. A simultaneous PCR for β-actin was also conducted on each cDNA sample as an internal control as described above. Amplified Oct-4 (697 bp), Scl (201 bp), and β-actin (234 bp) products were resolved on 2% agarose gel and visualized by ethidium bromide staining.

RESULTS

In Utero Transplantation and Delivery

cyESCs stably expressing GFP were used in this study [17]. In the setting of allogeneic transplantation, GFP was used as a genetic tag to track transplanted cell progeny. We employed the OP9 stromal cell coculture method instead of the embryoid body formation method to facilitate the hematopoietic differentiation [19, 23, 24] (Fig. 1A, 1B). According to the flow cytometric analysis, CD34, CD31 (platelet/endothelial cell adhesion molecule-1 [PECAM-1]), CD144 (VE-cadherin), and VEGFR-2 (Flk-1) were all upregulated on day 6 but decreased thereafter (Fig. 1C–1E, 1G). Among the markers examined, CD34 is a widely used surface marker of hematopoietic stem cells in both human and monkey subjects [25–27]. The others are key markers of hemangioblasts (which generate endothelial and hematopoietic lineages) in both mice and humans [14, 28]. Cells positive for both VEGFR-2 and VE-cadherin emerged on day 6 (Fig. 1H). CD45, however, was not detected until day 12 (Fig. 1F). Despite the hemangioblast marker expression on day 6, the hematopoietic *Scl* gene was upregulated at this time point as assessed by RNA PCR (Fig. 1I), implying that the hematopoietic commitment might have already occurred on day 6 [29, 30]. We therefore designated the day 6 cyESC-derived progenitor cells as putative hematopoietic precursors. The time course profiles presented here were similar to those of hESCs [14, 24]. The GFP expression was stable during the 6-day culture (Fig. 1A, 1B) and afterward (data not shown).

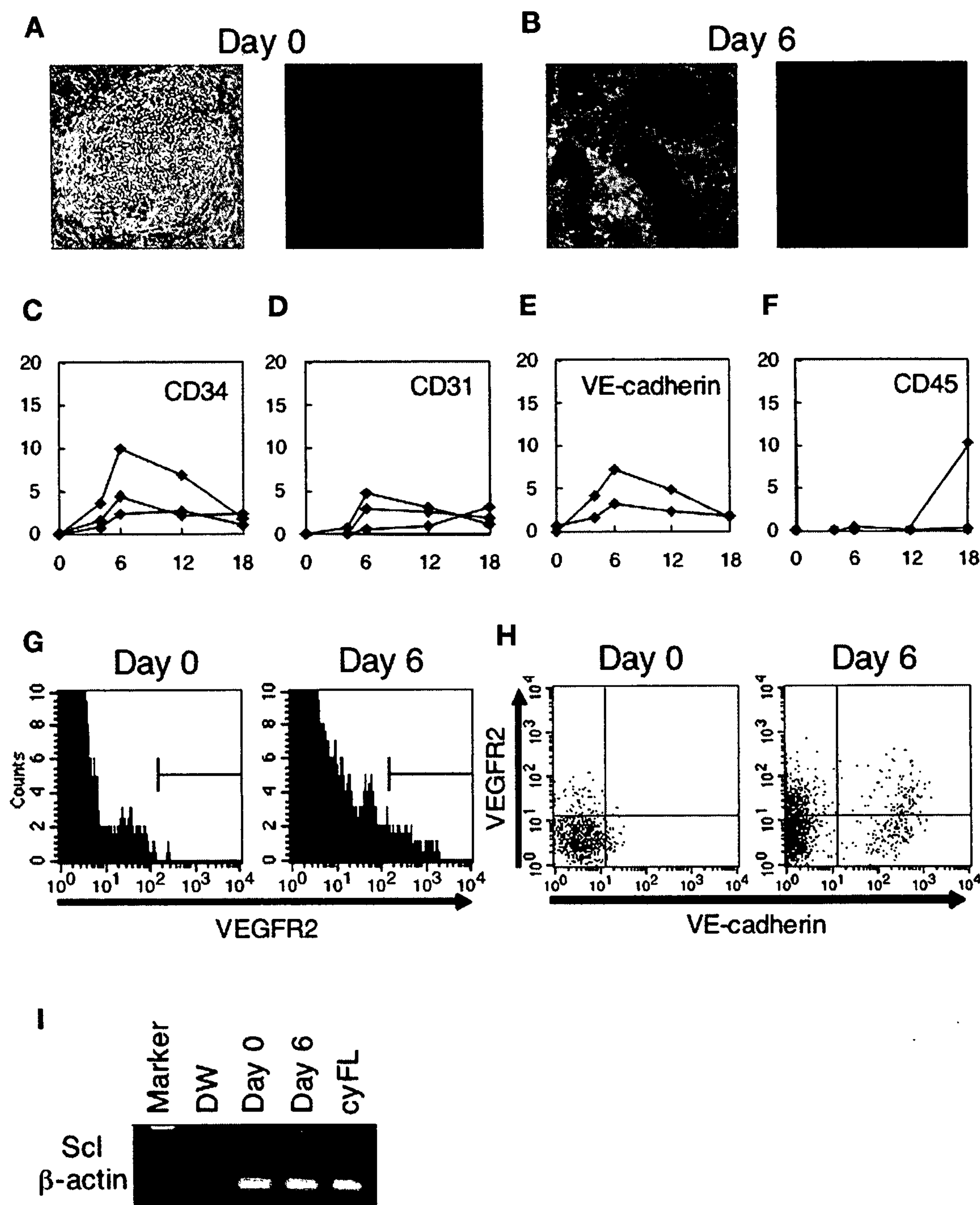

Figure 1. Flow cytometric analysis during the in vitro differentiation of cyESCs. Undifferentiated cyESCs expressing green fluorescent protein were cultured on OP9 cells with multiple cytokines (see Materials and Methods). **(A)**: Cells on day 0 are shown in bright (left) and dark (right) fields. **(B)**: Cells on day 6 are shown in bright (left) and dark (right) fields. **(C)**: Cells on days 0, 4, 6, 12, and 18 were stained for CD34. **(D)**: Cells on days 0, 4, 6, 12, and 18 were stained for CD31. **(E)**: Cells on days 0, 4, 6, 12, and 18 were stained for VE-cadherin. **(F)**: Cells on days 0, 4, 6, 12, and 18 were stained for CD45. The vertical axis shows the fraction (percentage) of cells that were stained positive. **(C–F)**: Results of two or three independent experiments are shown. **(G)**: Although cells on day 0 already express low levels of VEGFR-2, a VEGFR-2^{high} population did not emerge until day 6. **(H)**: Dot-plot profiles for VEGFR-2 and VE-cadherin expression indicate that cells positive for both VEGFR-2 and VE-cadherin emerged until day 6. **(G, H)**: Representative results from three independent experiments are shown. **(I)**: The *Scl* gene expression was upregulated on day 6 to a level similar to that in the cynomolgus fetal liver as assessed by RNA polymerase chain reaction. Day-6 cells (putative hematopoietic precursors) were used for transplantation. Abbreviations: cyESC, cynomolgus embryonic stem cell; cyFL, cynomolgus fetal liver; DW, distilled water; VE, vascular endothelial; VEGFR, vascular endothelial growth factor receptor.