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

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

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

Innate immune responses

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

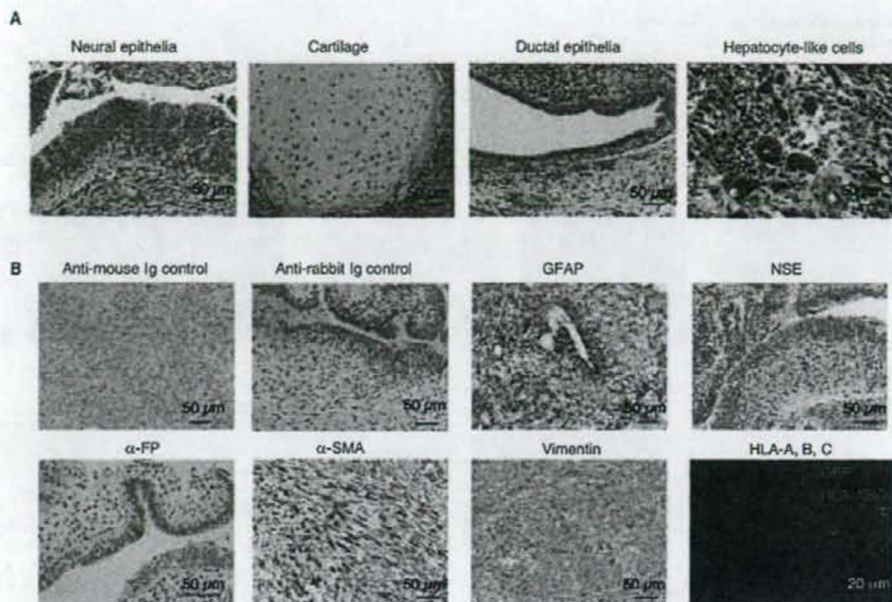

FIG. 2. Cynomolgus grafts in sheep contained mature tissue structures. (**A**) Hematoxylin & Eosin staining revealed that the grafts contained three germ layer cells with mature tissue structures. (**B**) Immunohistochemistry revealed that the grafts contained various kinds of mature cells derived from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

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

Adaptive immune responses

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

Regarding the humoral immunity (Fig. 5C), considerable levels of IgM against cyES cells were detected at 2 weeks post-transplant when the transplantation was conducted at 60 days of gestation (day 60 + 14). The slight increase in $CD45R^+$ fraction (as B cells) at 60 days of gestation might support the B cell development after 50 days of gestation (Fig. 5A). Anti-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

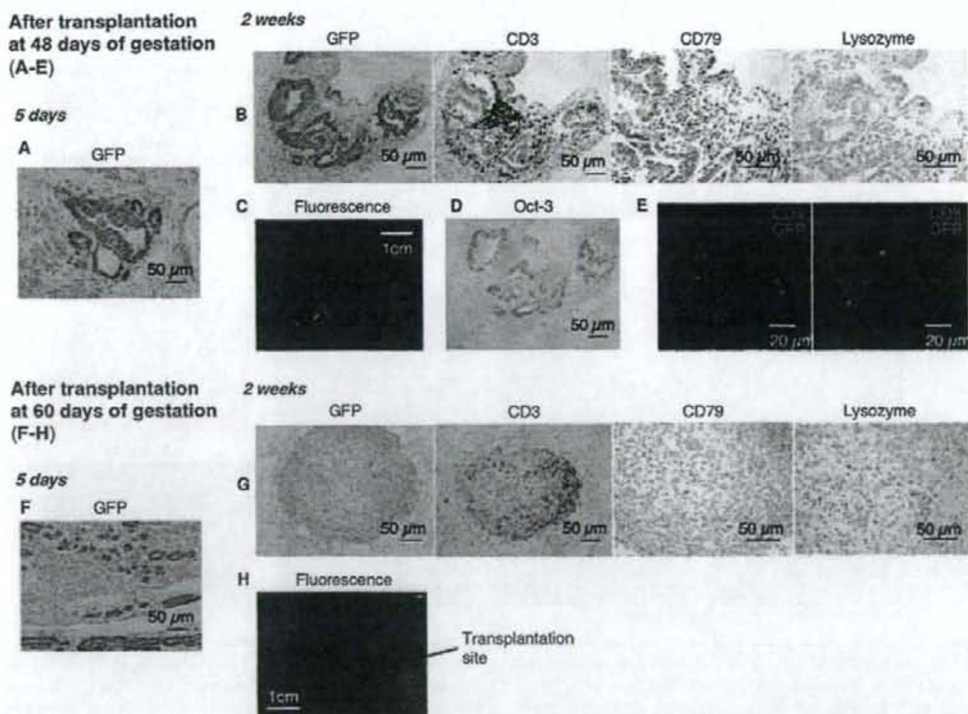

FIG. 3. Cynomolgus grafts in sheep during the fetal period. (**A–E**) When cyES cells expressing GFP were transplanted at 48 days of gestation, GFP^+ transplanted cell progeny were clearly detected both at 5 days (**A**) and at 2 weeks post-transplant (**B** and **C**). (**B**) According to the immunostaining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

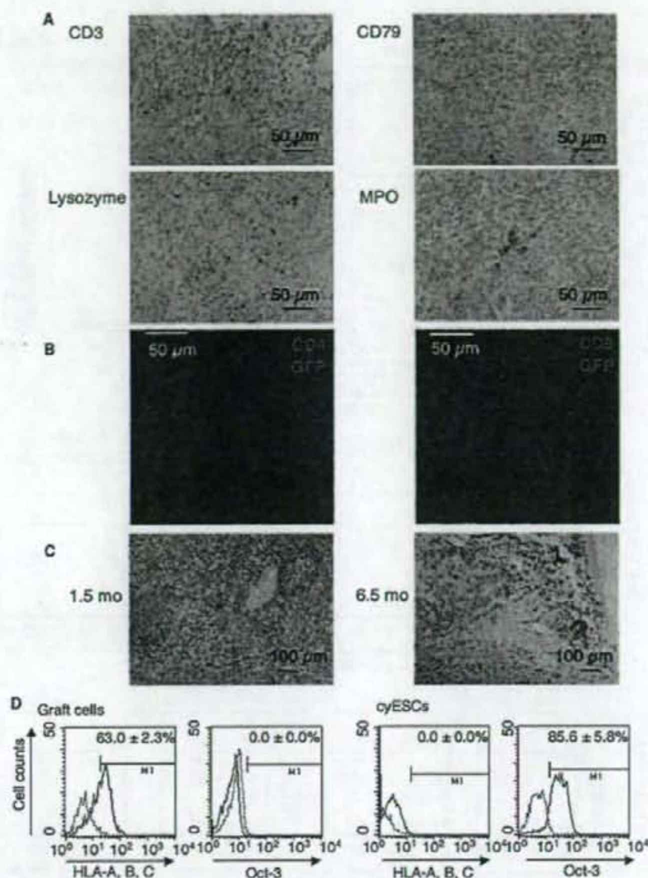

FIG. 4. Cynomolgus grafts in sheep after birth. (**A**) Immunostaining shows infiltration by T cells (positive for CD3), B cells (positive for CD79), macrophages (positive for lysozyme), and neutrophils (positive for MPO) in the cynomolgus-derived grafts in sheep after birth. (**B**) Most of the T cells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

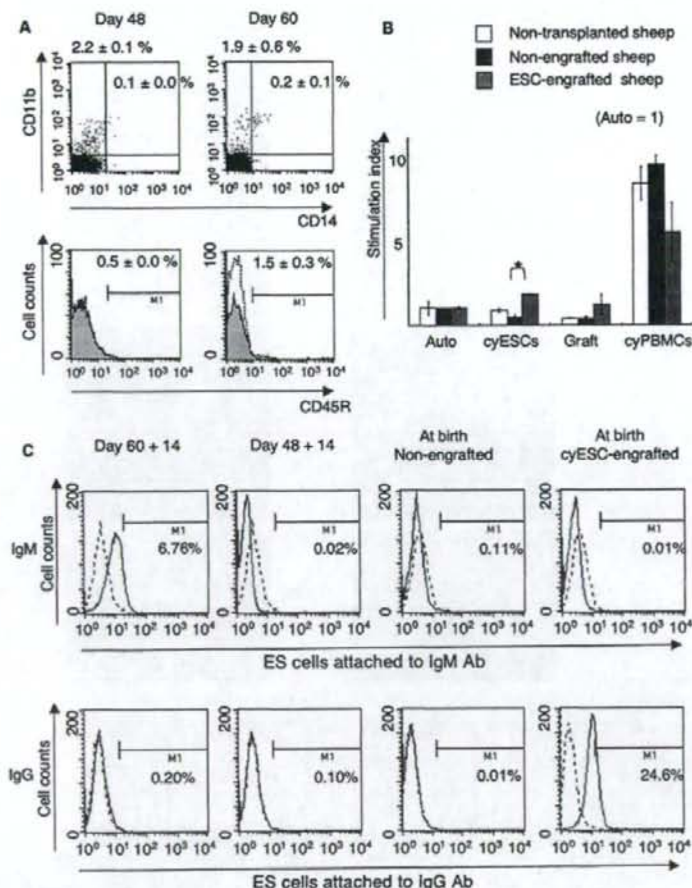

FIG. 5. Host immune responses. **(A)** Cell-surface antigens of fetal sheep peripheral blood leukocytes are compared between 48 days and 60 days of gestation. CD11b and CD14 double staining of the $CD5^-$ cells are shown (*upper*). There were no statistical differences in the percentages of NK cells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

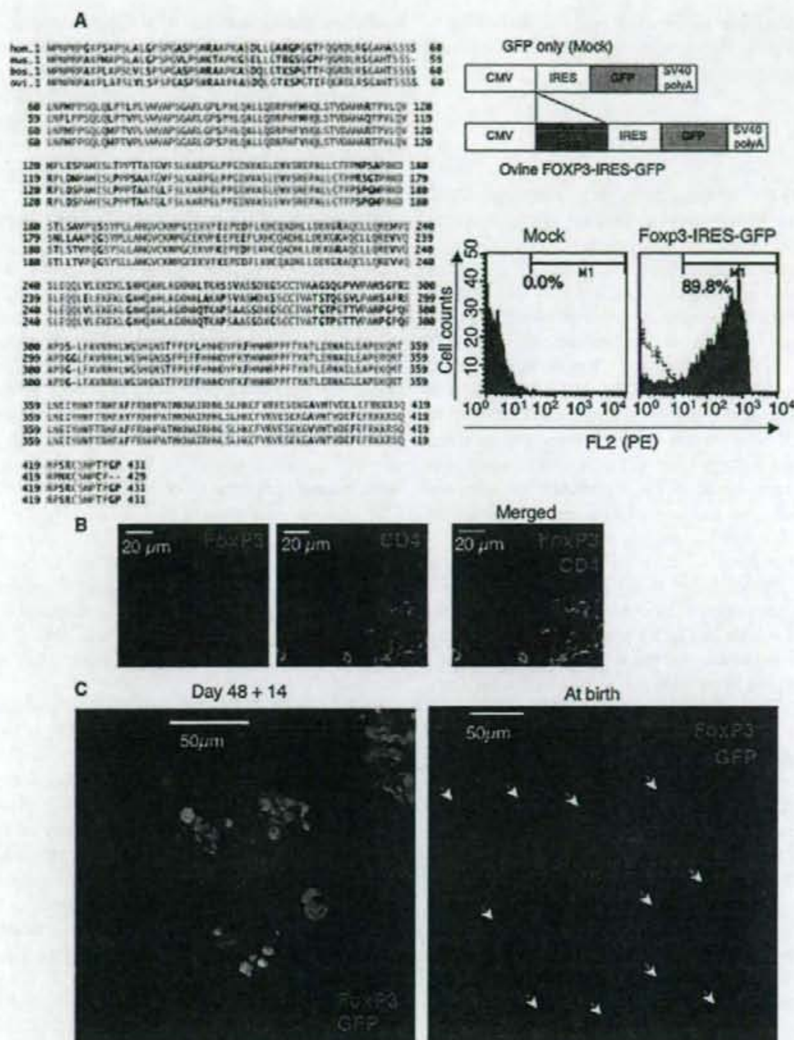

FIG. 6. Detection of Foxp3^{+} T_{reg} cells. (**A**) Amino acid sequences of Foxp3 in four mammals. Foxp3 of sheep (ovi) is compared with that of human (hom), mouse (mus), and cattle (bos) (GenBank accession numbers; NM014009, NM054039, and DQ322170, respectively). Conserved amino acids are indicated 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.

ACKNOWLEDGMENTS

We thank Norio Nakatsuji (Kyoto University, Kyoto, Japan) and Yasushi Kondo (Tanabe Seiyaku Co., Ltd., Osaka, Japan) for providing cyES cells. We acknowledge Chieko Ohno for technical assistance. This study was supported by grants (JMS 21st Century COE Program, High-tech Research Center Program, and KAKENHI) from the Ministry of Education, Culture, Sports, Science and Technology of Japan as well as grants (KAKENHI) from the Ministry of Health, Labor and Welfare of Japan.

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Received for publication July 6, 2007; accepted after revision November 19, 2007.

サルを用いた幹細胞研究

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臨 床 血 液　第49巻第4号 別刷

(2008年4月)

総 説

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Key words : Non-human primates, Large animals, Monkeys, Stem cells, ES cells, iPS cells

1. はじめに

1990年，アメリカでいわゆる「アニッサの事例」が起った。白血病の少女アニッサを助けるためには骨髄移植しかなかった。しかし適合ドナーが見つからない中，両親はもう一人子供を作る決心をした。その子の骨髄をアニッサに移植しようと。HLAが適合する4分の1の可能性に両親は賭けた。1990年妹マリッサが誕生した。幸いHLAが適合し，翌年妹マリッサからアニッサへの移植手術が行われ無事成功した。現在，姉妹とも健在だそうである。

さて，20XX年，アニッサはどのような治療を受けることになるか予想しよう（Fig. 1)。白血病に冒されたアニッサを助けるには組織が適合する骨髄移植しかないが，適合ドナーはやはり見つからなかった。しかし，両親はもはやもう一人子どもを作る必要はない。ES細胞治療が可能になったからである。まずアニッサの皮膚細胞を取り，その核を提供された卵子に移植する（体細胞核移植)[1)]。そこからできる胚盤胞からES細胞を取り出す。適当な方法によってES細胞を造血幹細胞に分化させ，それを移植する。あるいは，山中教授の技術を使って，皮膚細胞から人工ES細胞（iPS細胞）を誘導すれば，一気にES細胞までバイパスできてしまう[2~4)]。いずれの方法でも，自分の細胞から出来た造血幹細胞だから完全に適合する。アニッサは元気になって退院する。

外側の輪（Fig. 1）を使ったマウスの治療モデル（重症複合型免疫不全症，SCID）は2002年4月Cell誌に発表された[5)]。バイパス路のiPS細胞を利用するマウスモデル（鎌状赤血球貧血症）は2007年12月Science誌に発表された[6)]。いずれも病気の遺伝子に対して，ES細胞またはiPS細胞の段階で遺伝子治療（相同組換えによる遺伝子修復）を行なっている。この二つの報告は，アメリカのJaenischらのグループによる発表である。幹細胞治療に関して確かに目指すべきゴールは見えてきた。臨床応用もすぐそこまで来ているように考えられがちだが，本当にそうだろうか？ 本稿では，私どもの研究成果をもとに，臨床応用にあたっての問題点をみていきたい。

2. 造血幹細胞遺伝子治療の研究を振り返る

私は1995年アメリカの国立保健衛生研究所（NIH）に留学して以来，造血幹細胞遺伝子治療の研究に従事してきた。造血幹細胞遺伝子治療とiPS細胞治療は，似たところがある。どちらも幹細胞を利用した治療であり，どちらもレトロウイルスベクターで遺伝子を導入した細胞を体内に移植する治療である。したがって，造血幹細胞遺伝子治療の研究は，将来のiPS細胞治療（またはES細胞遺伝子治療）を考える上で参考になると思われる。

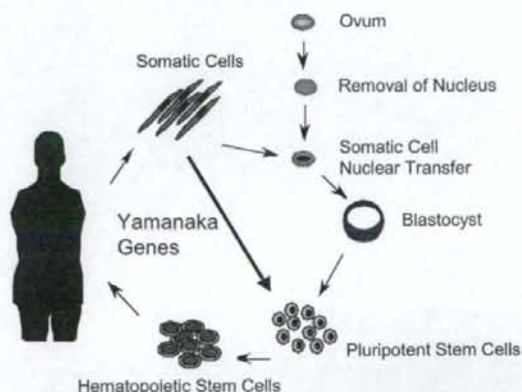

Fig. 1 Stem cell therapy in the near future.
Patient skin cells will be converted to pluripotent stem cells by somatic cell nuclear transfer[1)] or by four (or three) factors defined by Yamanaka[2,3)]. The pluripotent stem cells may be induced to differentiate into hematopoietic stem cells, and the cells can be used for therapeutic transplantation back to the patient.

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Table 1 Difference between mice and humans implicated from the study on hematopoietic stem cell gene therapy.
○ means good results. × means not so good results.

Disease	ADA Deficiency	Gaucher Disease	Fanconi Anemia	Chronic Granulomatous Disease	X-linked Severe Combined Immuno-deficiency
Mice (1980s)	○[7]	○[8]	○[9]	○[10]	○[11]
Humans (1990s)	×[12]	×[13]	×[14]	×[15]	–
1995	Orkin-Motulsky Panel Calls for Basic Research and Large Animal Study				
Humans (2000s)	○[16] Italy			○[17] Germany	○[18] France Excellent efficacy but caused leukemia[19]

この研究を振り返ってみたい（Table 1）。

1980年代，造血幹細胞を使ってマウスの血液疾患を遺伝子治療するという研究が行われた[7~11]。これらは大いに成功したため，気をよくしたアメリカは90年代に入ると早速ヒトに応用した[12~15]。ところが，その結果は芳しくなかった。業を煮やしたNIHは，1995年に「基礎研究重視・サル研究推進」を唱えるレポートを出した。

ちょうどこの時期にNIHに行った私は，この勧告に従い，サルを使った研究を始めた。そして，マウスの技術をヒト向けに改良することは，まったく別の技術を作ることだと知った。多くの研究者が地道な基礎研究を重ねた結果，2000年代になってようやく，いくつかのヒト疾患で治療成功例が出始めた[16~18]。なかでもX染色体性重症複合型免疫不全症（X-SCID）の治療成績は抜群で，骨髄移植以外，なすすべのなかった致死性の疾患から患者さんを救った。ところが，治療を受けた患者さんの半数近くに白血病という重い副作用が出た[19]。これはマウスの実験では確認されていない副作用だったので，研究者たちは驚いた。

なぜマウスでは白血病を確認できなかったのか？ 実はマウスが一生（2年間）かけて作る数の赤血球を，ヒトはたった1日で作っている[20]。これでは，たとえ100匹のマウスを一生追跡しても，ヒトの100日分にしかならない。これが，大型動物でないと腫瘍を検出しづらい理由の1つと思われる。以上，私自身が行なってきた造血幹細胞遺伝子治療の研究から学んだことは，ヒトにはマウスと異なる大型動物独特の生物学があるので，サル等の大型動物を用いた研究が重要であるということだ。

3. ES細胞治療のサルを用いた模擬実験

私どもは，マウスで成功した，前述のES細胞またはiPS細胞による個体造血の再生実験をサルで実施することを試みた。しかし，サルのiPS細胞はまだないし，サルES細胞を普通のサルに移植したのでは免疫拒絶されてしまい，実験にならない。免疫不全マウスのような免疫不全動物は，サルでは存在しない。そこで，サル胎仔への移植実験を考えた（Fig. 2）。胎仔（とくにfirst trimester）は，胸腺がないから免疫学的に未成熟であり，ヌードマウスに相当するといえる。たとえ異種の細胞を移植しても拒絶されることなく生着する[21]。胎仔への移植は，別のメリットもある。胎仔は日に日に大きくなるため，特別な前処置をしなくても，生着のためのスペースが自然に生まれ，前処置を特段必要としないことや，子宮内は無菌環境だから移植後の無菌管理が不要と言ったメリットである[22,23]。

未分化のままのサルES細胞を成体サルに移植してもそもそも生着しないし腫瘍も作らないが，サル胎仔に移植すれば期待通り奇形腫を作る[24,25]。重要なのは，腫瘍形成は注射針軌跡上の腹腔および胸腔に限られ，実質臓器に腫瘍はできないことである。すなわち，漏れた細胞が腫瘍を作る可能性が高い。漏れずに移植する技術が腫瘍形成予防のために実は重要である。

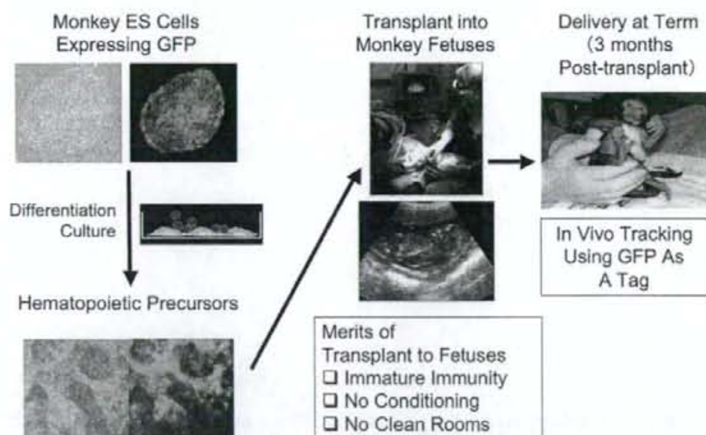

Fig. 2 Procedures for transplanting monkey ES cells into fetal monkeys.

興味深いのは，移植を受けたサル胎仔の各組織に ES 由来細胞のいわゆる「生着」が見られたことである[24]。前述の通り実質臓器には腫瘍は出来ないが，どの組織にも約 1% の移植由来細胞を認めた。しかも，周囲の細胞と同じ形態を示していたことから，移植した ES 細胞は，生着の場に応じて分化したか，または既存細胞と融合した結果，周囲と同じ表現型を得たものと考えられる。胎仔がレシピエントの場合，未分化 ES 細胞は本来，各組織へ生着しうるが，それが漏れた場合は奇形腫を作るらしい。こうしたことは，小型のマウスを使った実験からは知られていなかったことである。

次に，サル ES 細胞を試験管内で前造血細胞へ分化させてからサルの胎仔へ移植し，生まれたサルの体内で移植細胞の運命を調べた[25]。結果は，期待通り造血系を一部再構築できたものの，移植由来キメラ率は 4～5% と，マウスの成功例に比べるとそれほど高くなかった。今後は，マウスで報告された ES/iPS 細胞による造血再生技術を，霊長類向けに改良していく必要がある。

さらに問題なのは，全例で奇形腫が見られたことで，腫瘍形成リスクは高いと言わざるを得ない。分化培養後の細胞を移植したのに，なぜ腫瘍を形成したのか？ 実は，サル ES 細胞を 1 週間近く分化培養しても，40% ほどの細胞が未分化のままであることがわかった。残存した未分化細胞が腫瘍形成の原因の 1 つであると考えられた。したがって，腫瘍形成の予防のためには，未分化細胞の除去が鍵である。霊長類 ES 細胞の未分化表面マーカーである SSEA-4 の陽性細胞を除去してから移植すると，移植後の造血再生を損なうことなく，腫瘍形成は全く認められなかった。SSEA-4 は，臨床的なステムネス・マーカーといってよい。（マウス ES 細胞では SSEA-1 陽性だが，霊長類 ES 細胞では SSEA-4 陽性である。）

さて，造血系に分化させた場合ではなく，神経系に分化させた場合の結果はどうか。サルのパーキンソン病モデルを作製して，その線条体にサル ES 由来神経幹細胞を移植すると，1～2 ヶ月後にはドーパミン産生ニューロンの生着が確認できる。しかし，移植 5 ヶ月後には，腫瘍の形成が見られた。腫瘍は，やはり注射針の軌跡上に出来た。この腫瘍形成は，SSEA-4 陽性細胞を除去してから移植することで予防できた[26]。未分化マーカー SSEA-4 を用いるネガティブ・セレクション法は，ES 細胞を用いた移植・再生医療の安全性の向上のために普遍的な応用が期待できる。

ところで，サルで全例腫瘍を形成した同じ前造血細胞を，こんどは免疫不全マウスやヒツジ胎仔に移植するとどうなるか？ Table 2 に示した通り，これらの動物を用いた場合は，腫瘍形成が少ないことから，こうした異種移植実験では腫瘍形成リスクを過少評価する可能性がある。安全性の評価には，サルの同種移植実験が必要である。

4. ヒツジの利用

サル胎仔への移植実験は費用と手間がかかる。他の動物への移植も考えてみたい。マウスを使った実験結果は，そのサイズの故か，ヒトまで外挿できないことが多い。ヒトは単にマウスを大きくしただけではないのである。大型動物を用いる実験が必要な所以である[27]。

大型動物を用いる実験として，イヌがよく用いられてきた。アメリカなどではイヌの骨髄移植がペット医療として行われている。イヌのペットとしての長い歴史から，手法や試薬の多様性はヒトのそれに通じるものがあり，

Table 2 Formation of tumors post-transplant depending on recipients.

Cells	Fetal Monkeys	Immunodeficient Mice (NOD/SCID)	Fetal Sheep
Undifferentiated Monkey ES Cells	3/3	5/5	4/15
Monkey ES Cell-Derived Hematopoietic Precursors	3/3	2/5	1/10

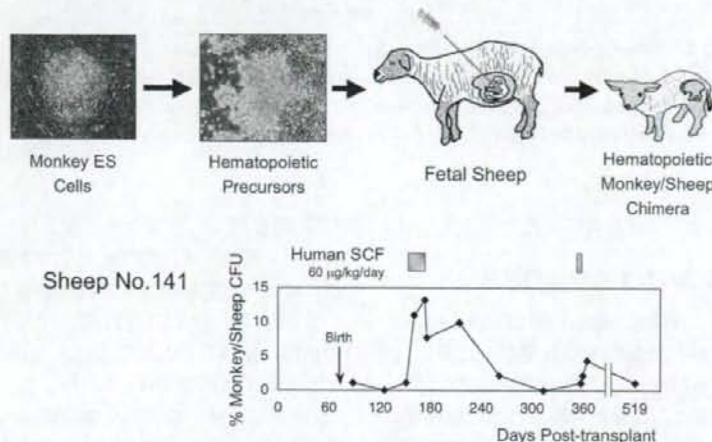

Fig. 3 In utero transplantation of monkey ES-derived cells into fetal sheep.
Monkey ES cells were induced to differentiate to hematopoietic precursors. The cells were transplanted into fetal sheep. After birth, marrow cells (colony-forming unit, CFU) were examined for monkey versus sheep hematopoiesis. Some lambs were administered with human stem cell factor (SCF) to selectively stimulate monkey hematopoiesis.

実験を行う上で有利である。ヒトの骨髄移植でノーベル賞を受賞した Thomas らのグループは，もともとイヌを用いた実験から骨髄移植の基礎を築いた。

しかし，譲渡犬の実験使用は禁止される傾向にあり，コロニー化されたビーグル犬が今後の実験使用の中心になる。血友病や筋ジストロフィーのコロニーも存在するので，特定の実験目的にはたいへん都合がよい[28, 29]。しかし，その場合の手間と費用はサルの場合とあまり変わらないことから，サルの代りの低コスト実験というわけにはいかない。

そういうこともあって，ES 細胞を移植するという名誉に与った大型動物は，サルの他は，今のところイヌではなく，ブタとヒツジである[30~34]。移植部位は，子宮内胎仔または虚血心筋が報告されている。私どもは，ヒツジ胎仔への子宮内移植を行なっている。ヒトとヒツジはよほど相性がいいらしく，ヒツジ胎仔へのヒト細胞移植実験は比較的古くから行なわれている。

なぜヒツジか？　そもそもヒツジ胎仔への幹細胞移植実験は，アメリカの Zanjani と Flake らによって始められた。彼らは，1990 年代，ヒト造血幹細胞をヒツジ胎仔に移植して，ヒトの血液をもつヒツジの作製に成功した[35]。ヒト造血幹細胞をさまざまな動物胎仔に移植して造血キメラを作ると，ヒトキメラ率には種差があって，ヒト－ヒツジ間で特に高い。しかもヒツジは流産率が低い。

私どもは，サル ES 細胞をヒツジ胎仔に移植する実験を行ない，肉眼的なサル／ヒツジキメラの作出に成功している[33]。また，サル ES 細胞を前造血細胞に分化培養後，ヒツジ胎仔に移植する実験を行ない，生後，約 1% のサル／ヒツジ造血キメラの作出に成功した (Fig. 3)[34]。ヒツジ胎仔への移植実験は，大型動物を用いた，幹細胞

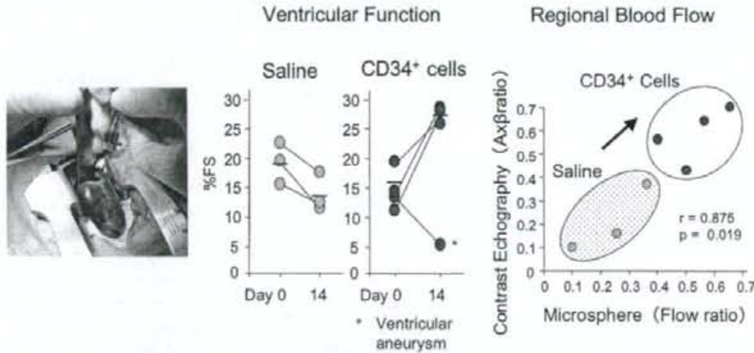

Fig. 4 Hematopoietic stem cells work well for myocardial infarction.
In a monkey myocardial infarction model, autologous CD34⁺ cells were transplanted to the infarcted heart. Significantly increased ventricular function and regional blood flow was observed. The regional blood flow was assessed by two independent methods.

の *in vivo* アッセイ系としてきわめて有用である。

5. 心筋梗塞に対する幹細胞治療

2001年，マウスの心筋梗塞に造血幹細胞を局所に移植した結果，移植細胞が内皮細胞や心筋細胞へと分化し，心機能の改善が認められたという報告があった[36]。さっそく世界各地で，患者さん自身の骨髄細胞を心筋梗塞の局所に移植する治療が行われ，大きな副作用もなく治療効果があがった[37~42]。

私たちは，いきなり患者さんに試す前にサルで試し，この治療がほんとうに効くことを確かめた（Fig. 4）[43]。しかし問題は，どうして効くのかである。多くの人は，それは移植した幹細胞が内皮細胞や心筋細胞に分化したからだと考えた。しかし私たちが調べてみたところ，移植細胞からこれらの細胞はできていなかった。実は移植した細胞（CD34⁺細胞）は，さまざまなサイトカインを分泌しており，これが血管新生を促しているらしいことがわかった。心筋梗塞の幹細胞治療は確かに効くが，移植した細胞は「幹細胞」として働くのではなく「サイトカイン工場」として働いていたのである。

6. 結　　語

私どもは，大型動物（サル・ヒツジ）を用いた前臨床研究を体系的に実施できる数少ない研究チームであり，真にトランスレーショナルな研究を推進してきた。本稿で述べた，最近の成果は，以下のようにまとめられる。

1）ES細胞の移植によってサルの造血を一部（2~5%）再構築できたが，腫瘍形成の危険性は非常に高い。しかしそれは，未分化マーカーSSEA-4を用いるネガティブ・セレクションによって予防可能である。

2）免疫不全マウスやヒツジ胎仔を用いる異種移植実験では，腫瘍形成の危険性を過少評価する。安全性の評価には，サルの同種移植実験が必要である。

3）サルES細胞を利用して，サルの血液をもつヒツジの作出に成功した。ヒツジは，幹細胞の *in vivo* アッセイ系として有用である。

4）心筋梗塞の幹細胞治療は確かに効くことをサルで示した。しかし移植細胞は，内皮や心筋に分化する「幹細胞」として働くのではなく，「サイトカイン工場」として働いていた。

ヒトは決してマウスを大きくしただけではない。今後も，サルやヒツジを用いて，マウスからヒトへの真の「橋渡し」研究を実施し，幹細胞治療の有効性と安全性をしっかり検証しながら，臨床応用につなげるための基盤技術を提供していきたい。

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ヒツジを用いたサル組織産生法

A Method for Generating Monkey Tissues Using Sheep

花園　豊*

移植医療においてドナーが絶対的に不足し，それが移植治療普及の足かせになっている。そこで，家畜動物にヒトの組織を作らせたらどうかと考えた。すなわち，ヒトの組織をもつキメラ家畜である。それに向けて筆者らは今回，サルES細胞をヒツジ胎仔に移植する実験を行い，肉眼的なサル/ヒツジキメラの作出に成功した。

1. はじめに

移植医療においてドナーが絶対的に不足し，それが移植治療普及の足かせになっているのは周知の事実である。そこで，家畜動物にヒトの組織を作らせたらどうかと考えた。すなわち，ヒトの組織をもつキメラ家畜である。筆者らは，ヒツジの体内で，ヒトES細胞を分化させてヒトの組織を作らせることを検討することにした。しかし，ヒトES細胞使用は制約が厳しいので，まずサルES細胞を使ってみた。

なぜヒツジなのか？　そもそもマウスでは小さすぎる。ヒツジは流産率が低いので，動物胎仔を利用する研究によく利用されてきた。しかし，将来の実用化を考えると，ヒトとの解剖学的類似性，繁殖面（多産・発育の早さ），無菌化の点で，ブタの方がヒツジより有利かもしれない。もっとも，宗教上の理由から世界人口の3分の1はブタを食べられないから，ヒツジで実用化をねらう価値は十分ありそうだと考えた。

2. キメラを作る

キメラはギリシャ神話に登場する動物である。それは，テュポンとエキドナの娘で，ライオンの頭，ヤギの胴体，ヘビの尻尾を持つという（図1）。リュキアに住み，カリア王アミソダレスに育てられたが，ペガソスに乗る英雄ベレロポンに退治された。しかし，なにも外国の神話を持ち出さなくても，日本にもこの種の伝説はある。それは，鵺（ヌエ）と言って，「平家物語」に登場し，サルの顔，タヌキの胴体，トラの手足を持ち，尾はヘビで，「ヒョーヒョー」という鳥のトラツグミの声に似た大変に気味の悪い声で鳴いた，とされる（図1）。鳴き声までキメラであるところはギリシャ神話よりリアルと言えよう。

一方，伝説ではなくて，実在するキメラ（またはヌエ）として，以下の三者が知られている。

①接ぎ木によるキメラ植物
②初期胚間移植によるウズラとニワトリのキメラ
③ES細胞を利用したキメラマウス

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図1　伝説上の動物：キメラとヌエ
1つの動物に2つ以上の異種動物の組織が組み合わさっている。

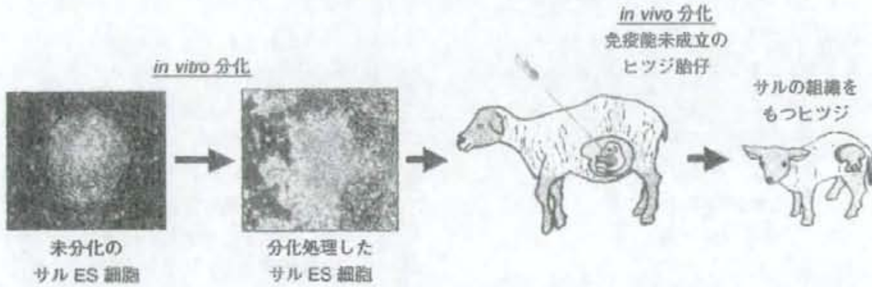

図2　まずサル ES 細胞を試験管内で適宜分化させる。その細胞を，ヒツジ胎仔に移植する。胎仔の体内で移植細胞の増殖・分化がうまく進めば，サルの組織をもったヒツジが生まれるというスキームである。ヒツジ胎仔は免疫系が未熟で，移植細胞が拒絶されないというのが前提である

今回の筆者らのキメラ作製法は，②と③の折衷になるだろう。図2にその方法を示す。まずサル ES 細胞を試験管内で適宜分化させる。その細胞を，ヒツジ胎仔に移植する。胎仔の体内環境の中で，移植細胞の増殖，分化，成熟を進ませる。うまくいけば，サルの組織をもったヒツジが生まれるというスキームである。ヒツジ胎仔はまだ免疫系が未成立で，移植したサルの細胞が拒絶されないというのが前提にある。今回はサル ES 細胞を使ったが，ヒト ES 細胞を利用すれば，ヒツジにヒトの組織を作らせることができるかもしれない。そうなればいろいろな医療応用が考えられるだろう。

しかし，本当にこんなことが可能なのか，まず未分化のサル ES 細胞を移植して，生着するかどうかを調べた[1]。全部で 15 頭 36 か所に未分化の