リカではヒト胚研究をめぐる論議が再燃した。

2001年8月9日，ジョージ・ブッシュ大統領はNIH「ヒト多能性幹細胞を使用する研究に関するNIHガイドライン（National Institutes of Health Guidelines for Research Using Human Pluripotent Stem Cells）」が初めてヒトES細胞研究に助成金を提供することを発表した。ただし，対象となる研究は8月9日以前に作られた受精胚由来の細胞株を使用するものに限定された。ヒトES細胞を樹立することと，すでに樹立されているヒトES細胞を利用して研究することを区別し，樹立されているヒトES細胞の研究利用のみ連邦政府の研究助成を認めるかたちとなっている。従って，NIHにリストされているヒトES細胞株は，すべて2001年8月までに樹立されたものである。最近でもハーバード大学が民間資金により17種類のヒトES細胞を作ることに成功したという報道があり，わが国（京都大学）で樹立された3株など，それ以降に世界で樹立された細胞株を考慮に入れると，すでにかなりの数のヒトES細胞株が存在するようである。

3. わが国の状況

わが国では，2001年9月に文部科学省から「ヒトES細胞の樹立及び使用に関する指針」が公布・施行された。この指針の策定は，内閣総理大臣の諮問機関である科学技術会議生命倫理委員会と1999年に発足した同ヒト胚小委員会によってまとめられた「ヒト胚性幹細胞を中心としたヒト胚研究に関する基本的考え方」（2000年3月）に従い，パブリック・コメントを経て指針案が作成され，総合科学技術会議およびその下に設置されている生命倫理専門調査会における検討を踏まえたものである。これによって，不妊治療の過程で余剰となったヒト初期胚（凍結胚）のうち夫婦から研究利用へ無償による提供についての同意が得られた場合に限ってのみES細胞を樹立することが認められることになった。すでに樹立されているヒトES細胞を使用する研究だけでなく，樹立研究そのものを認めるという点においては，NIHガイドラインとは異なっている。

指針では，ヒトES細胞の使用に関して，研究目的は当面医療の発展等につながる基礎研究に限定し，個体産生は勿論のことES細胞から再生した組織や細胞を人体へ移植するなどの研究は認められていない。併せて，医療に用いるための医薬品の製造や，医薬品の安全性試験等に用いるためのヒトES細胞の大量供給など医療関連分野への使用も現時点では行わないこととしている。

研究の実施開始の手続きや進め方にも厳しい基準が設けられ，受精卵の提供やES細胞の作製・利用にかかわるすべての機関に倫理審査委員会の設置を義務づけ，個別の研究について研究の妥当性を綿密に作成された計画をもとに研究機関内倫理審査委員会と国の二重の審議を受けることが決められている（図1）。研究成果は原則として公開，指針に反した場合，研究機関名や内容などが公表されることになっている。

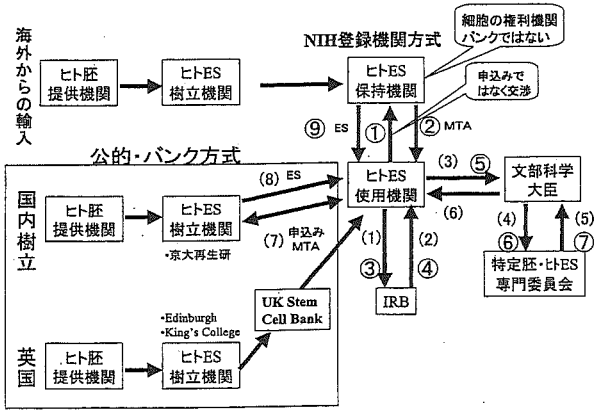

図1　ヒトES細胞使用までの手続き

表2　大臣確認されたヒトES細胞の使用計画

使用計画	使用機関	大臣確認日	細胞の由来
血管発生・分化機構の解析と血管再生への応用	京都大学	2002年 4月	Monash大
血管発生・分化機構の解析と血管再生への応用	田辺製薬	2002年 6月	Monash大
中枢神経系の再生医学の基礎的研究	慶応義塾大学	2002年 6月	Monash大，WiCell
造血細胞への分化誘導法の開発	東大医科研	2002年12月	WiCell
ES細胞由来造血幹細胞による造血の再生	東京大学	2002年12月	WiCell
維持と分化に関する研究	信州大学	2002年12月	WiCell
血液細胞の分化誘導系の確立	岐阜大学	2003年 4月	Monash大
心筋細胞の再生医学の研究	岐阜大学	2004年 8月	Monash大
パーキンソン病モデルサルにおける移植効果及び安全性評価	田辺製薬	2004年 3月	Cellartis
パーキンソン病モデルサルにおける移植効果及び安全性評価	自治医科大学	2004年 3月	Cellartis
神経細胞，感覚系細胞への分化誘導	理化学研究所	2004年 3月	京大再生研
脂肪細胞，中胚葉系幹細胞への分化誘導	理化学研究所	2004年 3月	京大再生研
ヒトES細胞の維持培養を可能にするシグナル因子の研究	理化学研究所	2004年 3月	京大再生研
安全かつ簡便な新規培養技術の開発研究	京大再生研	2004年 7月	京大再生研
遺伝子導入法の開発と遺伝子改変技術の確立	京大再生研	2004年 7月	京大再生研

この指針を受けて，文部科学省は2002年4月，京都大学が申請していたヒトES細胞の樹立計画を承認したのに続き，同大学が申請していたヒトES細胞から血管の内皮細胞や壁細胞に分化できる細胞を取り出し血管を再生させる使用研究を承認した。今までに文部科学大臣の確認が得られている使用計画は15件で，内10件は，アメリカ，オーストラリア，スウェーデンなどから輸入された細胞を用いるものである（表2）。国内で唯一ヒトES細胞の樹立計画の政府の承認が得られている京都大学再生医科学研究所では，2003年3株の樹立に成功し，2004年3月より分配を始めた。すでに5件の国産ヒトES細胞を用いた研究が進められており，国産ヒトES細胞を用いた研究は今後一層盛んに行われるようになるものと思われる。使用計画の研究対象は，神経系に関するものが4件と一番多く，血液（3件），血管（3件），心筋（1件）などの研究がつづいている。指針は施行から3年が経過し，現在，特定胚およびヒトES細胞研究専門委員会によって指針の見直し作業が行われている。

4. 医療への応用

受精卵では完全な条件を備えた子宮という理想的な“培養器”の中で，細胞の増殖と分化が統制されて進み，三次元的な形態形成が行われる。ES細胞では分化増殖を試験管内で行う場合，三次元的形態形成のメカニズムが未解明な上，分化誘導に関わる諸条件が完全にはわかっていないため，細胞から直接立体的な組織を作ることはできない。また肝臓や腎臓などほとんどの臓器は複数の種類の細胞から出来上がっており，これらを再構成させることや，臓器に血管を導入することは非常に難しく，今後さまざまな検討が必要である。

臓器そのものを作ることは無理でも，ES細胞から生体と同じ機能を持った細胞を作ったり，作った細胞を病気の治療に活用することは可能である。ヒトの体は多種類の細胞からできているが，病気の中には特定の細胞の変性や機能不全で引き起こされるものも多い。例えば，脳の一部の神経細胞

が変性脱落してしまうためにおこるパーキンソン病や，脊髄の中の神経線維が事故などで断ち切られた脊髄損傷，心臓の心筋細胞の壊死を引き起こす心筋梗塞，膵β細胞の機能低下に基づく糖尿病などである。これらの患者に正常細胞を移植することにより病気を治そうとするのが細胞移植（補充）療法である。ES細胞から作り出した神経細胞をパーキンソン病患者や脊髄損傷患者に移植したり，インシュリンを分泌する膵臓のランゲルハンス島細胞を作り出して糖尿病患者の治療に利用するといったことも現実的に臨床応用を視野に入れ想定されている。

米ジェロン社は，ヒトのES細胞から作ったオリゴデンドロサイトを脊髄損傷の患者に移植して治療する臨床試験を2005年より始める計画を明らかにしている。同社ではすでに8種類の細胞をヒトES細胞から作り出すのに成功し，このうち神経細胞（パーキンソン病），心筋細胞（心疾患），骨細胞（骨粗鬆），β細胞（糖尿病），軟骨細胞（骨関節炎），血液細胞（血液疾患）の6種類は動物実験での効果を確認中という。

5. 臨床応用への課題

ヒトES細胞を再生医療に臨床応用する場合の課題は何であろうか？

移植医療で最も重要な問題となるのは，ヒトES細胞の分化によって生じた細胞に対して起きる可能性のある免疫拒絶反応である。脳内への移植など一部の例を除いて一般的には重要な課題になる。その解決策としては，主要組織抗原複合体（MHC）遺伝子の一部を破壊したES細胞株の作製や，さまざまなMHCのタイプの受精卵からES細胞を分離して集めるES細胞バンク構想が考えられる。これらは免疫拒絶反応を完全に抑えることは不可能としても，免疫抑制剤と共用することによっての対処が可能になるであろう。

この他究極の拒絶反応回避技術として，患者の体細胞（たとえば皮膚細胞）の核を除核した受精卵に移植して，受精卵と同じように発生させたクローン胚からES細胞を作り出す方法が考えられている。クローン羊ドリーを作り出した体細胞核移植技術（クローン技術）の応用である。ウシでは体細胞の核を別のウシの除核未受精卵に移植してクローン胚をつくり，それをもとに心筋細胞や腎臓の細胞などを作った後，これらを体細胞を取り出した元のウシに移植した場合，拒絶反応が起こらないことが確認されている[7]。また，マウスではすでに核移植卵からES細胞の作製に成功しており，作製されたES細胞が正常発生する能力をもつことが示されている[8]。

ヒトクローン胚研究は，2001年，米国アドバンスト・セル・テクノロジー社が行ったのが最初の報告で，作製したクローン胚は6細胞期の段階で止まっており，ES細胞樹立のために必要な胚盤胞期胚まで発生が進まなかった。しかし彼らは，第2減数分裂を終えた未受精卵をサイトカラシンB処理とカルシウムイオノフォアを用いて単為発生させることにより，胚盤胞まで発生が進むことが明らかにしている[9]。2004年2月には，韓国ソウル大学などの研究チームが，ヒトクローン胚からヒトES細胞を作製することに成功した[10]。彼らは同意を得た16人の女性から242個の卵母細胞の提供を受け，このうち176個の卵母細胞の核を取り除いて卵母細胞と同じ人の体細胞を移植し，クローン胚を作製した。このうち30個が子宮に着床できる胚盤胞期胚と呼ばれる段階まで分裂が進み，さらにその中の20個の胚盤胞期胚から最終的に1株のES細胞を作製することに成功している。

ヒトクローン胚の作製は，韓国では2003年末にクローン人間づくりを禁止する生命倫理法が成立したものの，医療研究目的に限ってヒトクローン胚の作製が認められている。ドイツではヒトクローン胚の作製は禁止，アメリカでも政府予算を使った研究は禁止されている一方，今まで全面禁止していたフランス議会が2004年7月，ヒトクローン胚の研究を5年間の期間限定で容認する生命倫理法を採択した。わが国でもヒトクローン胚研究については，それまで「ヒトに関するクローン技術等の規制に関する法律（2001年6月施行）に基づく「特定胚の取り扱いに関する指針」によって禁じられていたが，2004年7月政府の総合科学技術会議は，再生医療などへの応用が期待されるヒトクローン胚について，基礎研究の解禁を決定した。イギリス政府は2004年8月に国立ニューキャッスル大学が申請していた人のクローン胚からES細胞を作る研究を承認したと発表した。イギリスは2001年に世界で初めて人のクローン胚を作成する研究を容認していたが，許可を与えたのはこれが初めてである。このようにヒトクローン研究はソウル大学の成功以降，世界的に大きく動き出した感はあるが，成功率はまだまだ低く，研究には多くのヒト卵子を必要とすることや，胚を新規に作製することの懸念等，問題点は多い。

再生医療を現実のものとするためには，免疫拒絶問題以外にも，ヒトES細胞を思い通りに目的の細胞へ導くための方法が確立されなければならない。また，目的とする特定細胞だけを確実に分離する技術も重要となる。仮にES細胞が混じっていたりすると移植後の体内で奇形腫を形成したり，思わぬ細胞を作り出してしまう恐れがあるからである。また現状では，ヒトES細胞の培養にフィーダーとしてマウス胎児由来線維芽細胞が使用されていたり，培養液としてウシ血清が使用されていたりするケースがあるが，異種タンパク質や未知ウイルスの混入等を避ける意味でも，こうした動物製剤を使用しなくても行える培養系技術の開発といったことも安全性を確保する観点から必要な課題と考えられる。

さらに，ES細胞の実際の臨床応用を考えたときには，どの段階まで分化した細胞を患者の体内のどこにどれだけ移植すれば効果があるかや，どのような副作用があるかなど，有効性や安全性を検討する前臨床的試験が不可欠となる。通常このような場合，モデル動物を利用することになるが，ES細胞やモデル動物がマウスのものであったりした場合には，実験結果がヒトを十分反映しないこともしばしば指摘されており，このような場合，ヒトに近縁なサルおよびサルES細胞を用いた前臨床的検討が有用となる。サルのES細胞株は，Thomsonらの研究グループ以外には，オレゴン大学のWolfらが樹立したアカゲザルES細胞株[11]と筆者らの樹立したカニクイザルES細胞[5]の数株が報告されているのみであり，ヒトES細胞に比べるとサルES細胞を用いた研究報告例も少ない。しかし，サルES細胞のモデルサルへの移植は，同種間移植の評価系として，ヒトES細胞の臨床応用に貴重な情報をもたらすものと思われる。

6. ヒトES細胞研究のその他の用途

ここで，ヒトES細胞の利用について再生医療以外の用途についても少しだけ言及してみたい。

(1) 発生・分化機構や疾患原因解明のための基礎的研究

いうまでもなくヒトES細胞は，発生・分化の分子機構を解明する研究において直接的な情報を提供してくれる。ヒトの発生初期の未解明の現象が原因で新生児の先天性欠損や自然流産につながる胎盤異常が起きると考えられているが，ヒトES細胞を試験管内で研究することで，このような問題を引き起こす遺伝子・分子および細胞現象を解明し，これを防止する方法を見つけ出すことができるかもしれない。また，発生初期の染色体異常が及ぼす影響の解明にも利用できる。初期小児癌の多くが胎生期に原因があるとされているが，ES細胞の研究によりこの癌の発生過程を追跡するような研究も可能である。

(2) 創薬研究への活用

ヒトES細胞の分化によって得られる各種機能細胞を創薬研究開発に活用することも極めて実現性の高い利用法と考えられる。現状の創薬研究では，臨床試験に先立って，その有効性や安全性を評価する目的で，動物に薬剤を投与することによる生体内試験や，動物細胞を使用する生体外（試験管内）試験など数多くの前臨床試験が行われている。しかし，動物やその細胞を用いた試験では，ヒトの細胞に与える影響を予測することが困難な場合もある。ヒトES細胞に由来する機能細胞は真にヒト細胞そのものであり，生体内の細胞とよく似た反応を示す可能性が高く，薬効・薬理作用

や毒性など薬剤開発の評価系として，より安全で安価なモデルを提供できるようになるものと考えられている。

(3) 遺伝子工学上の新技術の開発

マウスES細胞ではすでに行われてきたことではあるが，ヒトES細胞において試験管内で遺伝子改変や遺伝子導入（特定の遺伝子発現やタンパク質発現）を行うことができるようになれば，生命科学の進展において有益な情報を提供し，遺伝子治療法をはじめとして医療に多大な貢献をもたらすであろう。

7. おわりに

ヒトES細胞は再生医療への応用のみならず，疾病原因の解明，創薬開発への利用等において多大な可能性を有することは確実と考えられるが，ヒトES細胞の研究はまだ緒についたばかりといっても過言ではなく，現状では重要な技術的課題が多く残されている。再生医療の実現に向けては発生生物学，分子生物学以外にも，遺伝子工学，組織工学などの幅広い分野における研究が併せて進められる必要がある。

また，ヒトES細胞は，生命の萌芽としてのヒト初期胚に人為的操作を加えることによって初めて得られるものであることから，生命倫理という観点でも大きな課題を有している。ヒト胚ならびにヒトES細胞に対する考え方は個々人の生命観により様々であり，あらゆる立場の人の意見を束ねることは難しいかもしれない。しかしながら，それを研究に使用すること自体が倫理的な観点ら極めて慎重に行うべき行為であることについては論を待たないであろう。研究者は研究を推進するにあたり，常に社会の声に耳を傾けながら，どのような研究を何の目的で行っているのかを社会に情報発信し，それについて社会全体が議論を重ねることが，再生医療を実現し，その恩恵を受けるために必要なことではないだろうか。

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Transplantation of neural cells derived from retinoic acid-treated cynomolgus monkey embryonic stem cells successfully improved motor function of hemiplegic mice with experimental brain injury

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Abstract

We induced neural cells by treating cynomolgus monkey embryonic stem (ES) cells with retinoic acid. The treated cells mainly expressed . IIItubulin. They further differentiated into neurons expressing neurofilament middle chain (NFM) in elongated axons. Half of the cells differentiated into Islet1+ motoneurons in vitro. The monkey ES-derived neural cells were transplanted to hemiplegic mice with experimental brain injury mimicking stroke. The neural cells that had grafted into periventricular area of the mice distributed extensively over the injured cortex. Some of the transplanted cells expressed the neural stem/progenitor marker nestin 2 days after transplantation. The cells expressed markers characteristic of mature motoneurons 28 days after transplantation. Mice with the neural cell graft gradually recovered motor function, whereas control animals remained hemiplegic. This is the first demonstration that neural cells derived from nonhuman primate ES cells have the ability to restore motor function in an animal model of brain injury.

Keywords: Motor neuron; Nonhuman primate; Embryonic stem cells; Hemiplegia; Differentiation

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31

In Vivo Tumor Formation From Primate Embryonic Stem Cells

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Summary

To achieve human embryonic stem (ES) cell-based transplantation therapies, allogeneic transplantation models of nonhuman primates would be particularly useful. In this chapter, we describe an example of this model. We prepared cynomolgus ES cells genetically marked with the green fluorescent protein. The cells were transplanted into the allogeneic fetus because the fetus is immunologically premature and does not induce immune responses to transplanted cells. In addition, fetal tissue compartments are rapidly expanding, presumably providing space for engraftment. At 3 mo posttransplantation, a fluorescent teratoma, obviously derived from transplanted ES cells, was found in the fetus. However, transplanted cell progeny were also detected (approx 1%) in multiple fetal tissues. The cells were solitary and indistinguishable from surrounding host cells as assessed by *in situ* polymerase chain reaction. Transplanted cynomolgus ES cells can engraft in allogeneic fetuses. The cells will, however, form a tumor if they "leak" into an improper space, such as the thoracic cavity.

Key Words: Allogeneic transplantation; genetic marking; green fluorescent protein; immunological tolerance; *in situ* PCR; *in utero* transplantation; primate embryonic stem cells; teratoma.

1. Introduction

Because human embryonic stem (ES) cell lines have dual abilities to proliferate indefinitely and differentiate into multiple tissue types *(1,2)*, human ES cell-based transplantation therapies are considered to hold a great potential in the treatment of a variety of diseases and injuries. To address the safety and efficacy of these therapies, allogeneic transplantation models of large animals, especially nonhuman primates, would be useful. However, it has been difficult to transplant primate ES cells or their derivatives into allogeneic hosts. There are two major reasons for this. First, the efficient and stable marking of primate ES cells has been difficult. It is necessary to distinguish transplanted allogeneic ES cell progeny from surrounding host cells. Second, the immune

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rejection of transplanted cells must be circumvented for a sustained engraftment. The cells would otherwise be cleared by immune responses.

We have previously reported highly efficient gene transfer into cynomolgus ES cells using a lentivirus vector derived from the simian immunodeficiency virus *(3)*. Lentiviral transgene expression in ES cells is stable, with minimal levels of transcriptional silencing *(4,5)*. In addition, cynomolgus ES cell sublines stably expressing green fluorescent protein (GFP) were established after electroporation of a GFP-expressing plasmid *(6)*. By using such cynomolgus ES cells genetically modified to express GFP, it is now possible to distinguish transplanted allogeneic ES cell progeny from surrounding host cells as GFP will serve as a good genetic tag.

The early gestational fetus is a good recipient with which to circumvent immune rejection because the immune system is premature *(7,8)*. Furthermore, in the animal fetus, "space" would be relatively available for engraftment as compared to the adult because of the rapid expansion of fetal tissue compartments. Thus, transplanted cells could engraft without conditioning of recipients, such as by irradiation or immunosuppressive treatment.

In this chapter, we show a method to transplant nonhuman primate (cynomolgus macaque) ES cells *(9)* into xenogeneic immunodeficient mice to form teratoma. In addition, we show methods to transplant nonhuman primate (cynomolgus macaque) ES cells stably expressing GFP *(3,6)* into the allogeneic fetus *in utero* and to examine the in vivo fate of transplanted cells using GFP as a genetic tag. At 3 mo after the allogeneic *in utero* transplantation, a fluorescent tumor, obviously derived from transplanted ES cells, was found in the thoracic or abdominal cavity. Notably, transplanted cell progeny were also detected (approx 1%) in multiple fetal tissues. The cells were solitary and indistinguishable from surrounding host cells as assessed by *in situ* polymerase chain reaction (PCR). Thus, transplanted cynomolgus ES cells can engraft in allogeneic fetuses. However, the cells will form a tumor if they "leak" into an improper space, such as the thoracic and abdominal cavities *(10)*.

2. Materials

2.1. Cells

1. Cynomolgus ES cells stably expressing GFP (*see* Chapters 20 and 21, this volume).
2. Mouse embryonic fibroblasts from CD-1 (also referred to as ICR) (Charles River, Wilmington, MA) or BALB/c mice (Charles River).

2.2. Teratoma Formation in Immunodeficient Mice

1. 6- to 8-wk-old non-obese diabetic/severe combined immunodeficient (NOD/SCID) mice (Jackson Laboratory, Bar Harbor, ME) (*see* **Note 1**).
2. Hanks' balanced salt solution (HBSS; Invitrogen, Carlsbad, CA; cat. no.14025-092).
3. Dulbecco's modified Eagle's medium/nutrient mixture F-12 1:1 mixture (DMEM/F12) (Invitrogen, cat. no. 11330-032).
4. ES cell-qualified fetal bovine serum (Invitrogen, cat. no. 10439-024).
5. 10,000 IU/mL penicillin-10,000 µg/mL streptomycin (100X; Invitrogen, cat. no. 15070-063).
6. 200 m*M* L-glutamine (100X; Invitrogen, cat. no. 25030-081).
7. 2-Mercaptoethanol (Sigma, St. Louis, MO; cat. no. M3148).

8. Culture medium for primate ES cells: DMEM/F12 containing 15% ES cell-qualified fetal bovine serum, 2 m*M* L-glutamine, 100 IU/mL penicillin-100 µg/mL streptomycin, and 0.1 m*M* 2-mercaptoethanol.
9. 0.25% trypsin in HBSS (2.5% trypsin 10X liquid; Invitrogen, cat. no. 15090-046).
10. 1% bovine serum albumin (BSA fraction V; Sigma, cat. no. A4503) in HBSS.

2.3. Teratoma Formation in Allogeneic Fetuses

1. Anesthetic and surgical facilities for primates (including ultrasound and inhalation anesthesia equipment) *(11)*.
2. A time-dated pregnant cynomolgus monkey of 50- to 70-d gestation (*see* **Note 2**) *(12)*.
3. Ketamine hydrochloride (Ketalar® 50; Sankyo, Tokyo, Japan).
4. Isoflurane (Forane®; Dainippon Pharmaceutical, Osaka, Japan).
5. A percutaneous transhepatic cholangiography (PTC) needle (22-gage, Sonoguide PTC needle type B; Hakko Medical, Nagano, Japan; cat. no. 22412210).
6. A 1-mL syringe (Terumo, Tokyo, Japan; cat. no. SS-01T) filled with graft cells (10^5–10^7 cells in 200–500 µL).
7. A 1-mL syringe (Terumo, cat. no. SS-01T) filled with normal saline (for flushing).

2.4. Sample Preparation

1. 4% paraformaldehyde (Wako, Osaka, Japan; cat. no. 169-18432) and 8% sucrose (Wako, cat. no. 192-00012) in phosphate-buffered saline (PBS; Invitrogen, cat. no. 10010-023).
2. OCT compound (Tissue Tek series; Sakura, Zoeterwoude, Netherlands; cat. no. 4583) containing 10% sucrose.

2.5. In Situ *PCR*

1. A PTC100 Peltier thermal cycler (MJ Research, Waltham, MA).
2. 20 µg/mL proteinase K (Sigma, cat. no. 39450-01-6) in PBS.
3. 0.1% Triton X-100 (Sigma, cat. no. T8787) in PBS.
4. A slide frame for *in situ* PCR (slide seal; Takara, Shiga, Japan; cat. no. 9066 [25 µL] or cat. no. 9067 [65 µL]).
5. 50 µL Digoxigenin dNTP labeling mix (Roche, Basel, Switzerland; cat. no. 1277065).
6. Rabbit anti-Digoxigenin polyclonal antibody, horseradish peroxidase labeled (Dako, Glostrup, Denmark; cat. no. P5104) diluted (1:100) in 2% BSA and 5% horse serum (Invitrogen, cat. no. 16050-130) in PBS.
7. A Vector SG substrate kit (Vector, Burlingame, CA; cat. no. SK-4700).
8. Kernechtrot solution (0.1% Kernechtrot in aluminum sulfate; Muto, Tokyo, Japan; cat. no. 4087).

3. Methods

3.1. Teratoma Formation in Immunodeficient Mice

1. Wash ES cells with HBSS twice and add 0.25% trypsin to the dish at 37°C for 3 min. Neutralize trypsin with ES culture medium and make a suspension of ES cell clumps.
2. Transfer the cell suspension into a 50-mL conical tube, centrifuge it at 140*g* for 4 min, and resuspend the pellet with 20 mL 1% BSA/HBSS.
3. Centrifuge the cell suspension again at 140*g* for 4 min and resuspend the pellet with an appropriate volume of 1% BSA/HBSS (10^6 cells in 150–200 µL per injection site).
4. Aspirate the ES cell suspension into a 1-mL syringe with a 23-gage needle and inject the suspension into NOD/SCID mice subcutaneously (*see* **Note 3**).

5. Resulting tumors will be palpable at 8–13 wk after the injection. Expose, observe, and excise tumors.
6. Fix tumor samples (5 × 5 × 3 mm) at 4°C for 4 h in 4% paraformaldehyde and 8% sucrose in PBS and embed the samples in paraffin for histological examination. To prepare fresh frozen samples, embed samples (5 × 5 × 3 mm) in OCT compound containing 10% sucrose, freeze them in liquid nitrogen, and store them at −80°C.

3.2. Teratoma Formation in Allogeneic Fetuses

3.2.1. Anesthesia

1. Prepare a pregnant monkey around the end of first trimester (50–70 d; full term 165 d) (*see* **Note 2**).
2. Give the monkey 10 mg/kg ketamine hydrochloride intramuscularly. Secure the monkey on a table and monitor maternal heart rate by electrocardiography (*see* **Note 4**).
3. Induce and maintain anesthesia by inhalation of isoflurane (1.5–2%) mixed with 100% oxygen via a mask.

3.2.2. In Utero *Transplantation*

1. Shave whole abdomen and sterilize the surface with iodine solution (from **Subheading 3.2.1., step 3**).
2. Determine fetal position by transabdominal ultrasound with a 7.5-MHz convex probe (*see* **Note 5**).
3. Let an assistant secure the other side of the uterus while an operator holds the transducer parallel to the intended course of the needle.
4. Select an optimal entry site into the uterine cavity, avoiding the placental tissue.
5. Insert a 23-gage PTC needle through the maternal skin and uterine wall into the amniotic cavity and then into the desired site (e.g., peritoneal cavity, brain, or liver) under continuous ultrasound guidance (*see* **Note 6** and **Fig. 1**). A small push of an injector can visualize a tip of the needle on echocardiography.
6. Let an assistant gently inject the cells (200–500 µL) and flush the needle with 100 µL normal saline. The operator should focus on keeping the tip of the needle in an appropriate position.
7. Confirm adequate heart beats after the procedure (*see* **Note 7**).

3.2.3. Caesarian Section

1. Prepare the pregnant monkey after transplantation as described in **Subheading 3.2.1.** *In utero* transplantation is usually done around the end of the first trimester (50–60 d) (*see* **Subheading 3.2.2.**). The full term is 165 d; therefore, the *in utero* incubation time of transplanted ES cells is about 3 mo.
2. Expose the gravid uterus through a midline incision and deliver the fetus through a low transverse hysterotomy (*see* **Note 8**).
3. Clamp and divide the cord. Remove the placenta and cord. Close the uterus and abdomen with absorbable sutures.
4. Insert a small catheter (24-gage intravenous catheter) into the umbilical vein and irrigate the newborn with normal saline to completely wash out fetal blood for mercy killing. Open the chest and abdomen, observe the whole body, and excise tumors (*see* **Fig. 2A–C**). Collect tissues.
5. Fix tissue samples (5 × 5 × 3 mm) at 4°C for 4 h in 4% paraformaldehyde and 8% sucrose in PBS and embed the samples in paraffin for histological examination. To prepare fresh

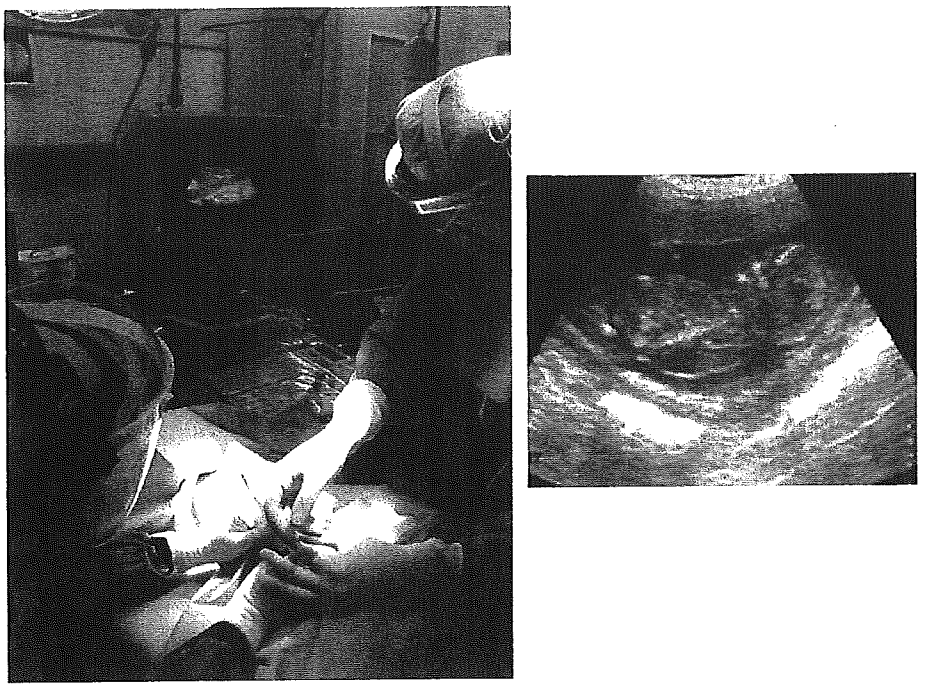

Fig. 1. *In utero* transplantation. Pregnant monkeys were anesthetized by intramuscular administration of ketamine hydrochloride (Ketalar). Cynomolgus ES cells genetically modified to express GFP (10^6 cells/fetus) were injected into the fetal abdominal cavity or liver through a 23-gage needle using an ultrasound-guided technique around the end of the first trimester (**left**). The full term is 165 d. The weight of the fetus at the time of transplantation was estimated at 20 g, which is equivalent to that of an adult mouse (**right**).

frozen samples, embed samples (5 × 5 × 3 mm) in OCT compound containing 10% sucrose, freeze them in liquid nitrogen, and store them at −80°C.

3.3. In Situ *Detection of Transplanted Cell Progeny*

You may examine tissue sections for in vivo fate of transplanted cell progeny by *in situ* PCR, which amplifies marker (GFP) sequences ***(10,13)***. It is especially useful when it is difficult to identify cells by staining specific surface markers, when GFP fluorescence is hampered by the high autofluorescence of tissue samples, or when the transgene expression is shut down ("silenced") in vivo.

3.3.1. Cell Wall Permeabilization

1. (Optional) If a tissue section is embedded in paraffin, then dewax it by dipping the slide in xylene three times, each for 10 min, and then in 100% ethanol three times, each for 10 min. Air-dry the slide.
2. Soak the slide in 20 μg/mL proteinase K/PBS and incubate it at 37°C for 10 min (*see* **Note 9**).

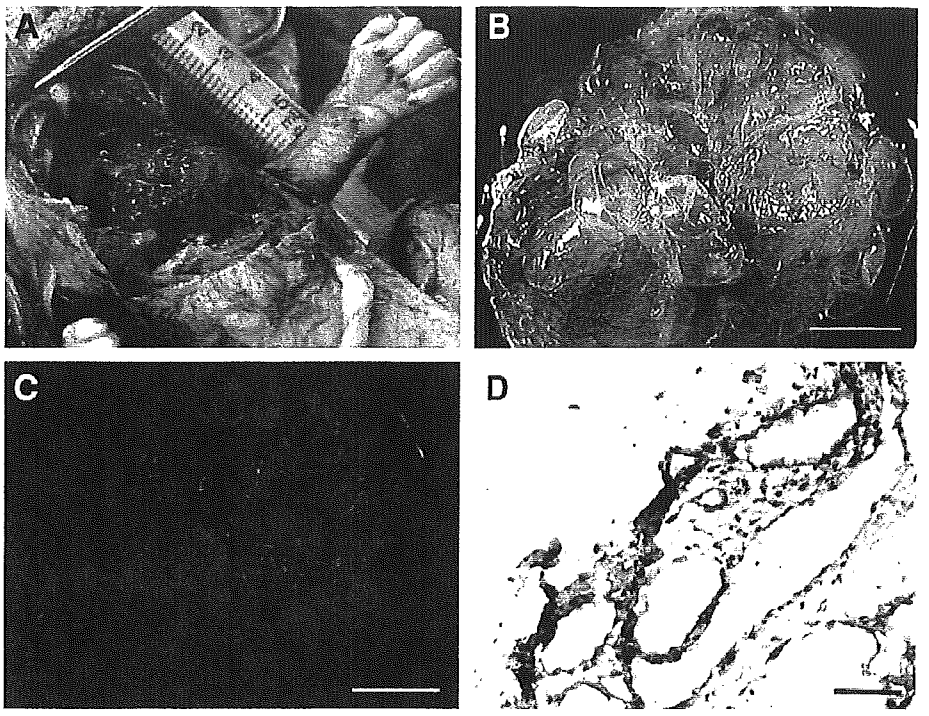

Fig. 2. Teratoma formation in a cynomolgus fetus after transplantation of allogeneic ES cells. (**A**) A tumor (4 × 3 × 2.5 cm) was detected in the thoracic cavity 3 mo after allogeneic transplantation of ES cells expressing GFP. The tumor was observed in (**B**) a bright field and (**C**) a dark field (**C**) under a fluorescence stereomicroscope. GFP was expressed in the tumor, clearly indicating that the tumor was derived from transplanted ES cells. (**D**) The GFP gene was also detected in the tumor cells by *in situ* PCR (stained black). Bar in **B** and **C** = 1 cm. Bar in **D** = 50 μm.

3. Soak the slide in 0.1% Triton X-100/PBS for 5 min and wash it with PBS twice, each for 5 min.
4. Soak the slide in 95% ethanol for 10 min and then in 100% ethanol twice, each for 10 min, to remove proteins and air-dry it.

3.3.2. In Situ *PCR*

1. Attach a slide frame to the slide (from **Subheading 3.3.1., step 4**) and incubate it at 95°C for 5 min.
2. Apply a master mix of *in situ* PCR to the slide at room temperature (*see* **Note 10** and **Table 1**).
3. Cover the slide with a film (*see* **Note 11**).
4. Place the slide upside down in a PTC100 Peltier thermal cycler and start a cycling program (i.e., 94°C for 1 min and 55°C for 2 min with 15 cycles; *see* **Note 12**).

3.3.3. Detection

1. Remove the slide frame gently after PCR (from **Subheading 3.3.2., step 4**) and soak the slide in two changes of PBS for 5 min each.
2 Dropwise add an horseradish peroxidase-labeled anti-Digoxigenin solution (diluted 1:100 with 2% BSA and 5% horse serum in PBS) onto the slide and incubate it at 37°C for 2 h.

Table 1
***In Situ* PCR Reaction Mixture**

Master mix	Volume per reaction	Final concentration
10X PCR buffer (Mg^{2+} free)	2.5 µL	1X
25 m*M* $MgCl_2$	4.5 µL	4.5 m*M*
dNTPs mixture (2.5 m*M* each)	3 µL	420 µ*M* dATP
Digoxigenin (DIG) DNA labeling mix (Roche)	3 µL	420 µ*M* dCTP
		420 µ*M* dGTP
		378 µ*M* dTTP
		42 µ*M* DIG-dUTP
Forward primer (10 µ*M*)	2 µL	0.8 µ*M*
Reverse primer (10 µ*M*)	2 µL	0.8 µ*M*
Takara Taq polymerase (5 U/µL)	0.8 µL	0.16 U/µL
Water	7.2 µL	NA
Total volume of master mix	25 µL	NA

NA, not applicable.
Primer set for the GFP sequence: 5′-CGT CCA GGA GCG CAC CAT CTT C-3′ and 5′-GGT CTT TGC TCA GGG CGG ACT-3′.

3. Soak the slide in two changes of PBS for 5 min each.
4. Dropwise add a Vector SG solution onto the slide, stain it for 3–10 min, and gently wash it with water for 10 min.
5. Dropwise add a Kernechtrot solution and incubate the slide at room temperature for 1–2 min to stain nucleotides and gently wash it with water for 10 min.
6. Mount the slide with glycerol and observe it under a light microscope (*see* **Note 13** and **Fig. 2D**).

4. Notes

1. Although we use NOD/SCID mice (lack of B and T lymphocytes but presence of natural killer cells), SCID mice are usually used in many other laboratories to form teratomas from ES cells. NOD/SCID mice are more highly immunodeficient than SCID mice; thus, NOD/SCID mice may be better in the setting of xenotransplantation.
2. Cynomolgus or rhesus monkeys are the most appropriate to work with because of ES cell availability and their size. In the monkey fetus, "the window of opportunity" for successful tolerance induction may be earlier and narrower than thought ***(14)***. To avoid immune responses, transplantation at earlier days (around 40–50 d) may be better.
3. It is not necessary to disperse ES cell clumps to single cells when transplanting ES cells into mice (or other animals). We transplanted about 1×10^6 ES cells (corresponding to two confluent 60-mm dishes) per site in mice. There is, however, considerable variation among reports: from 10–15 clumps (200 cells) per site ***(2)*** to 5×10^6 cells per site ***(15)***. ES cells are usually transplanted subcutaneously into the hind leg muscle, testis capsule, or abdominal cavity. In our experiments, a teratoma was formed in any site. It is recommended to choose injection sites you can observe easily from the outside and from which you can easily excise tumors.
4. For ultrasound-guided transplantation operations, endotracheal intubation is not necessary.
5. We prefer a small convex transducer rather than a big linear transducer because of the small size of the monkey fetus. Although a needle adapter is available, we prefer the freehand technique.

6. You may puncture transplacentally when the placenta is located anteriorly. Bleeding from the placenta usually stops spontaneously. However, we recommend every effort to avoid this approach by manipulation.
7. The survival rate with this *in utero* transplantation technique is currently 100%, excluding those fetuses that died from massive teratoma formation.
8. Uterine atony requiring oxytocin administration is quite rare in primates.
9. The treatment with proteinase K may need longer time depending on samples.
10. The amount of master mix per slide is 25 µL for Takara cat. no. 9066 and 65 µL for cat. no. 9067.
11. Slides are attached to the Takara slide seal kit. Be careful not to trap air under films.
12. The PCR conditions should be optimized for each *in situ* PCR.
13. The results should be observed within the same day. On the following day, the tissue would peel off, making examination difficult.

Acknowledgments

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Hematopoietic Microchimerism in Sheep After In Utero Transplantation of Cultured Cynomolgus Embryonic Stem Cells

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Background. Although directed differentiation of human embryonic stem (ES) cells would enable a ready supply of cells and tissues required for transplantation therapy, the methodology is limited. We have developed a novel method for hematopoietic development from primate ES cells. We first cultured cynomolgus monkey ES cells in vitro and transplanted the cells in vivo into fetal sheep liver, generating sheep with cynomolgus hematopoiesis.

Methods. Cynomolgus ES cells were induced to mesodermal cells on murine stromal OP9 cells with multiple cytokines for 6 days. The cells (average 4.8×10^7 cells) were transplanted into fetal sheep in the liver (n=4) after the first trimester (day 55–73, full term 147 days). The animals were delivered at full term, and two of them were intraperitoneally administered with human stem-cell factor (SCF).

Results. Cynomolgus hematopoietic progenitor cells were detected in bone marrow at a level of 1% to 2% in all four sheep up to 17 months posttransplant. No teratoma was found in the lambs. After SCF administration, the fractions of cynomolgus hematopoiesis increased by several-fold (up to 13%). Cynomolgus cells were also detected in the circulation, albeit at low levels (<0.1%).

Conclusions. Long-term hematopoietic microchimerism from primate ES cells was observed after in vitro differentiation to mesodermal cells, followed by in vivo introduction into the fetal liver microenvironment. The mechanism of such directed differentiation of ES cells remains to be elucidated, but this procedure should allow further investigation.

Keywords: Primate embryonic stem cells, In utero transplantation, Hematopoietic microchimerism, Sheep.

(*Transplantation* 2005;79: 32–37)

A major barrier for most tissue or cellular transplantation therapies is the shortage of donors. Because human embryonic stem (ES) cell lines have dual abilities to proliferate indefinitely and differentiate into multiple tissue cells (*1, 2*), directed differentiation of human ES cells into functionally defied tissue types is a goal in providing an inexhaustible and potentially customized supply of transplantable cells or tissues. Clearly directed differentiation of ES cells is still in its infancy, and the methodology is quite limited. Many researchers have studied in vitro specific differentiation programs through manipulation of the cytokine milieu, cellular microenvironment, and conditional activation of specific gene expression (*3–5*). On the other hand, we and other groups have shown a line of evidence that undifferentiated ES cells respond to local cues after transplantation and differentiate into site-specific cells in rodent and nonhuman primate allogeneic transplantation models (*6, 7*). These studies have highlighted the importance of the in vivo local microenvironment for directed differentiation of ES cells. ES cells can be induced to differentiate into specific cells if exposed to the proper microenvironment. In this study, we have tried to use the in vivo fetal sheep liver microenvironment for hematopoietic development from primate ES cells.

Sheep in utero transplantation has been used as an assay system for human hematopoiesis (*8*). The fetal sheep is immunologically tolerant of allogeneic skin grafts or xenogeneic human hematopoietic cells before 75 days of gestation, which allows avoidance of the immunologic barriers present in postnatal models (*8–10*). In this model, long-term human/sheep hematopoietic chimeras have been established after the transplantation of human hematopoietic stem cells into the fetal sheep at a preimmune stage (*8*). It has also been reported that human mesenchymal stem cells engraft and show site-specific differentiation after in utero transplantation in sheep (*11*).

We have used nonhuman primate (cynomolgus monkey) ES cells (*12*) because this is the most faithful model for human ES cells for generating hematopoietic chimera in sheep. We first cultured cynomolgus ES cells in vitro to differentiate into mesodermal cells and introduced the cells into fetal sheep liver after the first trimester. Fetal liver is a hematopoietic organ at this stage of fetuses. We then examined the in vivo fate of transplanted cell progeny long term.

MATERIALS AND METHODS

Cell Preparation

Cynomolgus macaque ES cells (CMK6) were maintained on a feeder layer of mitomycin C (Kyowa, Tokyo, Japan) treated mouse (BALB/c, Charles River Japan,

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Kanagawa, Japan) embryonic fibroblasts, as described previously (*12*). The mouse bone-marrow stromal-cell line OP9 was maintained in α-minimum essential medium (Gibco, Rockville, MD) supplemented with 20% fetal calf serum, as previously described (*13*).

For the in vitro differentiation (induction of hematopoietic differentiation), ES cells were seeded onto mitomycin–C-treated confluent OP9 cell layers in culture dishes in Iscove's modified Dulbecco's medium (Gibco) supplemented with 8% horse serum (Gibco), 8% fetal calf serum, 5×10^{-6} M hydrocortisone (Sigma, St. Lois, MO), and multiple cytokines including 20 ng/mL recombinant human (rh) bone morphogenetic protein-4 (BMP-4; R&D, Minneapolis, MN), 20 ng/mL rh stem-cell factor (SCF, Amgen, Thousand Oaks, CA), 20 ng/mL rh interleukin-3 (Research Diagnostics, Flanders, NJ), 20 ng/mL rh interleukin-6 (Ajinomoto, Osaka, Japan), 20 ng/mL rh vascular endothelial growth factor (VEGF, R&D), 20 ng/mL rh granulocyte colony-stimulating factor (Chugai, Tokyo, Japan), 10 ng/mL rh Flt-3 ligand (Research Diagnostics), and 2 IU/mL rh erythropoietin (Chugai). During differentiation, media were changed every 2 to 3 days. After 6 days of culture, cells were dissociated with 0.25% trypsin (Gibco), collected with a cell scraper, washed with Hanks' balanced salt solution (HBSS, Gibco), resuspended in 0.4 mL of 0.1% bovine serum albumin/HBSS, and used for transplantation. Human cord-blood $CD34^+$ cells used in the present study were obtained at Jichi Medical School Hospital with informed consent.

Transplant Procedures

Pregnant Suffolk ewes (Japan Lamb, Hiroshima, Japan) were bred at the Utsunomiya University Farm. Fetal sheep at 55 to 79 days of gestation (full term 147 days) were used. Before transplantation, ewes were sedated with ketamine (10 mg/kg intramuscularly) and received a 0.5% to 1.0% halothane-oxygen mixture by inhalation by way of an endotracheal tube. The uterus was exposed through a midline laparotomy incision. Donor cells were injected into the fetus in the liver through the uterine wall using a 25-gauge needle under ultrasound guidance. After closure of the abdominal wall, penicillin and streptomycin were administered. The fetus was allowed to come to term. After birth, some lambs were intraperitoneally administered rhSCF at a dosage of 60 μg/kg once a day for 18 or 5 consecutive days.

Hematopoietic Progenitor Assay

To assess cynomolgus hematopoiesis in sheep, clonogenic hematopoietic colonies were produced by growing bone-marrow cells in methylcellulose with defined rh cytokines (Methocult GF+ and MegaCult-C, Stem Cell Technologies, Vancouver, Canada). Bone-marrow cells were aspirated from the iliac bone. From harvested bone-marrow cells, a leukocyte cell fraction was obtained after red–blood-cell lysis with ACK buffer (155 mM NH_4Cl, 10 mM $KHCO_3$ and 0.1 mM EDTA; Wako, Osaka, Japan). Cells were plated in triplicate at 1 to 5×10^4 cells per 35-mm plate. After 14 days, individual colonies were plucked into 50 μL of distilled water and digested with 20 μg/mL proteinase K (Takara, Shiga, Japan) at 55°C for 1 hour, followed by 99°C for 10 minutes. Each sample (5 μL) was used for polymerase chain reaction (PCR) amplification to detect cynomolgus-specific β2-microglobulin gene sequences. Nested PCR was performed. The outer primer set was 5'-GTC TGG ATT TCA TCC ATC TG-3' and 5'- GGC TGT GAC AAA GTC ACA TGG-3', and the inner primer set was 5'-GTC TGG ATT TCA TCC ATC TG-3' and 5'-GGT GAA TTC AGT GTA GTA CAA G-3'. Amplification conditions for both outer and inner PCR were 25 cycles of 94°C for 30 seconds, 58°C for 30 seconds, and 72°C for 30 seconds. Amplified products (135 bp) were resolved on 2% agarose gel and visualized by ethidium bromide staining.

PCR Southern Blotting

Cellular DNA was extracted from peripheral blood and bone-marrow cells after birth and subjected to the cynomolgus-specific β2-microglobulin PCR as described above. The PCR products were resolved on 2% agarose gel and transferred to Hybond-N+ (Amersham, Cleveland, OH). The membrane was hybridized with a radiolabeled cynomolgus-specific β2-microglobulin probe generated by PCR using the following primers: 5'-GTC TGG ATT TCA TCC ATC TG-3' and 5'-GGT GAA TTC AGT GTA GTA CAA G-3'. Radiolabeling of a probe was performed using a DNA labeling kit (Amersham).

RESULTS

In Vitro Culture and In Utero Transplantation

Cynomolgus ES cells were maintained on mouse embryonic fibroblast feeder cells. They form colonies on feeder cells (Fig. 1a). For induction of hematopoietic differentiation, undifferentiated ES cells were placed on murine stromal OP9 cells (*13*) in the presence of multiple cytokines including human BMP-4 and VEGF (*14*). The cell number increased by 10-fold at day 6, and cobblestone-like cells emerged at this time point (Fig. 1b). These cells were negative or only weakly positive for CD34 (hematopoietic) and CD31 (endothelial) markers as assessed by flow cytometry and had little clonogenic hematopoietic colony forming ability in culture (data not shown). These cells likely represent a differentiated population of mesodermal cells. We then transplanted the day-6 ES derivatives (average 4.8×10^7 cells, Table 1) into fetal sheep (n=4) by way of direct injection into the liver under ultrasound guidance after the first trimester (Fig. 1c).

Hematopoietic Microchimerism after Birth

The animals were delivered at full term. Marrow cells were harvested from the iliac bone and plated in methylcellulose. Clonogenic hematopoietic colonies (colony-forming units [CFU]) were thus produced to examine hematopoietic chimerism in the sheep (Fig. 2). Sheep bone-marrow cells generated colonies of clear hematopoietic morphology in this assay. Cynomolgus and sheep cells were equivalent in their colony-formation ability in this culture, and each colony was derived from a single cynomolgus or sheep hematopoietic progenitor cell. Therefore, the ratio of cynomolgus to sheep colony number is considered a chimeric fraction. To distinguish cynomolgus versus sheep colonies, we tried to immunostain colonies with antihuman class I, and more directly hematopoietic-specific antihuman CD45, but they reacted to sheep counterparts or generated a considerable nonspecific staining. We then conducted PCR for monkey-specific β2-microglobu-

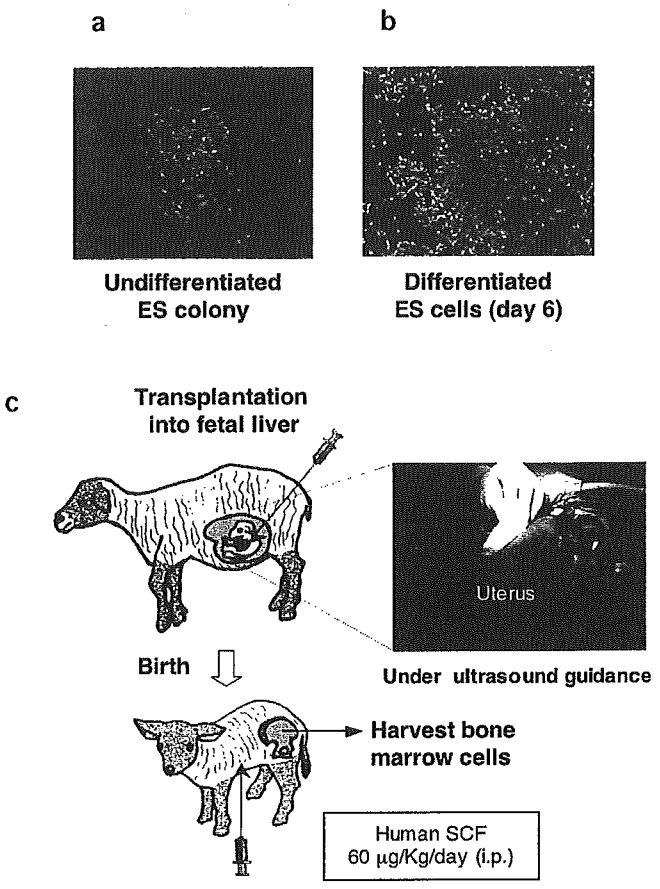

FIGURE 1. In utero transplantation of day 6 cynomolgus embryonic stem (ES)-derived cells. (a) Undifferentiated cynomolgus ES cells were maintained on mouse embryonic fibroblast feeder cells. (b) For hematopoietic induction, undifferentiated ES cells were placed on murine stromal OP9 cells in the presence of multiple cytokines. Cobblestone-like cells emerged at day 6. (c) The day 6 ES derivatives were transplanted directly into the fetal liver after the first trimester. After birth, marrow cells were harvested from the iliac bone and examined for cynomolgus versus sheep hematopoiesis. Some lambs were intraperitoneally (i.p.) administered with human stem-cell factor (SCF) to specifically stimulate cynomolgus hematopoiesis.

lin DNA sequences on DNA isolated from each colony (Fig. 2) (colony PCR). Cynomolgus CFU were clearly detected at a level of 1% to 2% in the bone marrow of all four sheep (Table 1). We repeated colony PCR and confirmed that data were reproducible. We detected both granulocytic and erythroid cynomolgus CFU. Cynomolgus megakaryocytic colonies were not detected, but it was no wonder considering the low frequency of cynomolgus megakaryocytic colony formation in vitro (*15*). The longest follow-up was 17 months after transplantation (14 months after birth), and cynomolgus CFU were still detectable. No lamb developed a teratoma.

Selective Expansion of Cynomolgus Hematopoiesis

To selectively stimulate cynomolgus compared with endogenous hematopoiesis in the sheep, we administered two animals with human SCF, a cytokine that does not cross-react to stimulate sheep hematopoietic progenitors (*16*). A time-course profile of the two animals (No. 141 and No. 182) is shown in Figure 3. In both sheep, in response to the human SCF administration for 18 days, the fractions of cynomolgus CFU increased several-fold (up to 13.2% at day 174 and 4.7% at day 112 posttransplantation, respectively). After cessation of SCF administration, the fractions fell to the original levels. Resumption of human SCF at the same dose but for 5 days produced a similar elevation in the chimeric fractions (up to 4.4% at day 364 and 8.8% at day 187 posttransplantation, respectively). No adverse effects associated with human SCF administration were observed. We did not examine antibody responses to human SCF in the sheep, but this was unlikely to occur because second trials of human SCF administration did work (i.e., increased the chimeric fractions) in both sheep.

Cynomolgus cells were also detected in the circulation, although the fraction was very low (<0.1%), even after human SCF administration as assessed by PCR Southern blotting (Fig. 4). The low levels of cynomolgus cells hampered the lineage analysis by flow cytometry. To detect cynomolgus T lymphocytes in the sheep, we collected the peripheral blood and tried to selectively expand cynomolgus T lymphocytes in the culture with human interleukin-2, antimonkey CD3 (FN-18), and antihuman CD28 (Kolt-2, cross-reacting to monkey CD28) (*17*). However, sheep lymphocytes were also stimulated to expand, and we failed to detect cynomolgus T lymphocytes.

Comparative Study of Repopulating Ability

Next, we transplanted human cord-blood hematopoietic stem cells ($CD34^+$ cells, average 1.8×10^6 cells) into fetal sheep (n=4) instead of day-6 cynomolgus ES derivatives. We used a $CD34^+$ fraction because it was widely used both in sheep in utero transplantation (*18*, *19*) and in clinical transplantation of hematopoietic stem cells (*20*), although some investigators used a human $CD34^-$ fraction for sheep in utero transplantation and obtained hematopoietic chimera as well or even better in serial transplantation experiments (*21*). For the present, however, we considered that the $CD34^+$ fraction was an appropriate standard with which to compare the repopulating ability of day-6 cynomolgus ES-derived cells as well. The chimeric fraction in bone-marrow CFU after in utero transplantation of $CD34^+$ cells was 1% to 9% (average 3.7%). Fractions of donor-derived cells in the peripheral blood were also very low (<0.1%) after the transplantation of $CD34^+$ cells. The average cell numbers necessary to achieve 1% chimerism in CFU after birth were estimated to be 4.3×10^7 and 6.0×10^5 for day-6 cynomolgus ES-derived cells and human cord-blood $CD34^+$ cells, respectively.

DISCUSSION

We have generated long-term hematopoietic microchimerism in sheep derived from ES cells. Achieving hematopoietic reconstitution from ES cells has been an enormous challenge. The difficulty is attributable to the developmental immaturity of ES-derived cells, which most closely resemble primitive embryonic yolk-sac hematopoietic progenitors (*22*). The processes governing embryonic versus adult blood formation are distinct, and mouse ES cells do not contribute

TABLE 1. In utero transplantation and donor-cell engraftment in sheep

Transplanted cells	Animals (sex)	In utero transplantation: Transplanted cell number per fetus	In utero transplantation: Gestational day at transplantation (Full term 147 days)	Donor cell-derived CFU in bone marrow* (months posttransplant): After birth	Donor cell-derived CFU in bone marrow* (months posttransplant): After human SCF administration	Average cell number necessary for 1% chimerism
Day 6 ES-derived cells	No. 57 (male)	5.0×10^7	67	1.1% (1/91) at 3.5 months	ND	4.3×10^7
	No. 55 (female)	5.0×10^7	55	1.1% (1/91) at 5 months	ND	
	No. 141 (male)	7.8×10^7	73	1.1% (1/91) at 3 months	13.2% (12/91) at 6 months	
	No. 182 (male)	1.4×10^7	66	1.6% (1/63) at 3 months	8.8% (8/91) at 6 months	
Human cord blood $CD34^+$ cells	No. 71-1 (male)	2.0×10^6	69	8.8% (8/91) at 1 months	ND	6.0×10^5
	No. 71-2 (female)	2.0×10^6	69	4.4% (4/91) at 3.5 months	ND	
	No. 99-1 (male)	1.5×10^6	79	1.1% (1/91) at 1 months	ND	
	No. 99-2 (female)	1.5×10^6	79	4.4% (4/91) at 2 months	ND	

* Percent cynomolgus CFU was calculated by dividing the number of CFU positive for the cynomolgus-specific β2-microglobulin gene sequence by the total number of CFU analyzed.

CFU, colony-forming unit; ND, not done; ES, embryonic stem.

to hematopoietic reconstitution in irradiated mice, unlike stem cells isolated from adult bone marrow (*23*, *24*). An effective approach to this obstacle has recently been reported. Genetically engineering mouse ES cells to express a specific transcription factor (HoxB4) or signaling molecule (STAT5) during a specific developmental window has resulted in hematopoietic reconstitution from ES cells in irradiated mice (*5*, *25*). However, the requirement for artificial over-expression of those genes is undesirable for clinical applications. Our method to develop hematopoietic engraftment from primate ES cells does not require genetic manipulation, and it is a combination of two steps: in vitro differentiation into mesodermal cells followed by in vivo development into hematopoietic cells in the proper microenvironment of fetal sheep liver.

To examine whether transplanted day-6 ES-derived cells engraft in other tissues, we did one more in utero transplantation experiment using same cultured cynomolgus ES cells (mesodermal cells at day 6) and delivered a fetus at 1 month after transplantation. Although cynomolgus CFU were detected in fetal liver and cord blood, cynomolgus cells were not detectable in any other tissues by a sensitive PCR after the fetal blood was completely washed out (data not shown). Therefore, our method appears to direct the fate of primate ES cells to the hematopoietic lineage.

We assumed that the initial in vitro culture of ES cells into mesodermal cells is crucial for successful engraftment in the fetal liver. In fact, when we transplanted undifferentiated (day 0) cynomolgus ES cells into fetal sheep (n=2), we failed to detect cynomolgus CFU or other nonhematopoietic cells in any fetal tissue at 1 month posttransplantation as assessed by a sensitive PCR (data not shown). Therefore, undifferentiated ES cells do not appear to engraft in fetal sheep, unlike adult mesenchymal or hematopoietic stem cells (*8*, *11*), but day-6 ES derivatives (mesodermal cells) can engraft and are susceptible to hematopoietic specification in the fetal-liver microenvironment. For the successful initial in vitro culture of ES cells, there are some points to be noted. First, we used stromal OP9 cells as a feeder. Second, we included BMP-4 and VEGF in the culture medium. The coculture with OP9 and inclusion of BMP-4 and VEGF promote hematopoietic differentiation of ES cells (*13*, *14*, *26–28*). Finally, we cultured cells in vitro for a relatively short period (6 days) to avoid over-maturation of cells (*14*). The xenogeneic fetal liver is able to provide such cultured ES cells with an adequate microenvironment for support of hematopoietic development (*29*). Factors present in the fetal liver responsible for the development remain to be elucidated.

On the other hand, several issues remain to be further investigated. Although the chimeric fractions increased by several-fold (up to 13%) after SCF administration, the increase was transient. To stably enhance the ES-derived chimeric fraction, an enrichment of cells responsible for the engraftment will be needed before transplantation, as suggested from Table 1 (see average cell numbers necessary to achieve 1% chimerism). Another issue is a very low level of donor-derived cells in the peripheral blood. Ours is quite different from previous human-to-fetal sheep experiments that demonstrated easily detectable peripheral blood chimerism (*16*, *19*). This has hampered the lineage analysis of cynomolgus cells in our sheep to obtain further evidence to support hematopoietic differentiation from ES cells. Low levels of pe-

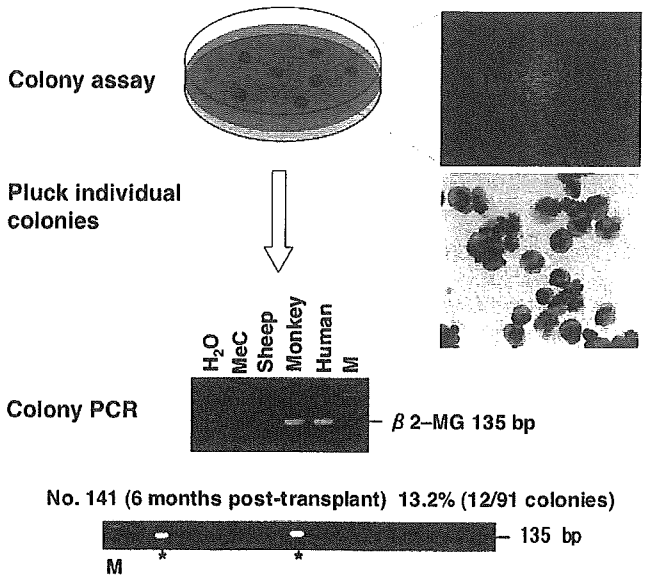

FIGURE 2. Assessment of cynomolgus hematopoiesis in sheep after birth. Bone-marrow cells were harvested from lambs and placed in methylcellulose. Hematopoietic colonies were thus formed. A cytospin specimen (stained with the Wright-Giemsa method) of plucked myeloid colonies demonstrated mature neutrophils. Each colony was derived from a single cynomolgus or sheep hematopoietic progenitor cell. To detect cynomolgus colonies, individual colonies were plucked and examined for cynomolgus-specific β2-microglobulin (β2-MG) sequences by polymerase chain reaction (PCR). PCR products were analyzed on 2% agarose gel. Plucked methylcellulose (MeC) alone (not containing colonies) and sheep colonies generated no bands by the PCR. Colony PCR was repeated at least twice. Representative colony PCR results of sheep No. 141 shown. *Bands positive for the cynomolgus-specific sequence. M, molecular weight marker.

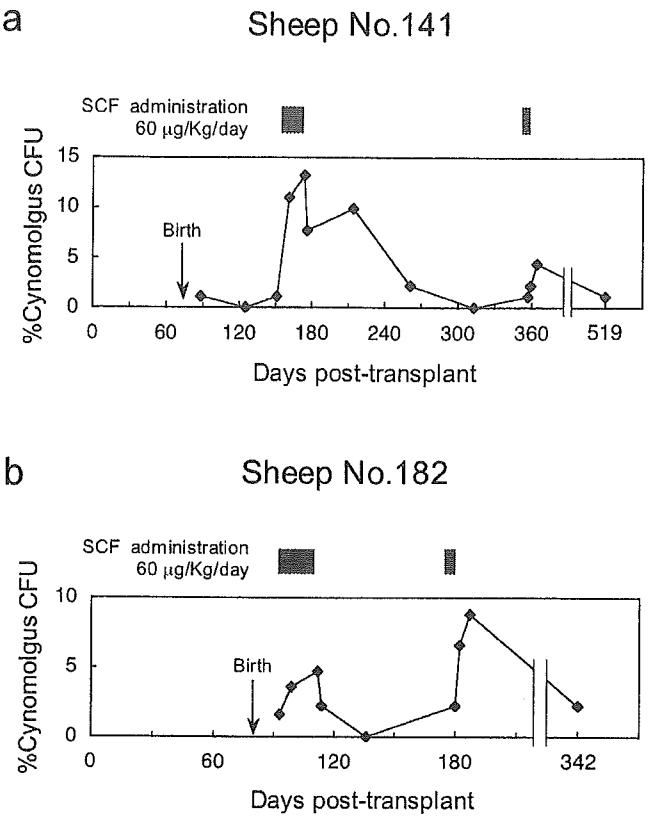

FIGURE 3. Time course of hematopoietic chimerism in the sheep receiving human SCF. (a) In sheep No. 141, human SCF was intraperitoneally administered at 60 μg/kg once a day from day 156 posttransplantation for 18 days. SCF administration was then stopped and tried again from day 352 for 5 days. (b) In sheep No. 182, human SCF was similarly administered from day 94 posttransplantation for 18 days, followed by a second administration at the same dose from day 175 for 5 days. Horizontal axis indicates days after transplantation. Vertical axis shows cynomolgus/sheep chimerism (a ratio of cynomolgus vs. sheep CFU in the bone marrow). Period of human SCF administration (*gray bars*).

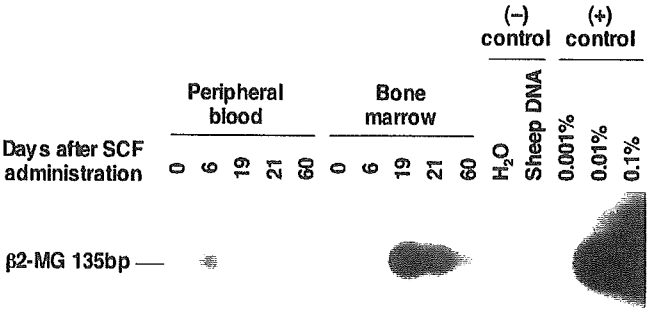

FIGURE 4. Detection of cynomolgus cells in the circulation. DNA was extracted from whole peripheral blood or bone-marrow nucleated cells after birth and subjected to cynomolgus-specific β2-microglobulin (β2-MG) PCR, and Southern blot analysis. Data before and after SCF administration shown. Positive controls show 0.001, 0.01, and 0.1% chimerism (cynomolgus to sheep). Cynomolgus cells were detectable after SCF administration, albeit at low levels (<0.1%).

ripheral chimerism were, however, also found after human $CD34^+$ cells were transplanted in our study. Therefore, the issue is not specific to transplanted ES cells, but is likely to be attributable to the experimental system. A possible explanation may be the different sheep species used in our study (Suffolk versus Dorset Merino). Another possible explanation is immune responses caused by relatively later gestational ages (in the second trimester, day 55–79) at transplantation in our study compared with other recent studies (day 40–45) (*19*). The immune response may have cleared xenogeneic cells from the circulation. The existence of microchimerism does not necessarily guarantee or predict tolerance in other systems (*30, 31*). "The window of opportunity" for successful tolerance induction may be earlier and narrower. To avoid sensitization, transplantation at earlier days may be more efficacious.

In conclusion, long-term hematopoietic microchimerism from primate ES cells is possible after in vitro differentiation to mesodermal cells, followed by in vivo transplantation into the fetal-liver microenvironment. We have used nonhuman primate ES cells in the current study, but if human ES cells are similarly used, human blood cells can be generated in sheep. This procedure should allow for further investigation.

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Embryonic Stem Cells

Improved Safety of Hematopoietic Transplantation with Monkey ES Cells in the Allogeneic Setting

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