た。この前核卵を体外培養したところ 17 個が2細胞期に等割し、胚盤胞へは 2 個が発生した。この胚盤胞から摘出した内部細胞塊を培養したところ、1個で単層状に outgrowth することが認められた。しかし、受精卵に由来するものと比較するとその成長が遅いことが観察された。これを継代処理したが、受精卵に由来する ES 細胞と類似したコロニーは再形成されなかった。

D. 考察

カニクイザルの未成熟および成熟個体から得られる卵において、その質にクローン胚を作製する上で何らかの差が有るか無いかを比較することと、免疫拒絶のないES細胞を樹立することを目的に、単為発生由来の ES 細胞樹立に着手した。

前者では、hCG 投与後 38 時間に回収した卵を使用したとき、成熟個体に由来する卵における除核率は高い傾向にあることが認められた。その後の発生においても、着床前期胚のより後期にあたる桑実胚への発生が成熟個体由来の卵を使用したときに確認された。これらのことは、hCG 投与によって誘導された卵の成熟からの時間が比較的に長いときには、除核および核移植卵の発生の面から、成熟個体に由来する卵を使用する方が良いことを示唆するものであった。また、hCG 投与後 28 時間に回収した卵を使用したとき、除核率においては、成熟個体および未成熟個体に差は認められず、核移植卵の発生においては、成熟個体由来の卵においてのみ、胚盤胞への発生が認められた。卵の成熟期間が短い間に回収された卵においても、未成熟個体よりも成熟個体に由来する卵を使用する利点が確認された。以上のことより、カニクイザルにおけるクローン研究に使用する卵としては、有意ではないものの、成熟個体に由来するものを利用するべきであることが明らかとなった。なお、マウスにおいては、未成熟個体から採取される卵の数は成熟個体よりも多いことが知られている。このため、サルにおいても同様な効果を期待したが、必ずしも卵の数が多く採取できることはなく、クローン研究に使用できる卵を確保するという利点が認められなかった。個々の実験をみると、未受精卵の核を除去できないこともあり、個体間におけるバラツキが大いに認められた。これは、遺伝的に均一なマウスと異なり、サル個体間の遺伝的多様性に一因があるものと考えられ、より効率的なクローン胚作出検討を行うために、1実験に複数頭のサルが準備されることも十分考慮される事項であると考えられた。

一方後者では、2個の単為発生に由来する胚盤胞への発生が確認され、これらの内部細胞塊をMEF 上で培養した。これらの内、1個で内部細胞塊の outgrowth を確認し、もう1個については outgrowth を確認できなかった。この outgrowth した内部細胞塊は、他の受精卵に由来するものと比べ、成長速度が遅いことが観察された。通常10ー14 日ほどで継代できる大きさに成長するのだが、単為発生由来のものでは、2週間を過ぎてもそれ程までの成長は認められなかった。この原因として、内部細胞塊の質あるいはメスゲノムのみで構成されていることに一因があると考えられた。これを改善す

るためには、良質な胚盤胞、特に内部細胞塊を形成させることが重要であり、単為発生誘起の方法および体外培養の方法をさらに改良する必要性が感じられた。また、核移植実験において、胚盤胞への発生はほとんど認められなかった。これには、核移植卵の作出方法、発生能あるいは体外培養環境など、いくつか要因が考えられるが、この単為発生卵の胚盤胞への発生が認められたことから、体外培養環境が、低い発生率の原因ではない可能性が示唆された。

E. 結論

卵を採取するカニクイザルの成熟および未成熟間において、クローン胚を作製、発生させる上で明確な差を認めるには至らなかったが、成熟個体に由来する卵を使用することの優位性が示唆された。また、単為発生由来 ES 細胞の樹立においては、胚盤胞、特に内部細胞塊の質の向上が必要であることが推察された。

F. 健康危険情報

なし

G. 研究発表

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H. 知的財産権の出願・登録状況　　なし

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分担研究報告書

カニクイザル体細胞由来 iPS 細胞樹立に関する検討

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研究要旨

体細胞に由来クローン ES 細胞は、再生医療などへ応用することで免疫拒絶が生じないことから、その効率的な作製方法の確立が要望されている。しかし、ヒトへの応用を考えた場合、卵を使用するため、その採取や生命の源となることから倫理的な議論となっている。このようなクローン ES 細胞と類似した人工多能性幹細胞(iPS 細胞)が樹立されたことから、カニクイザルにおいてもその樹立を検討し、ES 細胞様の形態を示す細胞株を得ることに成功した。本年はこの細胞株の性状解析を行い、多分化能を持つ ES 細胞との差異の有無を調べた。その結果、ES 細胞と同様な未分化マーカーの発現が免疫蛍光染色によって明らかとなった。また、多分化能として、三胚葉性の細胞種から成るテラトーマを形成できることも明らかと成った。このことは、カニクイザルにおいても、ヒトやマウスと同様な iPS 細胞が樹立できることを示し、カニクイザルを用いた再生医療などの基礎研究に利用できるものと考えられた。

A. 研究目的

体細胞由来のクローン ES 細胞は、体細胞を供与した個体と同一のゲノムを持つことから、クローン ES 細胞から分化する多様な体細胞を免疫拒絶を考慮せずに、供与個体に対して再生医療等の検討を行えるなどの利点がある。しかし、その作製には卵を利用するため、特にヒトにおいて倫理的な問題となっている。このようなクローン ES 細胞の代替になり得るものとして、人工多能性幹細胞(iPS 細胞)が樹立された。これは卵を使用せず、かつ体細胞の供与個体と全く同じゲノムを持つことから、倫理的な問題を回避する。このようなことから昨年、ES 細胞の形態に似た細胞株を得ることに成功した。ES 細胞と同様な未分化マーカーを発現していることも確認した。さらにこの性状を明らかにするために、詳細な解析を行った。さらに、体細胞の供与個体への移植が検討できるように、他の体細胞種から iPS 細胞の樹立を検討した。

B. 研究方法

レトロウィルスベクター法によってカニクイザル Oct3/4、Sox2、Klf4 および c-Myc 遺伝子を導

入して作製した新生児皮膚および胎児肝臓由来の iPS 細胞について、未分化マーカーの発現を次のように免疫蛍光染色で調べた。4%PFA 固定後、2%Toriton-X および 5%FBS を含む PBS で透過およびブロッキング処理をして、一次抗体として Oct3、Nanog、SSEA3、SSEA4 および TRA-2-54 を使用した。二次抗体として、Alexa 標識抗体を使用し、核のカウンター染色にヘキストを使用した。テラトーマ形成能を確認するために、未分化状態の細胞を免疫不全マウスの大腿部に移植した。移植後およそ 2 ヶ月に形成された腫瘍を摘出し、4%PFA 固定後切片を作製し、HE 染色により組織解析を行った。さらに、細胞株の染色体の正常性を調べるために、核型解析を行った。

また、帝王切開時に摘出される羊膜から細胞を回収し、羊膜由来細胞にレトロウィルスベクター法により Oct3/4, Sox2, Klf4, c-Myc の 4 遺伝子の導入を行い、iPS 細胞の誘導を検討した。

(倫理面への配慮)

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C. 研究結果

1株の新生仔皮膚由来および 5 株の胎仔肝臓由来の iPS 細胞を免疫蛍光染色により未分化マーカーの発現を調べたところ、全てで Oct3、SSEA4、Nanog および TRA-2-54 の発現を確認した。SSEA3 については、発現は確認されなかった。

免疫不全マウスに移植した1株の新生仔皮膚由来および 4 株の胎仔肝臓由来の iPS 細胞は、1 株の胎仔肝臓由来 iPS 細胞を除いた全てで奇形種を形成した。これら奇形腫を摘出し、組織学的な解析を行ったところ、全てで外胚葉、内胚葉および中胚葉の三胚葉性の細胞種から成るテラトーマであることが確認された(図 1)。また、胚様体を体外で接着培養させたところ、神経様の細胞、拍動する心筋様の細胞、網膜色素上皮様の細胞など多様な形態の細胞種に分化することも確認された(図 2)。

核型解析については、1株の新生仔皮膚由来および 3 株の胎仔肝臓由来の iPS 細胞のそれぞれ 50 個を調べたところ、前者で 41 個(81%)、後者で 39 個(78%)、41 個(81%)および 46 個(86%)で正常核型を示した。

羊膜由来細胞における iPS 細胞の誘導については成功に至っていないが、アルカリフォスファターゼ陽性細胞を持つコロニーが多数確認された。しかし、そのコロニーは陽性細胞および陰性細胞が混在して構成されていることが確認された。

D. 考察

本検討において、カニクイザル新生仔皮膚由来および胎仔肝臓由来細胞から作製した iPS 細胞の特徴を解析した。さらに、新規の iPS 細胞を樹立するために、羊膜由来の細胞を使用し、検討を行った。

免疫蛍光染色による未分化マーカーの発現は、カニクイザル、アカゲザル、ヒト ES 細胞など

と同様な発現が確認できた。SSEA3 については、発現が確認できなかったが、これはカニクイザル特有であり、他の霊長類では確認されている。これらのことから、樹立された iPS 細胞の未分化状態は維持されていることが明らかと成った。

さらに、多分化能の検討として、テラトーマの形成能を調べたところ、肝臓由来の 1 株を除いて全てで三胚葉性の細胞種から構成されており、多分化能を獲得していることが明らかと成った。テラトーマを形成しなかった 1 株においては、その理由は不明であるが、多分化能を獲得していない、あるいはマウス体内環境下の不適合などが一因にあるかもしれない。

また、iPS 細胞の核型は、調べられた全てで約 8 割以上の細胞が 42 本の染色体を持つ正常な状態にあることが確認され、その安定性が示された。

以上に述べた特徴は、他に報告されている霊長類の ES 細胞や iPS 細胞と同様な特徴を示しており、カニクイザルにおいても、それ自身の Oct3/4、Sox2、Klf4 および c-Myc 遺伝子を導入することで、iPS 細胞が樹立できることが明らかと成った。また、作製された iPS 細胞の染色体数もほとんどが正常であり、安定した核型を維持することが明らかとなった。

羊膜由来の細胞から iPS 細胞の作製を計画したが、現在のところ成功に至っていない。しかしながら、アルカリフォスファターゼ活性が陽性な細胞が確認されたことから、iPS 細胞を作製することが可能であることが推察された。

これらのことからカニクイザルにおいて、iPS 細胞を利用した免疫拒絶のない移植研究に前臨床研究に先だって利用できるものと考えられた。

E. 結論

ES 細胞と同様な性質を持つ iPS 細胞の誘導が、カニクイザル自身の遺伝子を利用することで可能であることを明らかにした。さらに、遺伝子レベルでの性状解析を行い、カニクイザル ES 細胞などと同様な性状を持つ細胞株であるかを調べる。カニクイザル iPS 細胞を利用した免疫拒絶のない移植研究などにヒト前臨床研究に先だって利用できるものと考えられた。

F. 健康危険情報

特になし

G. 研究発表

1.論文発表

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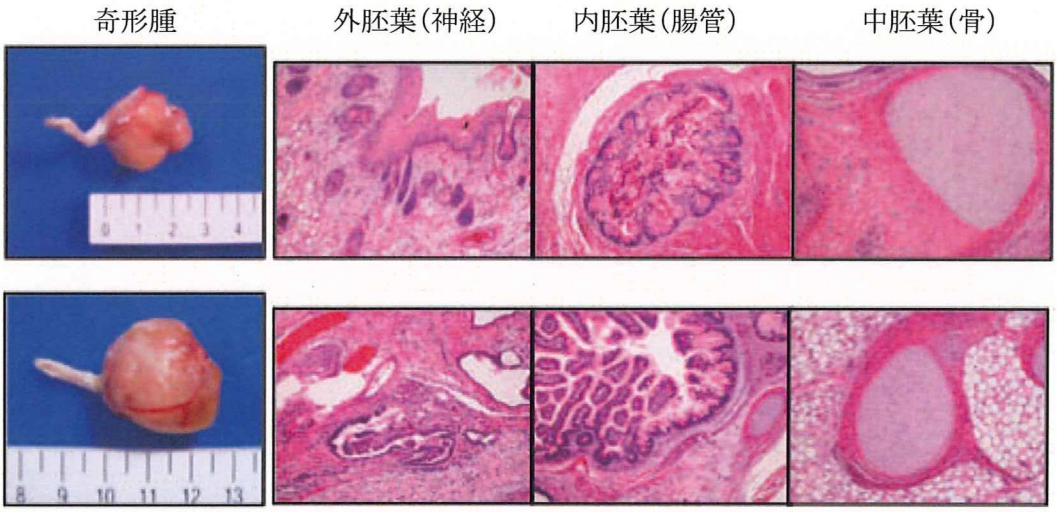
2.学会発表

なし

H. 知的財産権の出願・登録状況

なし

図 1. カニクイザル iPS 細胞から形成された奇形種およびその組織標本



上段:新生児皮膚由来

下段:胎児肝臓由来

図 2. カニクイザル iPS 細胞の体外培養下における自然分化

研究成果の刊行に関する一覧表

発表者氏名	論文タイトル名	発表誌名	巻号	ページ	出版年
Sakurai F, Nakamura S-I, Akitomo K, Shibata H, Terao K, Kawabata K, Hayakawa T, Mizuguchi H.	Adenovirus serotype 35 vector-mediated transduction following direct administration into organs of nonhuman primates.	Gene Therapy	16	297-302	2009
Tanaka Y, Ikeda T, Kishi Y., Masud S, Shibata H, Takeuchi K, Komur M, Iwanak T, Muramatsu S, Kondo Y, Takahashi K, Yamanaka S, Hanazono Y.	ERas is expressed in primate embryonic stem cells but not related to tumorigenesis.	Cell Transplant.	18	381-389	2009
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Miki H, Wakisaka N, Inoue K, Ogonuki N, Mori M, Kim JM, Ohta A, Ogura A.	Embryonic rather than extraembryonic tissues have more impact on the development of placental hyperplasia in cloned mice.	Placenta	30	543-546	2009

Kim J-M & Ogura A.	Changes in allele-specific association of histone modifications at the imprinting control regions during mouse preimplantation development.	Genesis	47	611-616	2009
Inoue K, Ogonuki N, Mekada K, Yoshiki A, Sado T, Ogura A.	Sex-reversed somatic cell cloning in the mouse.	J Reprod Dev	55	566-569	2009
Shimozawa N, Nakamura S, Takahashi I, Hatori M, Sankai T.	Characterization of a novel embryonic stem cell line from an intracytoplasmic sperm injection-derived blastocyst in the African green monkey.	Reproduction	139	565-573	2010
山海　直	医科学への応用を目的としたサル類の発生工学的研究	霊長類研究	24	357-366	2009

Gene Therapy (2009) 16, 297–302

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SHORT COMMUNICATION

Adenovirus serotype 35 vector-mediated transduction following direct administration into organs of nonhuman primates

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Adenovirus (Ad) serotype 35 (Ad35) vectors have attracted remarkable attention as alternatives to conventional Ad serotype 5 (Ad5) vectors. In a previous study, we showed that intravenously administered Ad35 vectors exhibited a safer profile than Ad5 vectors in cynomolgus monkeys, which ubiquitously express CD46, an Ad35 receptor, in a pattern similar to that in humans. However, the Ad35 vectors poorly transduced the organs. In this study, we examined the transduction properties of Ad35 vectors after local administration into organs of cynomolgus monkeys. The vectors transduced different types of cells depending on the organ. Hepatocytes and microglia were mainly transduced after the vectors were injected into the liver and cerebrum, respectively. Injection of the vectors into the femoral muscle resulted in the transduction of cells that appeared to be fibroblasts and/or macrophages. Conjunctival epithelial cells showed transgene expression following infusion into the vitreous body of the eyeball. Transgene expression was limited to areas around the injection points in most of the organs. In contrast, Ad35 vector-mediated transgene expression was not detected in any of the organs not injected with Ad35 vectors. These results suggest that Ad35 vectors are suitable for gene delivery by direct administration to organs.

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Keywords: *adenovirus serotype 35 vector; local administration; nonhuman primate; CD46*

Adenoviruses (Ads) are nonenveloped, double-stranded DNA viruses with icosahedral symmetry. To date, 51 human adenovirus (Ad) serotypes have been identified and classified into six species.[1,2] Among these serotypes, Ad serotype 5 (Ad5), which belongs to species C, is the basis of almost all the Ad vectors commonly used, including those used in clinical trials. Conventional Ad5 vectors have several advantages as gene delivery vehicles. However, it is now well established that the hurdles to Ad5 vector-mediated gene therapy are the high seroprevalence to Ad5 in adults and the refractoriness of cells lacking the expression of coxsackievirus-adenovirus receptor, which is a primary receptor for Ad5, to Ad5 vectors. Pre-existing anti-Ad5 immunity significantly decreases the transduction efficiencies of Ad5 vectors. Even when an Ad5 vector-based vaccine was administered locally into muscle, pre-existing anti-Ad5 antibodies reduced its efficacy.[3,4] A lack of coxsackievirus-adenovirus receptor expression renders the cells unsusceptible to Ad5 vectors at least *in vitro*. Important target cells for gene therapy, including hematopoietic stem cells and dendritic cells, often poorly express coxsackievirus-adenovirus receptor. In addition to these drawbacks, Ad5 vectors have high hepatic tropism. Even when Ad5 vectors are locally injected into a diseased area (for example, a tumor), they are drained from the injection sites into the systemic circulation and primarily transduce hepatocytes because of their high hepatic tropism; on the other hand, efficient transduction is obtained around the injection points. When Ad vectors carry a transgene that exerts cytotoxic effects on transduced cells, Ad vector-mediated hepatic transduction leads to severe hepatotoxicity.[5–7]

In contrast, human species B Ad serotype 35 (Ad35) vectors, which our group and several others have developed,[8–11] possess attractive properties that can overcome the drawbacks of conventional Ad5 vectors. First, Ad35 vector-mediated transduction is not hampered by anti-Ad5 antibodies, because Ad35 belongs to a different species (species B) than Ad5 (species C). Second, Ad35 vectors bind to human CD46 as a receptor.

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Human CD46 is expressed on almost all human cells, leading to broad tropism of Ad35 vectors in human cells, including coxsackievirus-adenovirus receptor-negative cells.[8,12] However, intravenous administration of Ad35 vectors resulted in inefficient transduction in the organs of human CD46-transgenic (CD46TG) mice and cynomolgus monkeys, which express CD46 in a pattern similar to that of humans.[13–15] These results indicate that CD46 does not successfully serve as a receptor for intravascularly injected Ad35 vectors and that Ad35 vectors are unsuitable for intravascular transduction. However, this property of Ad35 vectors would suggest a potential advantage, in that unwanted transduction would not occur in organs other than the organs targeted following direct injection of Ad35 vectors when draining from injected sites into the bloodstream. These properties suggest that Ad35 vectors would be suitable for gene transfer by local administration into the organs. In this study, we examined the transduction properties of Ad35 vectors following intraorgan administration in nonhuman primates, that is, cynomologus monkeys.

A previously constructed Ad35 vector expressing β-galactosidase (Ad35LacZ)[15] was locally administered at a dose of 1.5×10^{11} vector particles (VP) per point (high dose) or 3×10^{10} VP per point (low dose) in the following eight organs of two cynomolgus monkeys (designated no. 8 and no. 9; no. 8 received the high dose of Ad35LacZ and no. 9 received the low dose): liver, cerebrum, eyeball (vitreous body), quadriceps femoris muscle, pancreas, kidney, spleen and nasal cavity. Four days after administration, the tissues around the injection sites (approximately $40 \times 40 \times 10$ mm^3 with a central focus at the injection point) were collected and subjected to an analysis of β-galactosidase expression and histological pathology. The health condition of the monkeys was also monitored until necropsy.

Overall, both monkeys did well during the experiment. There were no apparent abnormalities in body temperature or heart rate, although no. 8, the high-dose monkey, exhibited slight reductions in blood pressure and body weight. Both monkeys apparently exhibited increased serum levels of aspartate aminotransferase and creatine phosphokinase on days 0–2 after injection. Mild decreases in hemoglobin levels and increases in levels of lactate dehydrogenase and C-reactive protein were also found in both animals. However, these changes were probably due to the operation. The levels of alanine aminotransferase, alkaline phosphatase, albumin, glucose, calcium, chloride and sodium in the serum were mostly within the normal ranges.

After the direct injection of the Ad35 vectors, the transduction profiles were assessed by immunostaining of β-galactosidase in the tissue sections; Table 1 summarizes the results. A detailed transduction profile in each organ is described below.

Liver

Direct injection of Ad35LacZ to the liver caused tissue damage around the injection site (Figures 1a and b). Infiltration of inflammatory cells, necrotic focus and regenerated bile duct epithelial cells were observed. Immunostaining of the liver sections revealed that hepatocytes were mainly transduced with Ad35LacZ in both no. 8 and no. 9 monkeys (Figures 2a and b). A higher level of β-galactosidase was expressed in the liver of no. 8 than in that of no. 9. The transduced cells were predominantly distributed around the injection point (approximately 1×1 mm^2) and were not found outside the periphery of the injection site. β-galactosidase was not expressed in the liver lobes, which were not injected with Ad35LacZ. β-galactosidase-expressing cells were mainly found on the border region between the normal and damaged areas. Direct injection of naked plasmid DNA or Ad5 vectors into mouse liver also resulted in the localized distribution of transgene-expressing cells around the injection points.[16,17] The liver would not allow dispersion of locally injected Ad vectors in the tissue.

Table 1 β-galactosidase expression in the organs following direct injection of Ad35LacZ into organs

	No. 8 (high dose)	*No. 9 (low dose)*
Liver	+++	+
Cerebrum	+++	+
Eyeball	+	−
Femoral muscle	+	+
Pancreas	−	++
Kidney	−	++
Spleen	−	−
Nasal cavity	−	−

+++, strong positive; ++, moderate positive; +, weakly positive; −, negative.

Cerebrum

Ad35LacZ was stereotaxically injected into the left frontal lobe of the cerebrum. After infusion of the high dose of Ad35LacZ, softening of the tissue, which appeared necrotic, was widely observed in the left basal ganglia (Figure 1c). Neutrophils were infiltrated into the necrotic area. In contrast, injection of a low dose of Ad35LacZ resulted in no apparent toxicity, although slight bleeding was found around the artery (Figure 1d). Transduced cells, which appeared to be microglia, were found around the softening regions of both no. 8 and no. 9 animals, although the latter had fewer transduced microglia (Figures 2c and d). There were no β-galactosidase-expressing cells in the right hemisphere of the brain, which was infused with phosphate-buffered saline buffer (data not shown).

Eye

Ad35LacZ was infused into the vitreous body for inoculation into the eyeball. The high dose induced invasion by inflammatory cells, including macrophages and neutrophils, into the ciliary body, iris and retina (Figure 1e). Necrotic changes were also found in all layers of the retina. The low dose caused similar damage to the eyeball. The high dose mediated transduction in the conjunctival epithelial cells (Figure 2e). β-galactosidase expression was not observed in other areas. After injection into the vitreous body, Ad35LacZ might be drained from it and transduce the conjunctival epithelial cells. Bora *et al.*[18] demonstrated that human CD46 was hardly expressed in eye tissues, suggesting that these tissues are refractory to Ad35 vectors. We did not find β-galactosidase expression in the eye of no. 9 animal. Phosphate-buffered saline injection did not result in

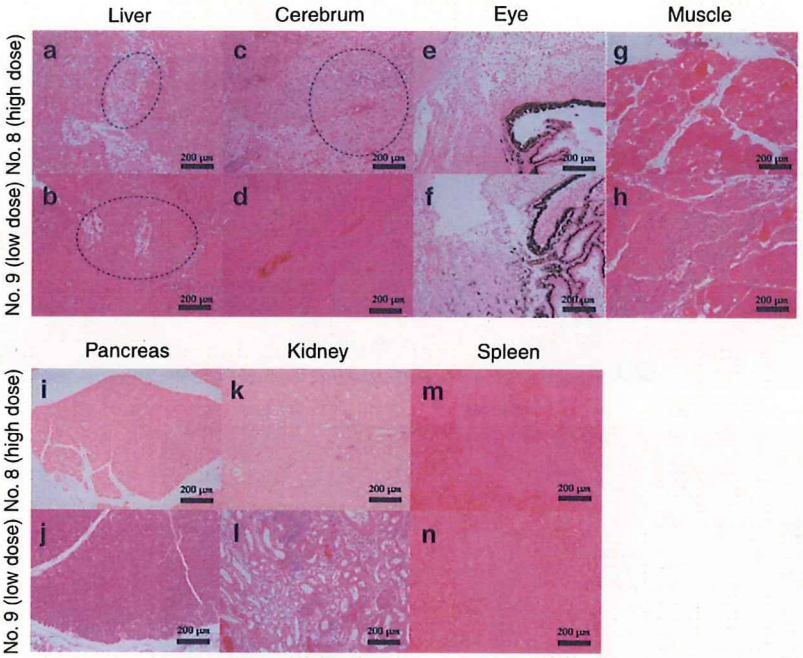

Figure 1 Tissue histology in the organs of cynomolgus monkeys 4 days after intraorgan injection of Ad35LacZ. (**a** and **b**) The liver, (**c** and **d**) cerebrum, (**e** and **f**) eyeball, (**g** and **h**) skeletal muscle, (**i** and **j**) pancreas, (**k** and **l**) kidney and (**m** and **n**) spleen. Young male cynomolgus monkeys (*Macaca fascicularis*) were housed and handled in accordance with the rules for animal care and management of the Tsukuba Primate Center and with the guiding principles for animal experiments using nonhuman primates formulated by the Primate Society of Japan. The animals (approximately 3 years of age, 1.9 and 2.2 kg) were certified free of intestinal parasites and seronegative for simian type-D retrovirus, herpesvirus B, varicella-zoster-like virus and measles virus. The protocol of the experimental procedures was approved by the Animal Welfare and Animal Care Committee of the National Institute of Biomedical Innovation (Osaka, Japan). The liver, cerebrum, eyeball, nasal cavity, pancreas, kidney, skeletal muscle and spleen of cynomolgus monkeys were each injected with Ad35LacZ suspended in 200 μl (100 μl for eyeball) of phosphate-buffered saline at a dose of 1.5×10^{11} vector particles (VP) per point (monkey no. 8) or 3×10^{10} VP per point (monkey no. 9). Four days after injection, tissue sections were hematoxylin–eosin stained by a routine method. Dotted-line circles in (**b**) and (**c**) indicate the necrotic area in the liver and the softening area in the cerebrum, respectively.

transgene expression or apparent abnormality in the eyeball (data not shown).

Femoral muscle

Severe inflammation did not occur after intramuscular injection of the high dose, although we found slight damage to the muscle fibers (Figure 1g). In contrast, the low dose induced more severe inflammation (Figure 1h). Infiltration of neutrophils and macrophages was seen in the muscle of no. 9. It is currently unclear why the low dose induced higher levels of damage. A slight difference in the injection point might affect Ad35 vector-induced inflammatory responses in the muscle. β-galactosidase expression was found only in the cells that appeared to be macrophages and/or fibroblasts located among the muscle fibers in both monkeys (Figures 2g and h). No muscle fibers expressed β-galactosidase in either monkey. It remains to be elucidated why intramuscular injection of Ad35 vectors mediated poor transduction in muscle fibers of cynomolgus monkeys. Ad35 vectors transduced the muscle following intramuscular injection in wild-type mice and in CD46TG mice.[12,14] The transduction mechanism and efficiencies of Ad35 vectors in muscle fibers might differ among species, and the muscle of nonhuman primates might be more refractory to transduction than that of rodents. Thirion *et al*.[19] demonstrated that Ad vectors would transduce human, rat and mouse primary muscle cells through different pathways. Danko *et al*.[20] reported that transgene expression levels by intramuscular injection of naked DNA were lower in dogs and nonhuman primates than in rodents. On the other hand, several studies demonstrated the utility of Ad35 vectors as vaccine vectors that express antigen by intramuscular administration in mice and nonhuman primates.[3,4] Macrophages and/or dendritic cells transduced with Ad35 vectors might play important roles in transgene-specific immune responses by intramuscular injection of Ad35 vectors.

Pancreas

Injection into the pancreas caused no severe damage to that organ in either monkey (Figures 1i and j). We did not find transduced cells in the pancreas of no. 8; in contrast, β-galactosidase was apparently expressed in exocrine acinar cells of no. 9 in the pancreatic lobules (Figures 2i and j). Chemiluminescence assay of β-galactosidase also revealed significant levels of β-galactosidase expression in the pancreas of no. 9 but not in that of no. 8 (data not shown). Wang *et al*.[21] also demonstrated that direct injection of conventional Ad vectors and adenoassociated virus vectors into murine pancreas achieved efficient transduction in acinar cells. Pancreatic acinar cells would be susceptible to Ad vectors.

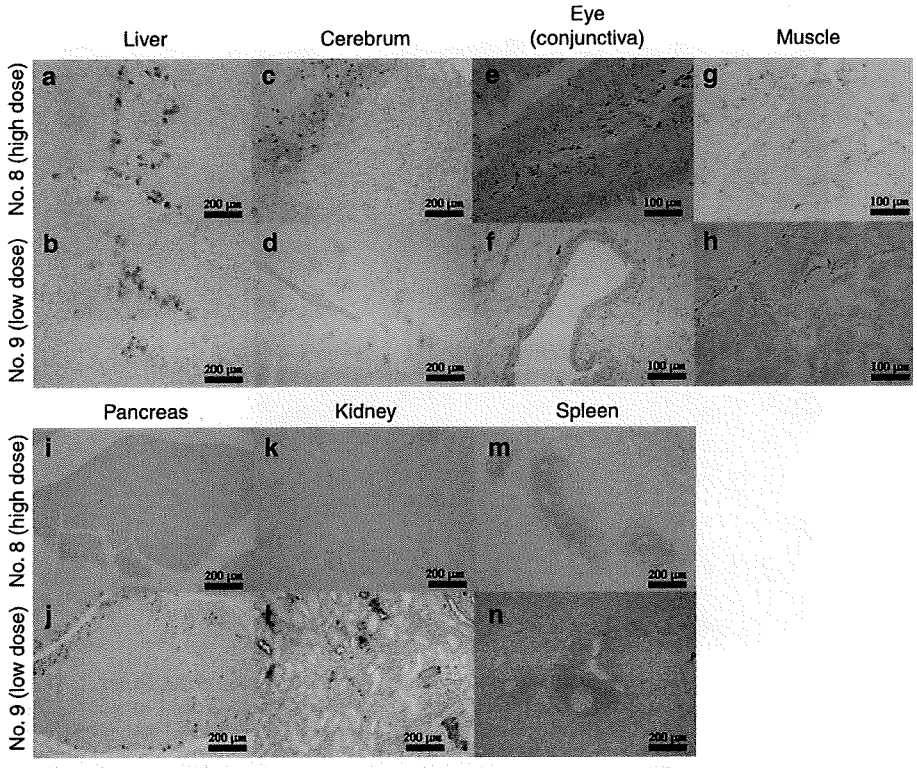

Figure 2 β-galactosidase expression in the organs of cynomolgus monkeys 4 days after intraorgan injection of Ad35LacZ. (**a** and **b**) The liver, (**c** and **d**) cerebrum, (**e** and **f**) eyeball, (**g** and **h**) skeletal muscle, (**i** and **j**) pancreas, (**k** and **l**) kidney and (**m** and **n**) spleen. Ad35LacZ was locally administered in the organs of cynomolgus monkeys at the low (3×10^{10} vector particles (VP) per point) or high dose (1.5×10^{11} VP per points) as described in Figure 1. Four days after injection, the tissues were collected for analysis of β-galactosidase expression and histological pathology. Immunostaining of β-galactosidase was performed using anti-β-galactosidase antibody (Abcam, Cambridge, UK).

Kidney

Ad35LacZ injection to the left kidney induced infiltration by inflammatory cells, including lymphocytes, into the interstitial tissue of the kidney (Figures 1k and l). The right kidney, which was injected with phosphate-buffered saline, did not exhibit β-galactosidase expression or inflammatory responses (data not shown). The high dose did not mediate β-galactosidase expression, but the low dose led to apparent transduction (Figures 2k and l). The renal tubular epithelial cells were mainly transduced with Ad35LacZ. In the kidney, compared with the other organs, transduced cells were more widely spread around the injection points. Refractoriness to the high dose and massive β-galactosidase expression by the low dose in the pancreas and kidney together form a major conundrum in this study. The differences in transduction efficiencies might be due to the slight differences in injection sites. Especially, Ad35LacZ may have been drained into the renal tubule of no. 9 following injection into the kidney, leading to efficient transduction in the renal tubule epithelial cells. Ad35 was originally identified in the kidney and causes cystitis,[22] indicating the tropism of Ad35 for renal epithelial cells.

Spleen and nasal cavity

Unexpectedly, direct injection of Ad35LacZ to the spleen did not induce inflammatory responses such as hyperplasia (Figures 1m and n). There was no β-galactosidase expression in the spleen of either monkey (Figures 2m and n). For transduction in the mucosal membrane of the nasal cavity, Ad35 vector suspensions were instilled into the nasal cavity of each monkey, but neither one showed β-galactosidase expression or cellular damage in the mucosal membrane of the nasal cavity (data not shown).

Other organs

β-galactosidase production in the lung, heart, thymus, bone marrow, lymph node, bladder and testis, which were not injected with Ad35LacZ, were examined by chemiluminescence assay. None of these organs showed detectable β-galactosidase expression (data not shown).

Next, we determined the blood concentrations of Ad35LacZ genome DNA in the blood using quantitative real-time PCR to examine whether or not Ad35LacZ locally injected to the organs was drained from the injection site into the bloodstream. The Ad35 vector DNA was detected in the blood as soon as 6 h post-injection, then gradually decreased (Figure 3). However, the blood-clearance kinetics of Ad35LacZ following intraorgan injection were slower than those following intravenous administration, which were previously reported,[23] although the total amounts of Ad35 vector doses in this study (no. 8: 1.5×10^{11} VP $\times$ 8 points; no. 9: 3×10^{10} VP $\times$ 8 points) were comparable to or lower than those in the previous study in which Ad35LacZ was intravenously infused in cynomolgus monkeys ($0.4–2 \times 10^{12}$ VP per kg, 1.88–2.96 kg).[23] Ad35 vector

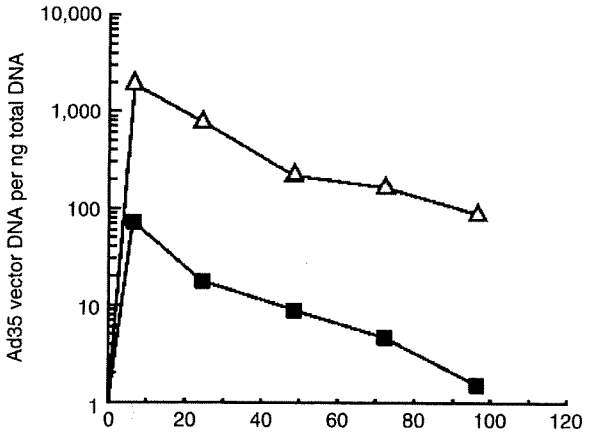

Figure 3 Blood concentrations of Ad35 vectors in cynomolgus monkeys following intraorgan administration. Ad35LacZ was locally administered in the organs of cynomolgus monkeys at the low (3×10^{10} vector particles (VP) per point, closed square) or high dose (1.5×10^{11} VP per points, open triangle) as described in Figure 1. Blood was collected at the indicated post-injection time points (6, 24, 48, 72 and 96 h post-injection). Total DNA, including Ad vector DNA, was isolated from the blood, and the Ad vector DNA contents were measured by quantitative TaqMan PCR assay, as previously described.[23]

DNA was still detectable 4 days after injection. These results suggest that Ad35 vectors or Ad35 vector DNA remaining in the injection sites might be gradually released from the injection sites and drained into the bloodstream.

Furthermore, to examine whether or not Ad35LacZ draining into the bloodstream was accumulated in the organs, we determined the Ad35 DNA contents in the portions of the liver and spleen that were away from the respective injection sites. The liver and spleen play crucial roles in the clearance of systemically injected Ad vectors. The Ad35 vector DNA was not detected in those portions of the liver in no. 9, but was detected in the portions of the liver in no. 8 and in those of the spleen in both monkeys (data not shown). These results suggest that Ad35LacZ or the Ad35 vector DNA draining into the systemic circulation would be taken up by the liver and spleen. We further assessed the Ad35 DNA contents in the lungs, heart, thymus and bone marrow, in which Ad35 vectors were not directly infused. Ad35 vector DNA was detected in the lungs and heart of no. 8 but not in those of no. 9 (data not shown). We did not detect Ad35 vector DNA in the thymus or bone marrow of either monkey. Considering that intravascularly injected Ad35 vectors did not efficiently transduce organs,[15] organs must not be transduced with Ad35LacZ, which is drained into the bloodstream and taken up by the organs.

In most cases of cancer gene therapy using Ad vectors, the vectors are administered directly to the tumor regions.[24–26] When used as vaccine vectors, on the other hand, Ad vectors are intramuscularly injected.[27,28] In addition, Ad vectors are intramyocardially injected in angiogenic gene therapy.[29,30] Thus, direct infusion of Ad vectors to organs is one of the most frequent application methods in clinical settings. However, there has been little information about the transduction properties of these vectors following direct injection into organs. This study demonstrated that different types of cells were transduced with Ad35 vectors depending on the organ after direct infusion into the organ. The differences in the histological structures and cell types comprising the organs would explain the differences in transduction properties of locally injected Ad35 vectors. This study provides important information for clinical study by intraorgan injection of Ad35 vectors, although the characteristics of the organs (structure, cell types and so on) differ different between normal tissue and diseased areas.

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ERas Is Expressed in Primate Embryonic Stem Cells But Not Related to Tumorigenesis

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The ERas gene promotes the proliferation of and formation of teratomas by mouse embryonic stem (ES) cells. However, its human orthologue is not expressed in human ES cells. This implies that the behavior of transplanted mouse ES cells would not accurately reflect the behavior of transplanted human ES cells and that the use of nonhuman primate models might be more appropriate to demonstrate the safety of human ES cell-based therapies. However, the expression of the ERas gene has not been examined in nonhuman primate ES cells. In this study, we cloned the cynomolgus homologue and showed that the ERas gene is expressed in cynomolgus ES cells. Notably, it is also expressed in cynomolgus ES cell-derived differentiated progeny as well as cynomolgus adult tissues. The ERas protein is detectable in various cynomolgus tissues as assessed by immunohistochemisty. Cynomolgus ES cell-derived teratoma cells, which also expressed the ERas gene at higher levels than the undifferentiated cynomolgus ES cells, did not develop tumors in NOD/Shi-*scid*, IL-2Rγ^{null} (NOG) mice. Even when the ERas gene was overexpressed in cynomolgus stromal cells, only the plating efficiency was improved and the proliferation was not promoted. Thus, it is unlikely that ERas contributes to the tumorigenicity of cynomolgus cells. Therefore, cynomolgus ES cells are more similar to human than mouse ES cells despite that ERas is expressed in cynomolgus and mouse ES cells but not in human ES cells.

Key words: Embryonic stem cell; ERas; Cynomolgus monkey; Tumorigenesis

INTRODUCTION

The ERas gene promotes the growth of and formation of teratomas by mouse ES cells by producing a constitutively active ERas protein and is not expressed in mouse ES cell-derived differentiated progeny or mouse tissues (22). Disruption of the ERas gene in mouse ES cells by homologous recombination results in a significantly reduced proliferation rate and a reduced tumorigenic potential without loss of pluripotency (22). Although the ERas gene is expressed in divergent species such as mice, dogs, and cows, it is not expressed in humans (1,12,17). Its inactivation is likely a relatively recent event in mammalian evolution. It is intriguing to speculate that some of the differences in the proliferation rate or other properties of mouse and human ES cells (9,13) are related to the differences in expression of this constitutively active ERas gene. It may also imply that the behavior of transplanted mouse ES cells does not accurately reflect the behavior of transplanted human ES cells and that the use of nonhuman primate models (11,21,25) would be more appropriate to demonstrate the safety (tumorigenicity) of human ES cell-based therapies (12). However, the expression of the ERas gene has not been examined in nonhuman primate ES cells (19,24). Here, we show that the ERas gene is expressed

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in cynomolgus ES cells unlike human ES cells. In addition, the ERas gene is widely expressed in adult cynomolgus tissues. However, its forced expression in cynomolgus cells even at high levels was not related to tumorigenesis. Therefore, cynomolgus ES cells are more similar to human than mouse ES cells despite that ERas is expressed in cynomolgus and mouse ES cells but not in human ES cells.

MATERIALS AND METHODS

Cell Culture and Differentiation

A cynomolgus ES cell line (CMK6) (19), its subline (CMK6G) stably expressing enhanced green fluorescent protein (EGFP) (20), and a human ES cell line (SA181, Cellartis AB, Göteborg, Sweden) (10) were maintained on a feeder layer of mitomycin C (Kyowa, Tokyo, Japan)-treated mouse (BALB/c, Clea, Tokyo, Japan) embryonic fibroblasts (MEFs) as previously described (10,19). Confluent ES cells were dissociated from the feeder layer using 0.1% collagenase type IV (Invitrogen, Carlsbad, CA, USA). Cynomolgus stromal cells were obtained from cultured adherent cells of cynomolgus bone marrow.

For neural differentiation from cynomolgus ES cells, astrocyte-conditioned medium (ACM) was prepared by culturing astrocytes obtained from mouse fetal cerebra in Dulbecco's modified Eagle's medium (DMEM)/F12 medium containing an N2 supplement (Invitrogen) (15). Colonies of cynomolgus ES cells (800–1000 μm in diameter) were plucked from the feeder layer using a glass capillary and transferred into nonadhesive bacteriological dishes each containing ACM supplemented with 20 ng/ml of recombinant human fibroblast growth factor-2 (FGF-2) (R&D, Minneapolis, MN, USA). The colonies were cultured for 12 days, giving rise to neural stem cells, which were plated onto poly-L-lysine/laminin (Sigma-Aldrich, St. Louis, MO, USA)-coated dishes and cultivated for 7 days in Neurobasal medium supplemented with 2% B-27 (both from Invitrogen), 20 ng/ml of FGF-2, and 20 ng/ml of recombinant human epidermal growth factor (R&D). After the medium was replaced with ACM and 14 days of culture, the neural stem cells differentiated into neurons (16).

ERas Cloning and Transfection

Based on the human ERas cDNA sequence in Genbank (accession No. NM 181532), the primer set 5′-CAT GGA GCT GCC AAC AAA GCC TG-3′ and 5′-TGT GTC CCT CAA AGC TAG TTG CCT-3′ was designed for the cynomolgus ERas' complete coding sequence. Total RNA was extracted from cynomolgus ES cells using the EZ1 RNA universal tissue kit (Qiagen, Hilden, Germany) with RNase-Free DNase Set (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, Foster, CA, USA). The sequence analysis was performed with Genetyx-Mac software (Genetyx Corporation, Tokyo, Japan). We previously constructed the plasmids pPyCAG-EGFP-gw-IP (expressing EGFP) and pPyCAG-EGFP-gw-IP-mouse ERas (expressing EGFP-mERas) (22). The cDNA encoding the human or cynomolgus ERas gene was inserted into pPyCAG-EGFP-gw-IP to construct pPyCAG-EGFP-gw-IP-human ERas (expressing EGFP-human ERas) or pPyCAG-EGFP-gw-IP-cynomolgus ERas (expressing EGFP-cynomolgus ERas), respectively. The insert was confirmed by DNA sequencing. The plasmids were transfected into cynomolgus stromal cells using Lipofectamin 2000 (Invitrogen). The transfected cells were selected in the presence of puromycin (5 μg/ml).

Transplantation

Cells (1×10^7/site) were transplanted into the thigh muscle of immunodeficient NOD/Shi-*scid*, IL-2Rγ^{null} (NOG) mice which were purchased from Central Institute for Experimental Animals (Kanagawa, Japan). Cynomolgus ES cell-derived teratomas were generated after in utero transplantation of cynomolgus ES cells into fetal sheep as described previously (23). Adherent cells from the teratomas were propagated in DMEM (Sigma-Aldrich) supplemented with 10% fetal bovine serum (FBS) (HyClone, Logan, UT, USA). All experiments 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.

Reverse Transcription (RT)-PCR

cDNA was prepared from each sample as mentioned above and subjected to PCR with the following primer sets: for Oct-4, 5′-GGA CAC CTG GCT TCG GAT T-3′ and 5′-TTC GCT TTC TCT TTC GGG C-3′; and for glyceraldehyde-3-phosphate dehydrogenase (GAPDH), 5′-CCC TGG CCA AGG TCA TCC ATG ACA AC-3′ and 5′-CCA GTG AGC TTC CCG TTC AG-3′. Amplification conditions were 30 cycles of 95°C for 60 s, 58°C for 60 s, and 72°C for 60 s. PCR without the initial RT was also conducted to rule out DNA contamination. For real-time quantitative RT-PCR, a QuantiTect SYBR Green PCR kit (Qiagen) and the ABI Prism 7000 (Applied Biosystems) were used, and amplification conditions were 40 cycles of 95°C for 60 s, 58°C for 60 s, and 72°C for 60 s. The gene expression levels were adjusted based on those of the internal control GAPDH.

Immunoblotting

Preparation of cell lysates and Western blot analyses were performed as described previously (22). Briefly, cells (1×10^7) were washed twice with ice-cold phosphate-buffered saline (PBS) and suspended in the buffer containing 10 mM Tris-HCl (pH 7.5), 1 mM $MgCl_2$, and the 1× Complete Protease Inhibitors (Roche, Basel, Switzerland), followed by incubation on ice for 30 min. These samples were disrupted with Dounce tissue homogenizer (Wheaton, NJ, USA) and added with 1 M NaCl to a final concentration of 150 mM. For lysis, these samples were added with a final concentration of 5% SDS, 1% NP-40, and 1% deoxicorate acids, followed by incubation on ice for 10 min. The samples were then centrifuged at $12,000 \times g$ for 30 min at 4°C, and supernatants were collected. The protein concentrations were measured by the Bio-Rad Bradford assay (BioRad Laboratories, CA, USA). An equal amount of protein (10 μg per lane) was electrophoresed on 12.5% polyacrylamide gel (Atto, Tokyo, Japan) and blotted onto a PVDF membrane (Immobiron; Millipore, MA, USA). The membrane was blocked by TBST containing 5% w/v Amersham ECL-Blocking Agent (GE Healthcare, Buckinghamshire, UK) and then incubated with rabbit antiserum against mouse ERas (22) overnight at 4°C. After washed with TBST, the membrane was incubated with anti-rabbit IgG HRP (Jackson Laboratory, CA, USA) at room temperature for 60 min. Signal was detected using Amersham ECL Plus Western Blotting Detection reagents (GE Healthcare) according to the manufacturer's protocol and visualized on the LAS 3000 mini (Fujifilm, Tokyo, Japan).

Immunohistochemistry

For the immunofluorescent staining of frozen sections, tissues were fixed with 4% paraformaldehyde. The sections were labeled with rabbit anti-serum against mouse ERas (22). The primary antibody (Ab) was detected with a Tyramide Signal Amplification Kit (Invitrogen). After nuclei were stained with DAPI (Donjindo, Kumamoto, Japan), the sections were observed with a confocal laser scanning microscope (Olympus, Tokyo, Japan). For immunohistochemistry, tissues were fixed with 4% paraformaldehyde and embedded in paraffin. To identify GFP-positive cells, the sections were stained with rabbit anti-GFP Ab (Clontech, Palo Alto, CA, USA), reacted with the Dako EnVision+ System HRP (Dako, Copenhagen, Denmark), and visualized with 3,3′-diaminobenzide tetrahydrochloride (Dojindo). Nuclei were counterstained with hematoxylin.

Flow Cytometry

The expression of GFP and ERas was analyzed using a FACS Calibur flow cytometer (BD Pharmingen, San Diego, CA, USA). To detect ERas, cells were fixed using fixation/permeabilization buffer (eBioscience, San Diego, CA, USA) for 2 h at 4°C and then incubated with Alexa Fluor 647 (Invitrogen)-conjugated rabbit antiserum against ERas for 60 min at 4°C. Data acquisition and analysis were performed using CellQuest software (BD Pharmingen). Fluorescence-conjugated, irrelevant Abs served as negative controls.

Cell Proliferation Assay

Total cell numbers and proliferating cell numbers were measured with Cell Counting Kit-8 (Dojindo) and with Cell Proliferation ELISA, BrdU (colorimetric) (Roche), respectively. For Cell Counting Kit-8, cells were seeded in 96-well plates at 5×10^3 per well and measured after 6, 24, 36, 48, and 80 h of incubation according to the manufacturer's instructions. For Cell Proliferation ELISA, cells were seeded in 96-well plates at 2×10^3 per well and measured after 24, 36, 48, 72, and 96 h of incubation. Significant differences were examined using the *t*-test.

RESULTS AND DISCUSSION

Cynomolgus Cells and Tissues Express ERas

We first cloned and sequenced the cynomolgus orthologue of the ERas gene from cDNA of the undifferentiated cynomolgus ES cells (Fig. 1A). The translated amino acid sequence showed a higher degree of homology to human than mouse ERas (99% vs. 75%) (Fig. 1B). We could not detect the ERas protein in cynomolgus ES cells by flow cytometry, implying its weak expression (data not shown).

Next, we examined the expression of the ERas gene in adult cynomolgus tissues by RT-PCR. Using a primer set to cover the entire coding region, the amplicons from all nine somatic tissues were the same size as that from cynomolgus ES cells (Fig. 2A). The PCR product was sequenced to confirm that it was the ERas gene. This clearly shows that a full-length version of the ERas gene is transcribed in cynomolgus tissues, despite that ERas is not expressed in mouse tissues (22).

We then examined the protein expression in adult cynomolgus tissues by immunohistochemistry. Immunoblotting revealed that the antibody reacts to human and cynomolgus ERas as well as mouse ERas, although the antibody was generated against recombinant mouse ERas (22) (Fig. 2B). In addition, it specifically reacts to ERas (25 kDa) and does not react to other Ras family proteins (N-, H-, or K-Ras; 21 kDa) in cynomolgus cells expressing these Ras genes (Fig. 2B). Using this antibody, we detected ERas-positive cells in all tissues tested (brain, thymus, intestine, and ovary) (Fig. 2C). At a higher magnification, it turned out that ERas is localized on the cytoplasmic membrane as expected (Fig.

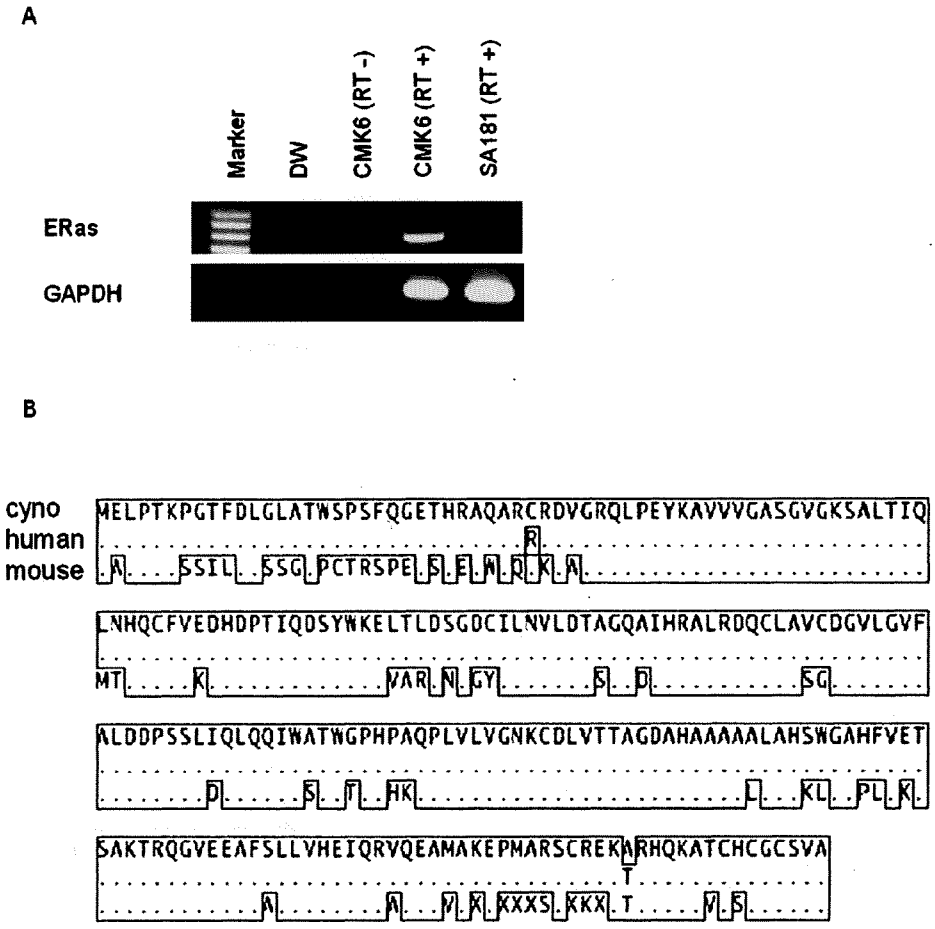

Figure 1. Cloning and sequencing of cynomolgus ERas. (A) The expression of the cynomolgus (cyno) ERas gene is detectable in cynomolgus ES cells (CMK6), but not in human ES cells (SA181), by reverse transcription (RT)-PCR. To exclude the possibility of genomic DNA contamination, PCR without the RT procedure (designated RT –) was also conducted. DW (distilled water) indicates no template in the reaction. RT-PCR of the GAPDH sequence is also shown as an internal control. (B) Amino acid sequences of cynomolgus, human (accession No. NM 181532), and mouse (accession No. NM 181548) ERas are shown. Amino acids identical to cynomolgus ERas are shown as dots and conserved amino acids are encircled with a solid line.

2D). We also tested mouse tissues, but ERas-positive cells were not detectable (Fig. 2C). Taken together, the ERas protein is indeed expressed in cynomolgus tissues, unlike in murine tissues.

The expression of the ERas gene becomes undetectable after the differentiation of mouse ES cells (22). We examined the expression of the ERas gene after the differentiation of cynomolgus ES cells. Cynomolgus ES cell-derived neurons and teratoma cells were examined for the expression of ERas as well as Oct-4, a pluripotent marker of ES cells. They were positive for ERas but negative for Oct-4 (Fig. 3A). Although cynomolgus ES cell-derived neurons were fragile and scarcely survived after dissociation from the culture dish, adherent teratoma cells could be cultured for more than six passages at a dilution of 1:4 to 1:8. Quantitative RT-PCR showed that the ERas gene expression levels were even higher in the cultured teratoma cells than in the undifferentiated cynomolgus ES cells (Fig. 3B). Then, we transplanted 1×10^7 cultured teratoma cells expressing the ERas gene (GFP-positive, passage 3) into the thigh muscle of NOG mice ($n = 3$), and examined the tumorigenicity of the cells. NOG mice were used as recipients, because they are more immunodeficient than other immunodeficient mice and transplanting to NOG mice is the most sensitive assay to detect tumorigenesis (7,14). However, no tumor developed after 2.5 months, although the transplanted cell progeny (GFP positive) were detected in every specimen (Fig. 3C). On the other hand, undifferentiated cynomolgus ES cells formed tera-

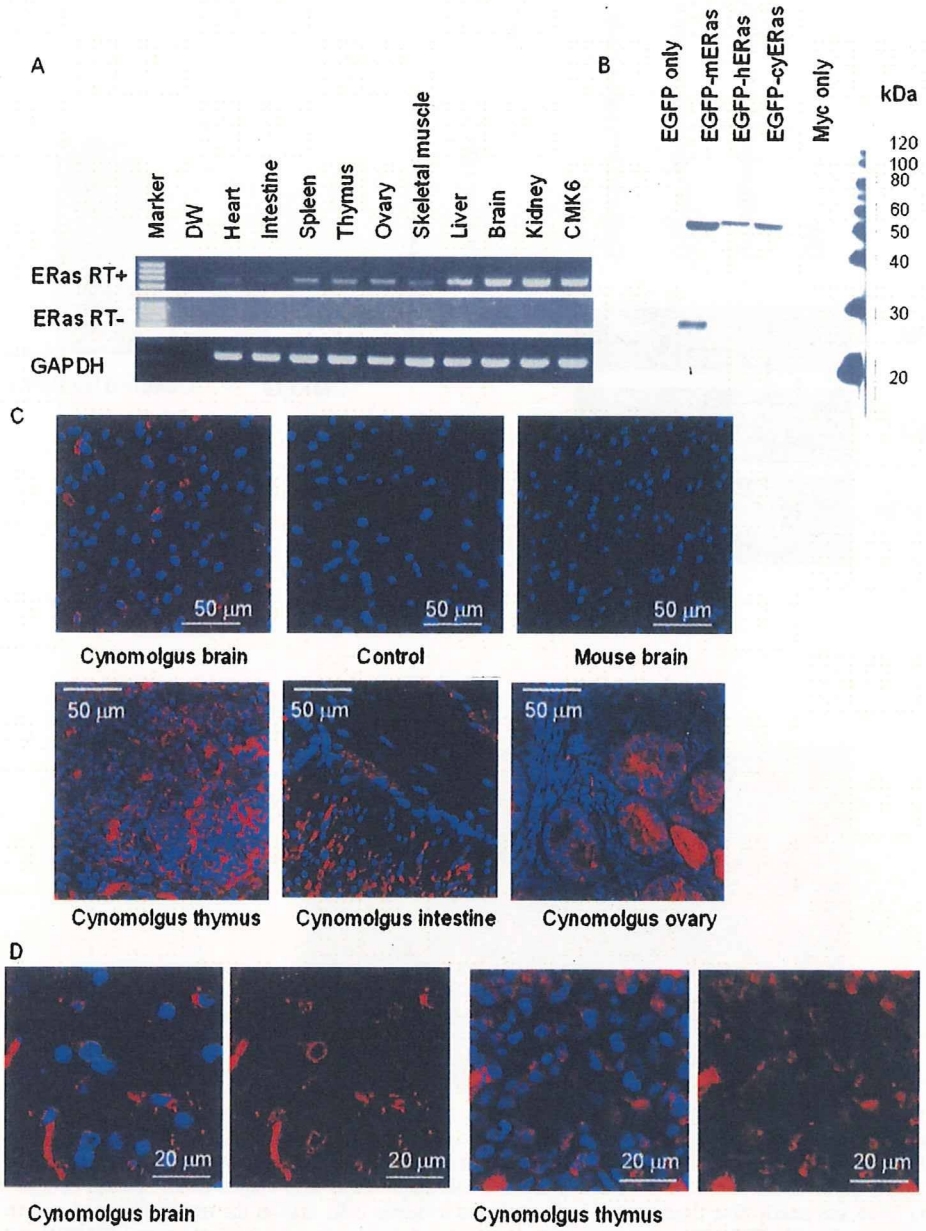

Figure 2. Cynomolgus tissues express ERas. (A) The expression of the cynomolgus ERas gene is detectable in various cynomolgus tissues by RT-PCR. To exclude the possibility of genomic DNA contamination, PCR without the RT procedure (designated RT –) was also conducted. DW indicates no template in the reaction. RT-PCR of the GAPDH sequence is also shown as an internal control. (B) Cynomolgus bone marrow stromal cells were transfected with the mouse ERas (mERas), human ERas (hERas), or cynomogus ERas (cyERas) gene fused with the EGFP gene, and immnunoblotted with the polyconal anti-ERas antibody. Although the antibody was generated against recombinant mERas (22), it reacts to hERas and cyERas as well as mERas. In addition, it does not react to cynomolgus other Ras family proteins (N-, H-, or K-Ras; 21 kDa). ERas (25 kDa) fused with EGFP (26 kDa) is detectable at 51 kDa. In mERas-transfected cells, ERas alone released from the fusion protein was also detected. (C) Using the anti-ERas antibody, ERas-positive cells (red) were detected in cynomolgus brain, thymus, intestine, and ovary. Control indicates the staining of cynomolgus brain without the primary ERas antibody. Although the primary antibody to ERas was originally developed for mouse ERas and should react more strongly to mouse than cynomolgus ERas, no positive signals were detected in mouse brain. (D) The ERas fluorescence with or without DAPI is shown at a higher magnification of both cynomolgus brain and thymus. ERas was detected on the cytoplasmic membrane.