つの処理方法で検討を行ったが、いずれにおいても胚盤胞への発生は認められなかった。昨年、Ionomycin(IM)＋ DMAP 処理では、胚盤胞への発生が認められたことを報告した。このため、クローン胚の活性化方法としては、Ionomycin＋ DMAP処理がより効果的なものであると考えられた。また、これら3つの処理間の違いは、活性化の引き金となる最初の処理(IM、IA および電気刺激)が違うだけである。しかし、これら処理の卵への効果としては、細胞内におけるカルシウムイオン濃度を上昇させ、活性化を誘起することである。この結果、卵の分裂中期での停止を誘導しているM期促進因子の活性を抑え、DMAP によりその再合成を阻害する。Ionomycin処理区において、胚盤胞が得られたことは、受精直後から初期の卵細胞質内で生じている現象を模倣でき、その結果、十分な発生能を誘起できたことが大きい要因の一つであるかもしれない。

次に薬剤による初期化誘導法の検討を行った。使用した TSA はマウスの核移植研究において、クローンマウスの作出およびクローン ES 細胞の樹立効率を上げるとの有効性が報告されている。ヒストン脱アセチル化酵素阻害剤である TSA は、遺伝子の転写を抑制する酵素の働きを阻害する作用を持つことから、クローン胚のドナーである体細胞核の遺伝子発現を初期胚時の状態に誘導するものと考えられる。今回、カニクイザル体細胞クローン胚に対して処理を行ったが、胚盤胞への発生は見られず、その効果が確認できなかった。しかし、マウスに対する有効性が示されていることから、カニクイザルにおいてもいくらかの効果を期待する事ができる。つまり、他の効果的と考えられる核移植関連技術と組み合わされることで、クローン胚の発生を改善することが期待できるものと考えられる。

受精卵を利用した核移植技術の検討に着手したところであるが、胚盤胞の前のステージである桑実胚への発生を確認する事ができた。これはヒトの不妊治療において廃棄される多精子受精卵をクローン研究に利用できるものとして、注目されている。今回カニクイザル体細胞核移植においては、一つの精子のみを顕微授精した受精卵を利用しているが、受精卵細胞質の基本的なレシピエントとしての能力に大差はないものと考えられる。また、精子によって正常な発生能を誘起された細胞質を利用している点も本法の利点として挙げられる。その結果、桑実胚へ発生したものと考えられ、本法はカニクイザルの体細胞核移植としても非常に期待できる技術である。しかし、受精卵の分裂中期を除核しなければならない。そのため、核膜の消失した状態であることから、その核の確認が非常に難しく、確実な除核法の確立という解決すべき課題を残した。今後さらに検討を重ね、本法の有効性を確認する予定である。

E. 結論

核移植によって構築されたクローン胚の活性化誘起法としては、Ionomycin＋ DMAP 処理が有効である。技術的にも検討を重ねる必要があるが、受精卵を利用した核移植方法は、カニクイザルの体細胞核移植において大いに期待できる成果が認められた。

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

厚生労働科学研究費補助金

(創薬基盤推進研究事業:生物資源・創薬モデル動物研究)

分担研究報告書

カニクイザル iPS 細胞樹立に関する検討

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研究要旨

体細胞由来クローン ES 細胞は免疫拒絶がない再生医療や遺伝性疾患の原因が解明できるものとして、その作製が大いに期待されている。しかし、卵の必要性から、ヒトにおいては倫理的な問題が大きい。最近、このクローン ES 細胞と同じ特徴を持つとされる人工多能性幹細胞(iPS 細胞)が樹立されたことから、ヒトと同じ霊長類であり、大型の実験動物であるカニクイザルにおいてもその細胞を作製することは、臨床応用に先だって安全性や効果に関した動物実験を行えることから、貴重な生物資源になり得る。本検討においては、その iPS 細胞をカニクイザルで作製することを目的とした。初期化を誘導する4つの遺伝子(Oct3/4, Sox2, Klf4, c-Myc)をレトロウィルスベクターによって、新生児皮膚由来細胞および胎児肝臓由来細胞に導入したところ、両者から ES 細胞のコロニーに非常に似た形態のコロニーを得た。これらの未分化マーカーの発現は、カニクイザル ES 細胞と同様な発現を示した。さらに、多分化能についても解析途中であるが、ES 細胞と同様な結果が得られている。以上、カニクイザルにおいても iPS 細胞が誘導できることを示唆するものである。

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的な拒絶を考慮せずに、供与個体に対して再生医療等の検討を行えること、あるいは疾患個体由来の細胞から作出されたときには、疾患原因の解明に利用できることなどの利点があることから、大変注目されている。しかし、クローン ES 細胞の低い作出効率をカバーするために、多くの卵子を必要とすることが、特にヒトにおいて倫理的な問題となっている。しかし、最近マウスやヒトの体細胞に人為的に遺伝子を組み込むことで、ES 細胞に類似した人工多能性幹細胞(iPS 細胞)が樹立された。つまりこれは卵を必要とせず、かつ体細胞を供与した個体と同じゲノムを持つことから、倫理的にも医学研究においてもセンセーショナルな報告であった。このため、本研究課題で作出することを目的とした体細胞クローン ES 細胞と同じ性格を持つとされるため、今年度からカニクイザル iPS 細胞の樹立も目的に追加した。クローン

ES 細胞と iPS 細胞は同じ性質を持つものと考えられているが、実際には確かめられていない。特に、人為的に誘導される iPS 細胞の性質や医科学研究へ応用した際の安全性・有効性は未知である。そのため、カニクイザルにおいて、クローン ES 細胞および iPS 細胞を樹立することで、両者の比較がヒトと同じ霊長類で、且つ大型実験動物であるカニクイザルで検討を行えるという利点がある。そこで、今年度はカニクイザル iPS 細胞の樹立を検討した。

B. 研究方法

iPS 細胞を誘導するために、体細胞の初期化を誘導する 4 つの遺伝子(Oct3/4, Sox2, Klf4, c-Myc)のクローニングを行った。これらの内、Sox2 および Klf4 は既にクローニングされていたため、残りの Oct3/4 および c-Myc をクローニングするために既に樹立されているカニクイザル ES 細胞をサンプリングした。次に、レトロウィルスベクターを産生させるために、Oct3/4, Sox2, Klf4 および c-Myc を個々に Plat-A 細胞に Fugene 6 を添加して、トランスフェクションした。また、遺伝子の導入効率を確認するために、別途 GFP 遺伝子も同様にトランスフェクションした。4 種の遺伝子のウィルス懸濁液を等量で混合し、ポリブレン加ウィルス混合液でカニクイザル体細胞を24時間培養した。カニクイザル体細胞には、新生児皮膚由来細胞(NSC)および胎児肝臓由来細胞(FHC)を用いた。数日後、両細胞ともに、マイトマイシン処理したマウス胎児線維芽細胞(MEF)上に播種した。

誘導された iPS 様細胞は ES 細胞と同様に培養および継代を行った。また、その未分化性を ES 細胞と同様な未分化マーカーで免疫蛍光染色を行って確認を行った。多分化能を確認するために、胚様体形成能およびその接着培養、さらにテラトーマ形成能についてはスキッドマウスへの移植を行った。

(倫理面への配慮)

本研究における動物実験は、独立行政法人医薬基盤研究所・動物実験委員会の承認を受けて実施した。また遺伝子操作については、独立行政法人医薬基盤研究所・組換え DNA 実験安全委員会の承認を受けて実施した。

C. 研究結果

カニクイザルにおいて、新規に Oct3/4 および c-Myc をクローニングした。これによって、体細胞の初期化を誘導する4つの遺伝子を整えた。実際に、レトロウィルスベクターによってカニクイザル体細胞への遺伝子導入効率を確認するために、これら4つの遺伝子導入とは別途 GFP 遺伝子の導入を行った。その結果、FHC で68%、また NSC で77%の GFP の発現を確認した。実際に、二種の体細胞において、ES 細胞に似た iPS 細胞様のコロニーが形成された。このコロニーをピペッティングで小塊にし、MEF 上に継代したところ、ES 細胞のコロニーに非常に類似したコロニーが形成された。これは iPS 細胞のコロニーと考えられたため、次に ES 細胞の樹立に際して行われている未分化性および多分化能の確認を行った。未分化性については、免疫蛍光染色により、Oct3, Nanog, SSEA4, TRA-60, および TRA-81 の発

現が両細胞種ともに確認できた。また、SSEA1およびSSEA3の発現は認められなかった。多分化能としては、まず胚様体形成を確認したところ、ES細胞と同様な形態の胚様体を形成した。さらに、これら胚様体を接着培養したところ、形態から判断して神経などの体細胞に分化していることが確認できた。テラトーマについては、現在免疫不全マウスに移植しており、その形成を確認中である。

これらの結果は、形成されたコロニーがES細胞と同様な性質を持つiPS細胞のコロニーであることを強く示唆するものである。

D. 考察

本検討において、体細胞クローンES細胞と同様な性質を持つと考えられるiPS細胞の樹立をカニクイザルで行った。

まず、初期化を誘導する4遺伝子の内、Oct3/4およびc-Mycを新たにクローニングすることに成功した。既にクローニングされていたSox2およびKlf4と併せて4つの遺伝子をNSCおよびFHCに導入を行い、iPS細胞の誘導が可能かどうかを検討した。しかし実際に遺伝子が導入されているか否かをiPS細胞が樹立できるか否かで判断することは正しいものではないと考えられることから、別途GFP遺伝子を導入して、その発現から遺伝子の導入効率を確認した。その結果、GFPの発現が比較的高い効率(NSC; 77%, HFC; 68%)で認められたことから、レトロウィルスベクターを介することで遺伝子が導入できることが確認された。

実際に両細胞種からES細胞に非常によく似た形態をもつコロニーが形成され、繰り返された継代によっても類似の形態を持つコロニーとして維持された。次にこれらの細胞株の特徴をES細胞と比較するために、未分化マーカーおよび多分化能を確認した。未分化マーカーについては、カニクイザルES細胞の発現様式と一致した。つまり、カニクイザルES細胞と同様な未分化状態にあることを示唆した。

次に、多分化能については、胚様体の作出とそれから各種体細胞への分化を確認すること、および免疫不全マウスへ移植し、三胚葉から成るテラトーマ形成を確認することが必要である。両者ともに現在確認中であるが、前者については一部確認することができた。すなわち、細胞株コロニーを浮遊培養したところ、ES細胞のものと類似した胚様体を形成することが確認できた。さらに、この胚様体を接着培養したところ、神経などの細胞種に分化していることが形態的に見ることができた。後者については、テラトーマ形成が確認されたところで組織標本を作製して、三胚葉性の細胞から成るものであるかを確認する。

以上、このように性状解析の途中であるが、非常にカニクイザルES細胞と同様な性状を示すことから、本検討によって誘導された細胞株はほぼiPS細胞であるものと考えられた。今後はテラトーマ解析、核型解析、遺伝子発現および導入遺伝子の組み込み状態などの解析を行う予定である。

E. 結論

iPS細胞の誘導をカニクイザルでも可能であることを示唆する結果が得られた。さらに、性状解析を行い、カニクイザルES細胞と同様な性

状を持つ細胞株であるかを調べる。

F. 健康危険情報

特になし

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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—Mini Review—

Reproductive Technologies and Related Studies in the Cynomolgus Monkey

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Abstract: *In mice, basic reproductive technologies, such as oocyte/sperm collection, embryo production, micromanipulation, and embryo transfer, have been established. With these technologies, production of transgenic mice has become routine. The cynomolgus monkey, which is one of the laboratory animals closest to human beings, has been used to obtain vaccine approval and for medical research. Therefore, production of transgenic animals in the monkey is a very significant subject. We describe herein the current state of related studies in addition to current findings regarding reproductive technologies.*
Key words: *Cynomolgus monkey, Non-human primate, Gamete, Micromanipulation, ES cells*

Introduction

The cynomolgus monkey (*Macaca fascicularis*) belongs to the group of catarrhine primates, the same as humans (*Homo sapiens*). Like humans, non-human primates such as cynomolgus, rhesus (*Macaca mulatta*) and Japanese (*Macaca fuscata*) monkeys, specifically Old World Macaques, have menstruation cycles, and the number of young delivered in these monkeys is usually one. Unlike rhesus and Japanese monkeys, which are seasonal breeders, the cynomolgus monkey and human are annual breeders, and the similarity between the two is very high. In many cases, microbiologically clean cynomolgus monkeys are used in various types of biomedical research, however, unlike mice, there are hardly any animal models for specific diseases in the monkey. In some cases, spontaneous, familial animals with the diseases under investigation are available.

Reproductive technologies, such as sperm/oocyte collection, embryo production, micromanipulation and embryo transfer, are necessary to propagate non-human primates with human pathology and to establish transgenic non-human primates. In view of the low production efficiency of transgenic animals, many oocytes or embryos and some recipients must be prepared for *in vitro* manipulations and for embryo transfer, respectively. Current reproductive technologies must be further modified and improved in the cynomolgus monkey. Of course, researchers must wait approximately 3 years for transgenic cynomolgus monkeys to be able to propagate after they are produced. However, such monkeys offer many benefits that more than offset the drawback of waiting such a long time until biomedical research can be carried out. In this review, we describe the current state of reproductive technologies and related studies (Fig. 1) that will greatly contribute to the development of biomedical research with regard to the cynomolgus monkey.

Basal Techniques for Manipulating Gametes

Many areas remain undeveloped with regard to basic reproductive technology for the monkey. Technological developments in these areas will greatly benefit researchers working in this field. Herein, we provide outlines of egg collection, sperm collection, *in vitro* fertilization (IVF) and embryo transfer.

Received: August 4, 2008
Accepted: August 18, 2008
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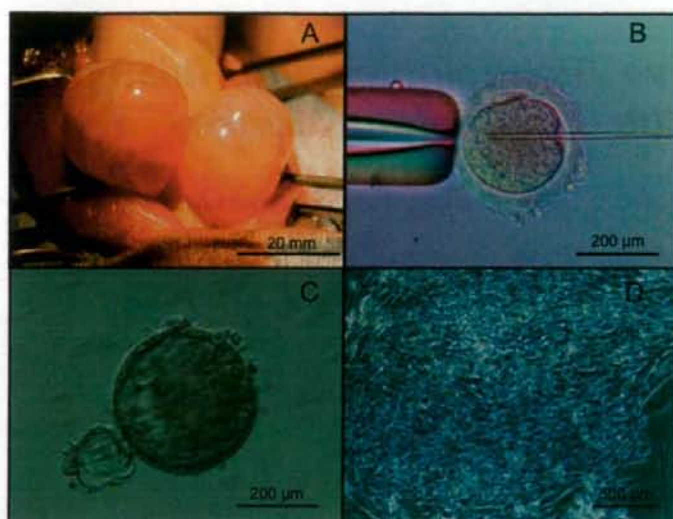

Fig. 1. Ovaries with developed follicles (A), ICSI (B), an ICSI-derived blastocyst stage embryo (C) and ES cells (D).
(A) Oocytes are collected from ovaries with developed follicles after hormone treatment. (B, C) Mature oocytes subjected to ICSI develop to blastocyst stage embryos. (D) ES cell lines are established from blastocyst stage embryos.

1) Collection of gametes and sperm cryopreservation

A newborn non-human primate was obtained by IVF and embryo transplantation in 1984. The technology for this procedure, however, remains unreliable, and overall, the results have been unsatisfactory. An important drawback of this technology is that the reproducibility and stability of the results for collecting good quality eggs is not perfect. Therefore, the uneven quality of the obtained eggs negatively impacts research results. Even if the same hormone processing is utilized, there are wide individual differences in the reactivity of the ovaries of monkeys. It is important in carrying out research to use animals with good-quality eggs that can be reliably obtained from hormone processing techniques. Although data concerning hormone processing have been reported since the 1980s, it is difficult to identify a particular technique that provides consistently reliable results.

The purposes of the hormone treatment are to induce follicle development and to mature it with regard to the egg in the follicle. We have executed some methods that use of variety of hormones such as FSH and recombinant FSH. It is possible that the effects of FSH are different in each lot because FSH is an animal extract that is subsequently refined. Moreover, it is thought that the molecular configuration of FSH differs in each type of animal, and it is uncertain whether or not the ovary reacts with FSH. Naturally, we agree that the reactivity is different in each type of monkey [1]. It has been confirmed that the ovaries of the cynomolgus monkey react to FSH very often and that FSH administration generates the growth of many ovarian follicles. Although only one ovarian follicle develops in a normal menstrual cycle, nearly 100 ovarian follicles develop with FSH administration. However, when we calculate the average number of eggs collected, the standard deviation is quite large, meaning that the individual differences are quite large. It is thought that the reason for this difference corresponds to the differences in the reactivity to FSH of each individual because individual differences are also observed when recombinant FSH is used. Moreover, the hCG is administered instead of endogenous LH, and it is necessary to determine the appropriate dosage of hCG for each type of animal. The appropriate hCG dosage for the monkey has not yet been determined, and the dosage used has generally been different for each researcher. Moreover, determining the timing of hCG administration is extremely difficult. The best time for hCG administration cannot be reliably determined

because the growth situation of the ovarian follicle is different in each individual even if the hormone administration method is the same. It is assumed that the eggs in the follicle develop from the GV stage to the MII stage in response to hCG administration. However, eggs can often be collected from other stages, such as the GV, MI and MII stages. These results indicate that there is a difference in the quality of granulosa cells even when they are within the ovary. Many problems continue to arise in hormone processing, which is a basic technology for egg collection crucial to executing the latest research.

The technology for sperm collection is reliable compared with that for egg collection. The animal is approached from the rectum, and electrical stimulation is given to the erection central nerve and ejaculation central nerve. This method is also effective under anesthesia, and it seems applicable to animals other than the monkey. It is also possible that the monkey can be trained to masturbate, as it has relatively high brain function. We gather sperm from cynomolgus monkeys by electrical stimulation of the rectum. The collected sperm is then used in reproductive experiments for IVF and intracytoplasmic sperm injection. In addition, cryopreservation technology is being used for monkey sperm development. Almost complete revitalization of sperm movement can be achieved after thawing, and IVF can be carried out successfully with these sperm [2].

This cryopreservation method is currently being used by various research laboratories. However, damage to the cell membrane of the sperm head has been observed by electron microscope after freezing and thawing [3]. It has been confirmed that this damage to the sperm head is similar to that occurring with the acrosome reaction, which is an indispensable phenomenon for fertilization. It therefore must be considered that the properties of the sperm head before and after freeze-thawing are different. However, it is possible to apply this method to various areas of research by providing accurate basal information for the sperm.

2) IVF and embryo transfer

It is well known that phenomena related to the acrosome reaction and capacitation of sperm are different in each type of animal, and it is unknown whether the present method used is the most suitable for the monkey. Furthermore, embryo transfer is also likely to be difficult. In 1984, the first newborn non-human primate was obtained by IVF and embryo transfer in monkeys [4–6]. This occurred 6 years after the same achievement in humans. Since then, 25 years have passed, but the number of research laboratories using this technology to actually obtain newborn monkeys by embryo transplantation remains limited. Considering the above-mentioned constraints regarding the technology, it is necessary to carry out basic technological development concurrently with more advanced research.

We have successfully carried out IVF in the cynomolgus monkey, African green monkey, and Japanese monkey [7–9], and a newborn cynomolgus monkey was obtained after the transfer of fertilized eggs to the oviducts of females and synchronization of the growth stages of the eggs and menstrual cycles of the females [10]. This procedure also included successful intracytoplasmic sperm injection (description following). Currently, fertilized eggs are generally created by IVF and intracytoplasmic sperm injection. Even if the results are considerably influenced by the quality of the eggs used and fertilization is confirmed, not all of the eggs develop well. Recently, more research has been carried out utilizing fertilized eggs. For instance, fertilized eggs are used for research regarding embryonic stem cells (description following) and gene manipulation. It is necessary to conduct this research from the perspective of also performing basic research.

The importance of these problems must be clear to all experienced researchers. However, because multiple factors are involved, including egg quality, composition of the culture solution and temperature, the problems are quite complex. There might be many researchers who do not evaluate the basic research because of a brilliant result reported in the monkey. However, this area of research cannot be avoided if we wish to continuously develop reliable technologies.

Progress cannot be achieved without carrying out basic research. It is necessary to conduct basic research that also supports continuing advanced research. The researchers must recognize enough that monkeys have a differentia and the individual difference and the result of showing of each individual is all true. It is necessary to synthesize and evaluate these results. Monkeys are important laboratory animals in medical research related to humans. Therefore, if there are individual differences between monkeys that affect research results, learning how to develop procedures that can handle these individual differences is significant for development of similar procedures in humans.

Micromanipulating Embryos and Gametes

As in the case of other mammalian species, manipulating the embryos and gametes of non-human primates under a microscope has attracted many researchers in the biomedical field. This type of study allows for exploration of the cellular and molecular basis of various assisted reproductive technologies (ART) and regenerative medicine in humans, while avoiding the ethical and practical issues of working with human oocytes and embryos. In this section, we review the development and present status of two micromanipulating techniques, microinsemination (ICSI) and nuclear transfer cloning, in non-human primates.

1) Microinsemination (ICSI)

Mammalian ICSI (intracytoplasmic sperm injection) started with use of the golden hamster by Dr. Yanagimachi's group in the 1970s [11, 12]. This species had been extensively used in the field of fertilization study because of the ease of superovulation, the clear cytoplasm of their oocytes and the easily visible acrosome reaction [13]. Their group demonstrated that a directly injected sperm head could transform into a male pronucleus and undergo DNA synthesis. However, the very strong developmental arrest of hamster embryos in vitro hampered further analysis of the developmental ability of the resultant embryos. The first ICSI babies in mammals were obtained in the rabbit in 1988 [14] and in the bovine in 1990 [15]. The birth of human ICSI babies has been reported as early as 1992 [16]. In primates, however, the development of ICSI techniques has not gone smoothly, probably because of the limited availability of fresh oocytes and recipient females for embryo transfer, which requires a large cohort of females at the appropriate reproductive ages. Fortunately, primate oocytes share several cellular and morphological similarities with human oocytes, and primate ART procedures are almost identical to those in humans. Therefore, primate ICSI may provide the best experimental model for the study of human ICSI without the complicated ethical and moral issues. In 1996, the first primate ICSI trials were reported by Hewitson *et al.*, who demonstrated that although the cellular process after ICSI basically mimics that occurring after IVF, occasional fertilization failure, including a lack of pronuclear formation and abnormal recondensation of chromosomes, may occur specifically in ICSI oocytes [17]. Subsequently, normal conception by ICSI in rhesus monkeys was reported by the same group; out of 14 embryos transferred, 5 developed to term, and 4 were born normally [18]. They argued that this rate was greater or equal to that reported in human clinics, although they raised some concerns about the behavior of the injected sperm nucleus, including abnormal sperm decondensation and remnants of sperm components inside the ooplasm. Following this success, ICSI in primates was put into practice, and the birth of babies was reported for rhesus monkeys and cynomolgus monkeys in other laboratories [19, 20]. ICSI is a very convenient way to produce fertilized oocytes and embryos, especially when the number of oocytes used is very limited. At present, therefore, monkey embryonic stem (ES) cells are primarily generated from ICSI-derived embryos [21–23].

Due to the advent of micromanipulation techniques, not only mature spermatozoa but also immature sperm cells (spermatids and spermatocytes) can be used as male gametes for conception. Studies with mice have played a leading role in the development of such microinsemination technologies using elongated spermatids, round spermatids and even secondary and primary spermatocytes [24–26]. The production of mouse offspring by round spermatid injection is easier than previously expected, and the technique has been routinely used in laboratories specializing in mouse ICSI [24]. The success of this technique is probably due to maternal (oocyte) inheritance of the microtubule organizing center (MTOC) in the mouse, which is completely different from the paternal (sperm) inheritance that occurs in other species [27]. Round spermatids in these species have not acquired the ability to form MTOC, and as Schatten's group predicted, this deficiency may lead to abnormal segregation of chromosomes at mitotic divisions. In fact, in rhesus monkeys, healthy offspring have been produced by injection of testicular spermatozoa or elongated spermatids, but not with round spermatids [28]. Only a mid-gestation fetus has been obtained by round spermatid injection in the cynomolgus monkey, and this fetus aborted at 103 days of pregnancy for unknown reasons (average pregnancy period: 165 days) [29]. This type of complicated development following round spermatid injection has commonly been reported in several species thus far; for example, rabbit embryos derived from round spermatids are associated with high rates of aneuploidy due, at least in part, to the inability of round spermatids to form MTOC after incorporation into the ooplasm [30, 31]. The incomplete capacity of round spermatids to activate oocytes might contribute to the poor development of round spermatid-

derived embryos. It is also possible that the genome of the round spermatid itself is not identical to that of mature spermatozoa because the male pronucleus from a round spermatid is prone to quick remethylation after fertilization, in contrast to the gradual demethylation occurring in the sperm-derived male pronucleus [32]. A small proportion of male-factor infertility in humans is thought to be affected by spermatogenic arrest at the round spermatid stage, although controversy remains regarding the accuracy of the investigation of testicular biopsy specimens [33]. If there are really cases that require round spermatid injection as a treatment, the safety and efficiency of this technique should be thoroughly clarified using primate models before its broad application to human clinics.

Genetically engineered animals offer opportunities for understanding the function of genes of interest, studying the pathogenesis and treatment of diseases and screening new chemicals for pharmaceutical purposes. An overwhelming majority of these animals are produced in mice due to the ease and efficacy of producing transgenic and gene-targeted mice. However, mouse models do not always provide sufficient information to extrapolate the data obtained to humans because the mouse and human are very different in certain physiological characteristics. It seems that primate models provide the best data in this respect, but conventional genetic modification technologies in the mouse, which allow for pronuclear DNA injection and generation of chimeric animals with embryonic stem (ES) cells, have not been successfully applied to primates. To overcome the low transgenic efficiency associated with pronuclear DNA injection, a method of retroviral mediated transgenesis into unfertilized oocytes followed by ICSI was developed in rhesus monkeys. In 2001, birth of a male baby carrying the green fluorescent protein (GFP) gene, named ANDi, was reported, although he did not express the characteristic green fluorescence for unknown reasons [34]. More recently, the technique was improved by using a lentiviral vector instead of a retroviral vector, and transgenic rhesus monkeys carrying mutant human Huntington gene were successfully produced [35]. The resulting transgenic monkeys showed important clinical features of Huntington's disease, including dystonia and chorea, which did not appear in mouse models. Thus, monkey transgenesis has become practically available based on efficient ICSI techniques.

2) Nuclear transfer cloning

Cloning animals using somatic cells shows great promise in the field of basic biology as well as for industrial and clinical purposes. However, the early studies of animal cloning by nuclear transfer were carried out using nuclei from preimplantation embryos as donors, probably because the reprogramming event of the blastomere nuclei is less complicated than that of somatic cell nuclei. This is also the case with monkeys; the first cloned rhesus monkey was produced from a 16-cell embryonic nucleus [36], while embryos from somatic cells developed only up to the 8-cell stage. Until now, no cloned monkey has been obtained by somatic cell nuclear transfer. However, since mouse ES cell lines were generated from somatically cloned mouse embryos (ntES cells [37]), cloning researchers in primatology have shifted their interest from cloning monkeys to the generation of ntES cells from cloned blastocysts as an experimental model for human regenerative medicine. Cloned mouse embryos develop into blastocysts very efficiently (30–70%), and many of these blastocysts contribute to ES cell establishment [38, 39]. It has also been demonstrated in mice that severe immunodeficiency can be cured completely by transplantation of hematopoietic cells differentiated from gene-transfected ntES cells [40]. However, it is extremely difficult to culture cloned monkey embryos to the blastocyst stage because most of them exhibit developmental arrest at the 8–16 cell stages. As this type of developmental failure has never been reported for other mammalian species, it may be a feature unique of primates, including humans. Only one group has thus far succeeded in generating ntES cells in rhesus monkeys, and the efficiency is still very low (2 lines from 213 reconstructed embryos) [41]. The very poor development of cloned monkey embryos may be attributed to chromosomal instability due to the removal of NuMA, nuclear mitotic apparatus protein, at the time of enucleation [42]. However, their consistent developmental arrest at certain cleavage stages (8–16 cell stages) is reminiscent of the so-called "developmental block" at the maternal to zygotic transition. It is very probable that the cloned embryos fail to activate zygotic genes due to incomplete reprogramming of the somatic donor genome. Thus, cloned primate embryos may suffer from genetic as well as epigenetic insufficiencies. It has recently been reported that genomic reprogramming of reconstructed embryos can be enhanced by treatment with chromatin-modifying chemicals, including histone deacetylase inhibitors [43]. At present, there are many chromatin-modifying chemicals, and each has its own functional mechanisms; some, therefore, may have a significant

effect on primate nuclear transfer. Trials of these types of chemicals require further effort, but this kind of primate research may open up a new area of regenerative medicine in humans.

ES Cells

The ES cell line in mammals was established from mouse blastosyst embryos for the first time in 1981 [44, 45]. The somatic and germ cells of ES cell origin were included in progeny produced from mouse embryos into which ES cells were injected. Using this property, many transgenic mice, including gene-targeted mice, have begun to be produced. However, the introduction of ES cells in the human and monkey has attracted attention as a tool in medical applications not intended to produce transgenic animals. Moreover, many basic and application studies for regenerative medicine have begun to be carried out because induced pluripotent stem (iPS) cells similar to ES cells have recently been established in humans, but not monkeys [46]. It is important that the safety of this technique in monkeys be established before direct application to humans. Herein, we primarily review ES cells in the cynomolgus monkey.

1) Establishment and characteristics

Primate ES cell lines were established in the rhesus monkey for the first time in the United States in 1995 [47] and were subsequently established in humans in 1998 [48]. In Japan, they were established in the cynomolgus monkey in 2001 because the monkey is widely used for biomedical research [22]. Monkey ES cell lines are different from mouse ES cell lines but are very similar to human ES cell lines [49–51]. In addition, the diversity of the genetic background of the embryos that are the origins of the ES cell lines may create delicate differences in properties among primate ES cell lines [52–54].

Blastocyst stage embryos derived from *in vivo* or *in vitro* fertilization (including ICSI) are used to establish cynomolgus monkey ES cell lines. Each inner cell mass (ICM) is isolated by a method involving either a combination of antiserum and complement (immunosurgery) or injection needles. Isolated ICM is cultured on a mouse embryonic fibroblast (MEF) cell monolayer. The adherent ICM is gradually extended as a colony. By day 8–10 of each culture, the colony is passaged onto a fresh MEF cell monolayer. The passage is performed by dividing the colony into some clusters with collagenase-based solution and injection needles. Primate ES cells usually fail to form colonies following dissociation into single cells when using trypsin-based solution. Consequently, maintenance of cynomolgus monkey ES cell lines is performed by dividing the colony into small clusters with pipetting following dissociation with either collagenase-based solution or low concentrations of trypsin and collagenase-based solution. It has recently been reported that treatment of single human ES cells with the Rho-associated kinase inhibitor, Y-27632, greatly improves colony development [55]. This result is important for researchers attempting large-scale culture in primate ES cells because a large number of differentiated cells of ES cell origin are necessary for regenerative medicine.

Characterization of ES cells involves consideration of the following three key factors: self-renewal, pluripotency and normal karyotype. In particular, pluripotency, which means the ability to differentiate to various cells, is a very important property when considering medical applications. Pluripotent ES cells must be in an undifferentiated state, which can be shown by confirming expression such as for Oct3/4, Nanog, Sox2, SSEA1, SSEA3, SSEA4, TRA-1-60, TRA-1-81, and alkaline phosphatase. These expression patterns differ between primates and mice. In primates, all of the above except for SSEA1 are expressed, while in mice, SSEA3, SSEA4, TRA-1-60 and TRA-1-81 are not expressed. However, the expression of SSEA3 varies in primates. Both rhesus monkey and human ES cells express SSEA3, but cynomolgus monkeys do not. These differences may not be important because it has been reported that the expressions of SSEA3 and SSEA4 are not essential to maintaining the pluripotency of ES cells [56]. Mouse ES cell lines should differentiate into germ cells, but in primates, this differentiation is not essential. Examining whether ES cells differentiate into germ cells via chimeras is difficult in primates because they have a long life cycle and only delivery one baby per pregnancy. In particular, it is impossible to ethically clarify this issue in humans. Instead, it is necessary to be able to develop teratomas. A teratoma is a tumor consisting of tissues from three embryonic germ layers (mesoderm, endoderm and ectoderm) that develops by transplantation of ES cells into immunodeficient mice. Recently, stem cells (EpiS cells) from the epiblast of postimplantation mouse embryos have been established [57, 58]. The colony morphology of these cells resembles that of primates. EpiS cells also have the ability to develop teratomas, but not to form chimeras. These findings may suggest

Table 1. Comparison of reproductive technologies and related studies in monkeys (Macaques), mice and rabbits

	Monkeys (Macaques)	Mice	Rabbits
Oocyte collection	Anesthesia Follicular aspiration	Euthanasia Ovulation	Anesthesia or Euthanasia Ovulation
Good quality oocytes	A few	Many	Medium
Sperm collection	Anesthesia Electroejaculation	Euthanasia Cauda epididymis	Using an artificial vagina
IVF	Difficult	Easy	Easy
ICSI	Difficult	Easy	Easy
Embryo culture	Difficult	Easy	Easy
ES cells	A little difficult	Easy	A little difficult
Embryo transfer	Difficult	Easy	Easy
ntES cell	Very difficult	Relatively easy	Difficult
Transgenic animals	Very difficult	Relatively easy	Difficult
Cloned animals	Very difficult (no report)	Difficult	Difficult
Contribution to medicine	Very high	High	High
Ethical issues	Very high	High	High

that primate ES cells do not have the ability to differentiate into germ cells. Therefore, the strategy of producing transgenic cynomolgus monkeys via chimeras may not be a wise choice.

2) Application

Some clinical applications have been advanced by translational research using cynomolgus monkeys. Thus, the expectations for regenerative medicine using ES cells have risen rapidly, and results have been reported in not only mice but also in monkeys. Future developments could lead to the availability of cells differentiated *in vitro* from ES cells for regenerative medicine. However, the pluripotency of ES cells involves a large risk. Teratomas may develop after transplantation into the body if undifferentiated cells are present in the population of differentiated cells. To avoid this risk, a previous study used a cell sorter to remove cells found to be positive for an undifferentiated marker, SSEA4, and the population of selected differentiated cells was then transplanted into cynomolgus monkeys. It was then confirmed that teratomas did not develop in the monkeys [59].

Moreover, the function of the transplanted cells has also been investigated. A transplant experiment using dopaminergic precursor cells from ES cells was conducted in cynomolgus monkeys with artificially induced Parkinson's disease. Transplantation resulted in improvement of symptoms [60]. This demonstrates that transplanted cells engrafted and differentiated into dopaminergic neurons. In addition, research to induce differentiation into cells such as hematopoietic or retina pigment epithelial cells has also been conducted using cynomolgus monkey ES cells [61, 62].

Next, we look at regenerative medicine from the perspective of immunology. Although it is relatively difficult for immunorejection to occur in the brain, it is often necessary to consider avoidance of this type of immunorejection. The nuclear transfer cloning technique (ntES cells), which we have already described, will contribute greatly to this end. Effective treatment free from immunorejection can be expected if various cells derived from the somatic cells of individuals are transplanted into the body. An examination of therapeutic methods using ntES cells has already been performed in mice [40]. Establishment of somatic cell-cloned ES cell lines has recently been reported in rhesus monkeys for the first time [41]. This report leads us to expect that these cells lines could be established in the cynomolgus monkey. Further developments in embryo technology for the cynomolgus monkey are necessary to produce novel biological resources for the medical sciences.

Conclusion

We reviewed recent findings regarding collection of oocytes and sperm, production of fertilized embryos, micromanipulation, and ES cells in the cynomolgus monkey, which is an experimental animal closely related to humans. Comparison of items contained in this review among monkeys (Macaques), mice and

rabbits is summarized in Table 1. We believe that the technologies related to the monkey are immature. However, the monkey, with its history of use for official approval of vaccines and preclinical studies, is indispensable to biomedical research and safety testing. The expectation of producing transgenic individuals is great for these types of monkey. It is hoped that to achieve this purpose, the various techniques related to gametes, embryos, and ES cells in the cynomolgus monkey described in this review will be investigated in detail and will continue to be improved.

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Article

Stable embryonic stem cell lines in rabbits: potential small animal models for human research

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Abstract

Although embryonic stem (ES) cell lines derived from mice and primates are used extensively, the development of such lines from other mammals is extremely difficult because of their rapid decline in proliferation potential and pluripotency after several passages. This study describes the establishment of rabbit ES cell lines with indefinite proliferation potential. It was found that the feeder cell density determines the fate of rabbit ES cells, and that maximum proliferation potential was obtained when they were cultured on a feeder cell density of one-sixth of the density at confluency. Higher and lower densities of feeder cells induced ES cell differentiation or division arrest. Under optimized conditions, rabbit ES cells were passaged 50 times, after which they still possessed high telomerase activity. This culture system enabled efficient gene transduction and clonal expansion from single cells. During culture, rabbit ES cells exhibited flattened monolayer cell colonies, as reported for monkey and human ES cells, and expressed pluripotency markers. Embryoid bodies and teratomas formed readily *in vitro* and *in vivo* respectively. These ES cell lines can be safely cryopreserved for later use. Thus, rabbit ES cells can be added to the list of stable mammalian ES cells, enabling the rabbit to be used as a small animal model for the study of human cell transplantation therapy.

Keywords: *blastocyst, embryonic stem cells, pluripotency, rabbit, teratoma*

Introduction

As embryonic stem (ES) cells are pluripotent and are able to self-renew indefinitely, they have potential as donors for cell transplantation therapy and as source material for the production of genetically modified animals (Wang and Zhou, 2003; Downing and Battey, 2004; Shufaro and Reubinoff, 2004). Although ES cell lines have been derived from mice, monkeys and humans and are used extensively, the development of such lines from other mammals requires further technical improvement. In pigs, for example, more than 30 attempts to establish ES cells have been published, but no cell lines are available because the culture conditions required to prevent spontaneous differentiation and senescence in inner cell mass (ICM)-derived cells have not been identified (for review, see Vackova *et al.*, 2007).

Laboratory rabbits have long been used in biomedical research. Recently, they have been used as experimental models for human diseases such as atherosclerosis, myocardial infarction,

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hypercholesterolaemia, hypertension, and bone and cartilage disorders (Rudert *et al*., 2002; Weekers *et al*., 2002; Bösze *et al*., 2003; Shiomi *et al*., 2004). Many of these diseases are lifestyle related, and their prevalence is increasing rapidly in developed countries. Rabbit models are important because the disease aetiologies exhibited by humans are more similar to those exhibited by rabbits than those exhibited by mice. Rabbits are larger than mice, which makes surgery easier and enables large samples to be obtained. Another advantage of the rabbit as a laboratory species is that reproductive techniques such as superovulation, IVF (Chang *et al*., 1959), sperm injection (Hosoi *et al*., 1998; Ogonuki *et al*., 2005), embryo cryopreservation (Kasai *et al*., 1992), sperm freeze-drying (Liu *et al*., 2004), and nuclear transfer cloning (Chesné *et al*., 2002; Inoue *et al*., 2002; Yang *et al*., 2007) are well established. Rabbit ES cells would be invaluable for the study of human diseases using gene-targeted technology and for testing stem cell therapies for human applications. Although many attempts have been made to derive ES cell lines from rabbits, none has been successful (Cole *et al*., 1964, 1966; Graves and Moreadith, 1993; Fang *et al*., 2006; Wang *et al*., 2007).

This study was undertaken to determine whether an efficient and reproducible technique for the establishment of rabbit ES cells could be developed by optimizing culture conditions. It was found that the density of feeder cells determines the fate of rabbit ES cells and that stable rabbit ES cell lines can be derived and propagated if the density of feeder cells is optimized.

Materials and methods

Derivation of rabbit ES cell lines

Mature Japanese White rabbits were purchased from Kitayama Labes (Nagano, Japan). Fertilized rabbit embryos were obtained from mature females that had been treated with 75 IU of FSH (Fertinorm P; Serono, Tokyo, Japan) and 100 IU of human chorionic gonadotrophin (HCG; Gonatropin; Teikoku Zoki, Tokyo, Japan) at an interval of 48 h or 72 h. The does were mated with fertile males immediately after HCG treatment. Twenty-two hours after mating, fertilized embryos (zygotes) were flushed from the oviducts using warmed HEPES-buffered RD medium (Carney and Foote, 1991) containing 4 mg/ml of bovine serum albumin (ICN Biomedicals, Irvine, CA, USA). The embryos were cultured in microdrops (16 µl, five embryos per drop) of fresh RD medium covered with mineral oil at 38°C under 5% O_2 and 6% CO_2 in air. In preliminary experiments, a number of embryos failed to hatch, or hatched only after a considerable delay; therefore, in subsequent experiments hatching was assisted by making a hole in the zona, using a Piezo micromanipulator, near the space between two blastomeres when the embryos reached the 2-cell stage. This facilitated hatching of blastocysts (see subsequent discussion). They were then cultured for 48 h until the early to middle blastocyst stages. Blastocysts escaped from the zona pellucida through the hole when medium was introduced into the perivitelline space using a Piezo micromanipulator (Yamagata *et al*., 2002). With this method, zona pellucida-free blastocysts were easily collected and could be used immediately for ES cell derivation. They were transferred to a four-well dish (Nunc, Roskilde, Denmark) and cultured on mitomycin C-treated ICR mouse embryonic fibroblast (MEF) cells at a concentration of 36 × 10^3 cells/cm^2. The culture medium (rESM) consisted of 78% DMEM/F-12 (Invitrogen, Carlsbad, CA, USA), 20% knockout serum replacement (KSR; Invitrogen), 2 mmol/l GlutaMax (Invitrogen), 1% non-essential amino acids, 0.1 mmol/l β-mercaptoethanol, 10^3 IU/ml ESGRO (murine leukaemia inhibitory factor; Invitrogen), and 8 ng/ml human recombinant basic fibroblast growth factor (Wako, Osaka, Japan). Six to 8 days after the initial plating, the outgrowth of the ICM was removed, dissected mechanically, and transferred to a four-well dish containing fresh feeder cells. Passage of ES-like cells was performed by incubating the cells with 0.05% trypsin for 1 min at room temperature, and mechanically disaggregating the resulting small clumps into single cells. Cells were then counted in a haemocytometer, resuspended, and plated at a density of 3 × 10^3 cells/cm^2 in culture medium. ES cell lines were obtained after five to eight passages. Fresh medium was added daily and cells were passaged every 3–4 days.

Detection of undifferentiated markers

Marker expression was analysed by fixing rabbit ES cells attached to the bottom of the culture plates in 4% paraformaldehyde for 15 min at room temperature, followed by and washing twice (5 min each) in phosphate-buffered saline (PBS) containing 0.05% Tween-20 (PBST). For permeabilization, cells were treated with 0.1% Triton X-100 in PBS for 10 min and washed twice with PBST. Cells were incubated in blocking solution (3% bovine serum albumin in PBS) for 30 min. The following primary antibodies were used: anti-SSEA1, anti-SSEA3, and anti-SSEA4 from Developmental Studies Hybridoma Bank (Iowa City, Iowa, USA), anti-Oct4 from Santa Cruz Biotechnology (Santa Cruz, CA, USA), and anti-NANOG from COSMOBIO (Tokyo, Japan). All antibodies were diluted in blocking solution (SSEA1 and Oct4, 1:500; SSEA3, SSEA4, and NANOG, 1:100) and incubated with samples overnight at 4°C. The next day, cells were washed three times with PBST and incubated with secondary antibodies at 4°C for 3 h. The following secondary antibodies were used: FITC-conjugated goat anti-mouse IgG from Zymed laboratories (San Francisco, CA, USA) and FITC-conjugated goat anti-mouse IgM, FITC-conjugated goat anti-rabbit IgG, and FITC-conjugated rabbit anti-goat IgG from Sigma (Saint Louis, Missouri, USA). Cells were washed three times with PBST, after which fluorescent signals were analysed.

To detect alkaline phosphatase activity, rabbit ES cells were stained using an alkaline phosphatase kit (Sigma) according to the manufacturer's protocol.

Karyotype analysis

Rabbit ES cells in the log growth phase were incubated with colcemid (final concentration = 100 ng/ml) for 4 h at 37°C in 6% CO_2. Cells were trypsinized and pelleted at 120 *g* for 3 min, resuspended in 6 ml of 75 mmol/l KCl, and incubated at 37°C for 15 min. Cells were centrifuged at 120 *g* for 3 min and then fixed using a 50% Carnoy's solution (acetic acid to methanol ratio = 1:3). The centrifugation and fixing steps were repeated three times. During the last repeat, cells were kept in the Carnoy's solution and dropped onto glass slides. Chromosome spreads were stained with Giemsa solution. At least 20 metaphase spreads were counted for each rES-like cell line.