through the degradation of viral proteins. Recently, MDM2 was also reported to ubiquitinate HIV-1 Tat protein and activate its transcriptional activity in a non-proteolytic manner [26]. Our experiment using MDM2 knockdown macrophages showed that HIV-1 replication in these macrophages was more efficient than in control siRNA-transfected macrophages. These data are consistent with MDM2 negatively regulateing HIV-1 replication through Vif ubiquitination (Fig. 5C). However, the replication efficiency of HIV-1 in MDM2 knockdown macrophages was only 2-fold higher and was slower than in control siRNA-transfected macrophages. This suggests the possibilities that the ubiquitination of Tat might work as a positive regulatory factor at an earlier phase of infection and that MDM2 might be involved in both positive and negative regulation of HIV-1 replication at different stages. Further studies on the detailed effect of MDM2 on HIV-1 replication are needed.

We also demonstrated that Vif can bind MDM2 directly. We also mapped the interaction domain of MDM2 with Vif to amino acids 168–320 which is located in its central acidic and Zn finger domains. This central domain is different from the primary p53-binding site of MDM2 which is located in its N-terminal region; however, this central deomain was recently reported as a second p53-binding site and was shown to be important for the regulation of p53 stability [27-30] (Fig. 2B &2C). Interestingly, several proteins including p300, $p14^{ARF}$, and pRB bind to the central domain of MDM2 and regulate the stability and function of p53 via MDM2 [28,31]. Thus, it is possible that Vif might affect the stability and function of p53. Indeed, we confirmed that Vif can stabilize p53 (*Izumi et al., unpublished data*), which could explain why the effect of MDM2 on p53 degradation was weaker than that on Vif as shown in Fig. 1A. A further study is under way to elucidate this new function of Vif (*Izumi et al., HIV-1 Vif induces G2 cell cycle arrest via the p53 pathway, unpublished*).

Finally, expanding evidence suggests that the ubiquitination system plays important roles in many aspects of HIV-1 replication including the degradation of A3G by Vif [9-11], the degradation of CD4 by Vpu [32], HIV-1 viral budding [33], Tat-mediated transactivation [26], and Vpr-induced G2 cell cycle arrest [34,35]. The functional linkage between Vif and MDM2 also suggests that ubiquitin processes such as the A3G/Vif interplay is highly complex. It is obvious that HIV-1 replication in target CD4+ T cells is strongly affected by the interplay of these proteins. From the viral point of view, this interplay might give an advantage to HIV-1 replication. One possibility is that MDM2 regulates cellular Vif levels appropriately, such as not to affect viral replication [36] but just enough to antagonize A3G. Recent studies suggest that the G-to-A mutations induced by A3G may not be the mechanism by which A3G restricts or controls viral replication [37] and that a partially effective Vif inhibitor may actually accelerate the evolution of drug resistance and immune escape [38]. The inhibitory activity of MDM2 toward Vif could be partially effective and therefore could lead to viral evolution of drug resistance and immune escape. More recently, Nathans et al. have reported a small molecule that specifically antagonizes Vif function and inhibits viral replication by targeting the A3G/Vif axis. This compound enhances Vif degradation only in the presence of A3G, but does not induce A3G degradation and rather stabilizes A3G. They suggested the possibility of a new proteolytic enzyme for Vif degradation and that their new compound interferes with Vif interaction with a host protein in a Vif-A3G-host protein complex, thereby making Vif less stable. The precise biological significance of this Vif-A3G-host protein complex requires future elucidation. Nevertheless, modification or intervention of such Vif-A3G-host protein interplay could lead to the development of new therapeutic strategies for HIV-1 infection.

Conclusion

MDM2 is a novel E3 ligase for Vif which induces the polyubiquitination and degradation of Vif to negatively regulate HIV-1 replication.

Methods

Plasmid constructs

Expression vectors for hemagglutinin (HA)- or FLAG-tagged MDM2, pCMV4/HA-MDM2 or pCMV4/FLAG-MDM2, and their mutants were constructed as previously described [19]. An expression vector for HA-tagged human APOBEC3G, pcDNA3/HA-hA3G [39], and HIV-1 reporter plasmids, pNL43/Δenv-Luc (WT) and pNL43/ΔenvΔvif-Luc (ΔVif) [8], were constructed as previously described. Expression vectors for FLAG-tagged Parkin and Cul5 (pcDNA3/FLAG-Parkin and pcDNA3/FLAG-Cul5, respectively) were constructed by the PCR method. Complementary DNA for HIV-1 Vif was also cloned into pDON-AI (TAKARA BIO INC.) and pDON/EGFP for expression of Vif and EGFP-fused Vif (EGFP-Vif). The subgenomic expression vector pNL-A1, which expresses all HIV-1 proteins except for *gag* and *pol* products, and its mutants expressing Vif deletion mutants were kind gifts from Dr. K. Strebel [22].

Co-immunoprecipitation assays

We performed an immunoprecipitation assay for protein-protein interaction *in vivo*, as described previously [8]. HEK293T cells were cotransfected with pCMV4/HA-MDM2 and pNL-A1 by the calcium phosphate method. Two days after transfection, cells were lysed in lysis buffer (25 mM HEPES pH7.4/150 mM NaCl/1 mM $MgCl_2$/0.5% TritonX-100/10% Glycerol) and complexes were immunoprecipitated with anti-MDM2 monoclonal antibody

(mAb) (SMP-14, Santa Cruz Biotechnology, Inc., Santa Cruz, CA and Ab-1, Calbiochem, EMD Biosciences, Inc, Darmstadt, Germany) and Protein A-Sepharose beads (Amersham Biosciences Corp.) at 4°C. The beads were washed with RIPA buffer (50 mM Tris-HCl pH8.0/150 mM NaCl/1% Triton-X 100/0.1% SDS/0.1% DOC) and analyzed by immunoblotting with anti-Vif mAb (#319) (A kind gift from Dr. M. Malim through the AIDS Research and Reference Reagent Program) [40] or anti-HA mAb (12CA5). To map the regions of MDM2 necessary for binding to Vif, HEK293T cells were cotransfected with expression vectors for a series of MDM2 deletion mutants together with pNL-A1. Complexes were immunoprecipitated with anti-HA mAb and analyzed by immunoblotting with anti-Vif mAb. To map the regions of Vif necessary for binding to MDM2, HEK293T cells were cotransfected with expression vectors for a series of Vif deletion mutants together with pCMV4/HA-MDM2. Complexes were immunoprecipitated with anti-Vif mAb and analyzed by immunoblotting with anti-MDM2 mAb. In all these experiments, transfected cells were treated with MG132 for 6 hrs prior to harvesting in order to stabilize both Vif and MDM2; otherwise we could not detect the expression of MDM2 because of its rapid degradation, as seen in Fig. 1A.

In vitro and in vivo ubiquitination assays

In vitro ubiquitination assays were carried out in ubiquitin reaction buffer (50 mM Tris-HCl/2 mM ATP/5 mM $MgCl_2$/2 μM DTT) with E1(200 ng), E2(Ubc5c)(150 ng), and GST-tagged ubiquitin (GST-Ub) (10 μg) as described previously [13]. MDM2 and Vif were expressed as GST-fusion proteins in Escherichia coli strain DH5α and BL21, respectively. The reactions were incubated at 30°C for 90 min. The samples were subjected to immunoblotting with anti-Vif mAb to detect GST-ubiquitin conjugated Vif.

For *in vivo* ubiquitination assays, HEK 293T cells were cotransfected with plasmids expressing Vif, FLAG-MDM2 or its mutants, and His-tagged ubiquitin (His-Ub) as indicated. Cells were treated with 10 μM MG132 for 6 hrs prior to harvesting. Forty-eight hours post transfection, cell lysates were affinity-purified with Ni-NTA-agarose beads (Invitrogen corporation, Carlsbad, CA) and analyzed by immunoblotting with anti-Vif mAb.

For production of RNAi within the cells, we used the pSuper vector as described previously [19]. pSuper-MDM2-1 contained the 19 nt derived from the *mdm2* cDNA (nt 404–422) as the target sequence. Double-stranded RNA containing scrambled 19 nt was used as a control. HEK293T cells were transfected with pSuper plasmids together with plasmids expressing Vif and HA-Ub. Cell lysates were immunoprecipitated with anti-Vif mAb followed by immunoblottimg with anti-HA mAb.

Single round infection assays with HIV-1 luciferase reporter virus

Luciferase reporter viruses with or without Vif were prepared by cotransfection of pNL43/Δenv-Luc (Wt) or pNL43/ΔenvΔvif-Luc (ΔVif) plus pVSV-G together with a mock vector or an expression vector for MDM2 or a mutant in the presence or absence of pcDNA3/hA3G by calcium phosphate as previously described [8]. The reporter viruses were adjusted according to p24 values and used to infect M8166 target cells. Productive infection was measured by luciferase activity and values were presented as percent infectivity relative to the value of each virus without the expression of hA3G.

Knockdown of MDM2 in macrophages and replication assays

Monocyte-derived macrophages (MDM) were cultured for 7 days from CD14+ monocytes isolated from the peripheral blood of an HIV-1-negative healthy individual. Electroporation with Stealth Select RNAi for MDM2 or Control (Invitrogen Corporation) was performed using the Nucleofector machine (Amaxa Inc., Gaithersburg, MD) according to the manufacturer's instructions. Twenty four hours after transfection, MDM were challenged with R5 HIV-$1_{JR\text{-}FL}$ at multiplicity of infection of 0.1 at 37°C for 3 hrs. The cells were cultured from day 4 to 21 after infection, and the concentration of p24 antigen in the supernatant was measured with an HIV-1 p24 antigen enzyme-linked immunosorbent assay [ELISA] kit (ZeptMetrix, Buffalo, NY).

Competing interests

The authors declare that they have no competing interests.

Authors' contributions

TI. designed research, performed research, contributed vital new reagents, analyzed data, and wrote the paper. ATK designed research, analyzed data, wrote the paper, and organized the research. KS, KIo, and MM prepared the materials and performed a part of the research. KIwai, HK, TS, MT, SI., and HA contributed vital new reagents. YK contributed vital new reagents, performed a part of the research, and analyzed the data. HH, KItoh, and JF designed the research, contributed vital new reagents, and analyzed the data. TU analyzed the data, drafted the paper, and organized the research.

Additional material

Additional file 1

Supplementary figure 1 – the stability of Vif protein in p53-/- MEF and p53-/-MDM2-/- MEF cells. *MEF cells were transfected with pDON/Vif or pcDNA3/HA-A3G. Twenty-two hours after transfection, the cells were treated with cycloheximide (CHX) for the indicated times, and cell lysates were subjected to immunoblotting with the indicated Abs.*

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Additional file 2

Supplementary figure 2 – immunopurified MDM2 induced the polyubiquitination of Vif in vitro. *(A) MDM2 as well as Cul5 induced the polyubiquitination of Vif. HEK293T cells were transfected with expression vectors for His-MDM2 and His-Cul5. His-tagged proteins were purified using Ni-NTA agarose and subjected to* in vitro *ubiquitination assays as described in a legend to Fig. 4A. Reactions were subjected to immunoblotting with anti-Vif Ab. Arrows indicate GST-Ub-conjugated Vif. Asterisks indicate non-specific bands associated with GST-Vif protein recognized by anti-Vif Ab, as they are seen in lanes 1 and 3. (B) MDM2 induced the polyubiquitination of Vif Wt but not that of Δ22 that was defective for binding MDM2. Filled asterisks indicate non-specific bands associated with GST-Vif protein, while white asterisks indicate those associated with GST-Vif Δ22.*

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Additional file 3

Supplementary figure 3 – the overexpression of MDM2 inhibited HIV-1 replication in the presence of A3F. *Single round infection assays were performed in the presence or absence of A3F as described in a legend to Fig. 5A. Values are presented as averages of more than 3 independent experiments.*

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MINI-REVIEW

Non-human primate surrogate model of hepatitis C virus infection

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ABSTRACT

More than 170 million people worldwide are chronically infected by HCV, which is the causative agent of chronic hepatitis C, cirrhosis, and finally liver cancer. Although animal models of viral hepatitis are a prerequisite for the evaluation of antiviral and vaccine efficacy, the restricted host range of HCV has hampered the development of a suitable small animal model of HCV infection. Use of the chimpanzee, the only animal known to be susceptible to HCV infection, is limited by ethical and financial restrictions. In this regard GBV-B, being closely related to HCV, appears to be a promising non-human surrogate model for the study of HCV infection. This review describes the characteristic of GBV-B infection of New World monkeys, and discusses current issues concerning the GBV-B model and its future directions.

Key words GBV-B, HCV, hepatitis C, monkey.

INTRODUCTION

Since HCV was identified as a major causative agent for non-A, non-B hepatitis in 1989 by Choo *et al.* (1), it has become evident that HCV is disseminated worldwide and is carried by an estimated more than 170 million people (2). In most advanced nations, the prevalence of HCV infection is roughly 1–2% and further dissemination is suppressed. By contrast, among developing countries the number of HCV-infected patients is still increasing due to iatrogenic exposure, including blood transfusion from unscreened donors and reuse or inappropriate sterilization of contaminated medical equipment, and injecting drug use (3). After HCV exposure, about 70% of individuals who exhibit acute infection progress to chronic liver disease, and many of these patients develop hepatic cirrhosis and hepatocellular carcinoma (2). Currently, the only treatment available for patients with chronic HCV infections is combination therapy with pegylated interferon and ribavirin. As the standard therapy is effective in only approximately 50% of patients with chronic HCV hepatitis, the other half of affected patients are still threatened by poor prognosis (4). It is therefore urgent to develop more effective therapeutics for HCV infection. At the same time, prophylactic vaccines are indispensable for prevention of further spread of HCV in developing countries, including reduction of the risk to health care workers of occupational transmission.

ANIMAL MODELS OF HCV INFECTION: RODENTS AND CHIMPANZEES

Research in infectious diseases will never progress without animal models. Because conventional small animals are not susceptible to HCV infection due to its limited host range, development of an effective prophylactic vaccine, as well as unveiling of the molecular mechanism of viral pathogenesis, has been hampered. Nonetheless, decades

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List of Abbreviations: ALT, alanine aminotransferase; CTL, cytotoxic T lymphocytes; GBV-B, GB virus-B; GE, genome equivalents; HCV, hepatitis C virus; HIV-1, human immunodeficiency virus type 1; SIV, simian immunodeficiency viruses; SIVmac, SIV derived from rhesus macaques.

of struggle have resulted in a number of animal models for HCV infection and hepatitis C.

Recently, a number of rodent models have been developed (for a review of this topic see reference 5). Rodent models, which permit HCV replication and involve transplantation of human hepatocytes, include immunotorelated fetal rat (transplantation of human hepatoma cell line Huh7 into newborn rats after prior injection of the same cells into pregnant females) (6) and immunodeficient mice such as trimera mice (7, 8) and uPA mice (9–11). These rodent models are highly useful for evaluating the efficacy of antiviral drugs and neutralizing monoclonal antibodies. In addition, a number of HCV transgenic mice have been developed. These enable direct characterization of the effects of expression of HCV genes on liver injury (5). These small animal models do not require costly facility for primates as mentioned later. While having a number of merits as mentioned above, these rodent models still have some limitations. For example the former models are not suitable for investigation of the pathogenesis of hepatitis C and the development of effective vaccine strategies, while in the latter models the proteins of interest are usually over-expressed as compared with natural HCV infection, and the integration site of the transgene may have an influence on the outcome of the study.

The chimpanzee model is the most straightforward since this animal can be experimentally infected with HCV. One third of HCV-inoculated chimpanzees develop chronic infection, while infection resolves in the remainder after an acute phase lasting 2–3 months, indicating that the chronicity rate in chimpanzees is somewhat lower than in humans (12). The chimpanzee model has been considered the primary choice for studying the relationship between the virus and host anti-viral immune responses, as well as for evaluating immunopathogenesis and the efficacy of prophylactic vaccination. However, irrespective of its benefits, many obstacles need to be overcome in order to use this model. For example in many countries it is illegal to employ the chimpanzee as an experimental animal, primarily due to ethical, (and secondly to financial), reasons. In fact, in 2004 the Dutch government decided to stop all research with chimpanzees at the biomedical primate research center in Rijswijk, Netherlands. As this was the only primate center in Europe where chimpanzees were used for biomedical research, this decision made chimpanzees unavailable as experimental animals in Europe. It is still possible to employ chimpanzees for biomedical research in some other countries, including the USA. However, the National Center for Research Resources of the National Institute of Health in the USA has recently decided not to continue to breed chimpanzees for research (13). It is estimated that the existing chimpanzees in the National Center for Research Resources will die within 30 years.

AIDS-related research has been one of the major purposes for using chimpanzees. However, due to the reasons mentioned above, as well as the endangered status of chimpanzees, nowadays AIDS scientists mainly make use of macaque monkeys infected with SIV as a non-human primate surrogate AIDS model (14, 15). The macaque/SIV model is useful since SIV is highly related to HIV-1, and induces AIDS-like diseases that are comparable to those of humans infected with HIV-1. Taking this into consideration, an alternative surrogate model which employs New World monkeys infected with GBV-B may be promising for future HCV/hepatitis C research.

GBV-B AS A NON-HUMAN PRIMATE SURROGATE MODEL OF HCV INFECTION

Among viruses so far known, GBV-B is the most closely related to HCV. However, due to a lack of epidemiological information as discussed below, GBV-B has been tentatively classified in the Hepacivirus genus of the Flavivirus family. Originally, Deinhardt *et al.* (16) found that some tamarins (genus *Saguinus*) developed hepatitis after inoculation with an inoculum obtained from a surgeon with the initials GB who had contracted hepatitis. After 11 passages in tamarins they obtained serum including GB agent(s), and were then able to achieve molecular cloning of GBV-A and GBV-B as flavivirus-like genomes (17) and to demonstrate GBV-B as an agent which could cause hepatitis in tamarins (18). Although it was unclear whether GBV-B originated from the GB inoculum or the tamarins themselves, later animal studies demonstrated that GBV-B is infectious for tamarins but not chimpanzees (19) and reciprocally that HCV is infectious for chimpanzees but not tamarins (20). These findings led to the retrospective conclusion that at least one of the tamarins employed for the *in vivo* passage study was persistently infected with GBV-B, and therefore GBV-B is probably a virus that originated in tamarins (20). However, GBV-B has not so far been isolated from additional tamarins, probably due to limited epidemiological analyses. Thus the natural host(s) and prevalence of GBV-B are yet to be determined.

CHARACTERISTICS OF GBV-B INFECTION OF NEW WORLD MONKEYS

Previous data have shown that a number of New World monkeys (parvorder *Platyrrhini*) including tamarins, the common marmoset (*Callithrix jacchus*) and the owl monkey (*Aotus trivirgatus*) are susceptible to GBV-B infection, as summarized in Table 1, although in tamarins peak concentrations of viruses in plasma are higher (10^7–10^{10} GE/ml) than in other monkeys (10^5–10^8 GE/ml) (21–29). In general, in any monkey species viremia

Table 1 Summary of characteristics of acute GBV-B infection in monkeys

Monkeys permissive of experimental infection	Tamarins (Genus *Saguinus*) Common marmoset (*Callithrix jacchus*) Owl monkey (*Aotus trivirgatus*)
Appearance of viremia	1–2 weeks post infection
Peak levels of viremia	Tamarins; 10^7–10^{10} GE/ml Marmoset and owl monkey; 10^5–10^8 GE/ml
Peak ALT levels	Approximately 200–500 IU/ml
Duration of viremia	2–3 months
Timing of seroconversion	A couple of weeks before clearance of viremia

persists for 2–3 months and is followed by clearance. GBV-B-infected monkeys with viremia usually develop self-resolving subacute hepatitis, as indicated by increases in the concentrations of serum enzymes such as ALT, gamma-glutamyltranspeptidase, and isocitrate dehydrogenase. Pathologically, degeneration and apoptosis of hepatocytes, as well as disruption and dilation of sinusoids, have been observed in the livers of GBV-B-infected tamarins with higher viremia and ALT activity (29). It is possible that GBV-B-specific CTL may cause the liver damage. However, a recent study reported that CTL are induced at a late stage of subacute GBV-B infection, and are inversely correlated with reduction in viremia (30). Since liver damage is usually found very early (1–2 weeks) after infection, when specific CTL are not observed, it is likely that viral replication in the hepatocytes leads directly to the early onset of cytopathic effects, while lower numbers of CTL may also contribute to cytotoxicity.

The clearance of viremia in the acute phase of GBV-B infection should require an effective antiviral immune response. In particular, in both GBV-B and HCV intrahepatic CTL appear to play a major role in viral clearance (30, 31). In addition, secondary GBV-B infection after clearance of the primary viremia induces a strong T cell response, leading to virtual absence of viremia, indicating that efficient memory is a key to protection from chronic viral infection (30, 32). In pre-immune chimpanzees antibody-mediated depletion of either CD4 or CD8 T lymphocytes affects their ability to control viral replication, resulting in prolonged viremia, demonstrating essential roles for both CD4 and CD8 memory in protection from viral persistence (33, 34).

On the other hand, the significance of humoral immunity in controlling GBV-B replication is still unclear. It is reasonable to assume that neutralizing antibodies also play important roles in the clearance of subacute viremia and protection from viral persistence. In the case of HCV, in one well characterized single-source outbreak of hepatitis C, viral clearance was associated with rapid induction of neutralizing antibodies in the early phase of infection, while chronic HCV infection was characterized by absent or low-titer neutralizing antibodies in this phase. Patients with resolution of infection were shown to exhibit broader cross-neutralizing activity of antibodies in the early phase of infection (35). In one chronic HCV patient who was followed up for 30 years, it has also been shown that HCV continuously escaped the host's immune system by repeated mutational changes, resulting in loss of recognition of the HCV envelope glycoproteins by antibodies (36). The fact that the sequences of envelope glycoprotein and specificity of neutralizing antibody change over time suggests that neutralizing antibodies exert selective pressure on HCV evolution. Thus, although neutralizing antibodies (and/or CTL) are not necessarily capable of controlling chronic viral infection, frequent escape from the antibodies needs so called fitness cost, resulting in the partial suppression of viral loads. Indeed, HCV-infected patients with primary antibody deficiencies have accelerated rates of disease progression (37).

Although features of the subacute phase of GBV-B infection are similar to that of HCV, a major defect of GBV-B infection as a surrogate model for HCV is that it is difficult to chronically infect monkeys. While as many as 70% of humans with HCV infection become chronically infected, only approximately a third of chimpanzees do so (2, 12). By contrast, only a few cases regarding chronic GBV-B infection have been reported so far. The best example was a case of a tamarin persistently infected with GBV-B (24); the monkey exhibited acute mild hepatitis with viremia (peak level; $\sim 10^9$ GE/ml), which reduced to a set point level (less than 10^4 GE/ml) at 16 weeks post infection, followed by a gradual increase in viremia which reached $>10^7$ GE/ml at 112 weeks post infection, along with a significant ALT increase. However, the viremia suddenly declined thereafter and became undetectable, in association with a reduction in antibody titer, and subsequent *in vivo* passage of virus obtained from the tamarin failed to reproduce persistent infection in other tamarins (24). In addition, immunosuppression of a GBV-B-infected tamarin by FK506 treatment, or infection of GBV-B with deletion of poly(U) tract in the 3′ UTR, reportedly resulted in relatively long-term persistent infection of GBV-B for up to 46 and 90 weeks, respectively (23, 27). These results indicate that GBV-B may have the potential for establishing chronic infection.

Furthermore, our recent study has demonstrated that among four common marmosets infected with GBV-B derived from a molecular clone pGBB (21), two developed long-term chronic infection for up to three years, with recurrent viremia in which plasma viral RNA levels fluctuated between undetectable and 10^5 GE/ml, which is equivalent to the case of chimpanzees chronically infected with

HCV (Iwasaki *et al.*, manuscript in preparation). Notably, the induction of antiviral antibody response as measured by anti-Core and -NS3 antibodies was delayed in both cases, followed by a gradual increase, and then sustained high antibody titers. This was in contrast with an abrupt and transient increase at the end of periods of subacute viremia in marmosets and tamarins with viral clearance. Whether a delayed antibody response is associated with persistent GBV-B infection remains to be determined.

Taken together, these findings indicate the similarity between HCV and GBV-B in regard to their ability to induce chronic infection, and also shed light on the further potential of GBV-B as a surrogate model for HCV.

FUTURE PROSPECT OF GBV-B SURROGATE MODEL

Although many questions are still to be addressed, accumulating evidence from extensive studies to date has greatly advanced the usefulness of the GBV-B as a surrogate model for HCV. The GBV-B model may be applicable for evaluating the feasibility and safety of anti-HCV vaccines employing novel viral vectors and gene therapy which creates RNA interference. For example, in a recent pilot study we showed that systemic administration of cationic liposome-encapsulated small interfering RNA to marmosets resulted in efficient regulation of GBV-B replication, indicating the usefulness of the surrogate model for proving the feasibility of RNA interference technology for future clinical application (38). This GBV-B model will also be helpful in identifying the virological and immunological factors which determine whether the outcome is acute resolving or chronic infection. While the GBV-B model appears to be valuable, development of an HCV/GBV-B chimeric virus would greatly expand the utility of the surrogate model, since it would enable us to directly evaluate antiviral vaccines and chemicals for HCV as a preclinical study. Rijnbrand *et al.* have reported that a chimeric GBV-B with 5′ untranslated region from HCV is infectious and causes hepatitis in tamarins (39). As recently demonstrated by Chevalier *et al.* (40), this will be a good model for evaluating the potential of small interfering RNA specific to HCV genome for future clinical application.

In regard to this, we may refer to an elegant precedent in the case of the macaques AIDS model. SIVmac is well known to efficiently infect, and result in the development of AIDS in macaques. Furthermore HIV-1, of which only 7% of the entire genome is derived from SIVmac, has been demonstrated to overcome the host range of authentic HIV-1, and to acquire the ability to productively infect macaque cells (41, 42). Instead of endangered chimpanzees, tamarins/marmosets which can be chronically infected with an HCV/GBV-B chimera (hopefully capable of inducing chronic hepatitis) should be the next generation of a promising non-human primate surrogate model for HCV infection, one which is similar to the macaques AIDS model. Whatever animals are used for pre-clinical study, it is important to keep in mind that results obtained from monkey models using either GBV-B or HCV/GBV-B chimera (as well as SIV or HIV/SIV chimera) may not necessarily be applicable to humans, because of potential differences in the molecular structure and/or mechanism by which antivirals and/or viral and host proteins function. Further characterization and understanding of the molecular biology and immunology of virus-host interactions will help in developing novel antiviral strategies.

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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.

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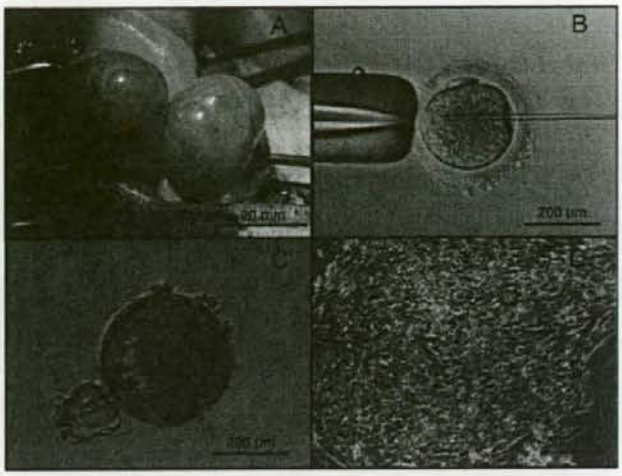

Fig. 1. Ovaries with developed follicles (A), ICSI (B), an ICSI-derived blastocyst stage embryo (C) and ES cells (D).
(A) Oocytes are collected from ovaries with developed follicles after hormone treatment. (B, C) Mature oocytes subjected to ICSI develop to blastocyst stage embryos. (D) ES cell lines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