Review

Transgenic nonhuman primates for neurodegenerative diseases
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Abstract

Animal models that represent human diseases constitute an important tool in understanding the pathogenesis of the diseases, and in developing effective therapies. Neurodegenerative diseases are complex disorders involving neuropathologic and psychiatric alterations. Although transgenic and knock-in mouse models of Alzheimer's disease, (AD), Parkinson's disease (PD) and Huntington's disease (HD) have been created, limited representation in clinical aspects has been recognized and the rodent models lack true neurodegeneration. Chemical induction of HD and PD in nonhuman primates (NHP) has been reported, however, the role of intrinsic genetic factors in the development of the diseases is indeterminable. Nonhuman primates closely parallel humans with regard to genetic, neuroanatomic, and cognitive/behavioral characteristics. Accordingly, the development of NHP models for neurodegenerative diseases holds greater promise for success in the discovery of diagnoses, treatments, and cures than approaches using other animal species. Therefore, a transgenic NHP carrying a mutant gene similar to that of patients will help to clarify our understanding of disease onset and progression. Additionally, monitoring disease onset and development in the transgenic NHP by high resolution brain imaging technology such as MRI, and behavioral and cognitive testing can all be carried out simultaneously in the NHP but not in other animal models. Moreover, because of the similarity in motor repertoire between NHPs and humans, it will also be possible to compare the neurologic syndrome observed in the NHP model to that in patients. Understanding the correlation between genetic defects and physiologic changes (e.g. oxidative damage) will lead to a better understanding of disease progression and the development of patient treatments, medications and preventive approaches for high risk individuals. The impact of the transgenic NHP model in understanding the role which genetic disorders play in the development of efficacious interventions and medications is foreseeable.

Background

Transgenic technology in biomedicine has opened a new era for animal modeling, which accelerates model development and results in better understanding of diseases as well as development of therapies for patients. The successful development of transgenic animal models for human diseases has led to remarkable breakthroughs that have significantly impacted the development of approaches to the diagnosis, treatment, and intervention of human diseases. Additionally, the models have clarified our understanding of disease mechanisms and the onset and course of pathology associated with disease [1-4]. For several reasons, the transgenic mouse is the most commonly used animal model: the availability of extensive information for particular strains, well developed techniques in handling gametes, embryos and surrogates; inexpensive and relatively limitless supplies, and short generation times [5]. Undeniably, the use of transgenic mice plays a very

important role in biomedical advancements and this role will continue to be critical. Transgenic rodents, developed with genetic defects similar to those seen in human patients, are widely used in biomedical research. Nevertheless, fundamental differences between rodents and humans limit the rodent from being the best model for all human diseases. These physiologic differences include life span [6], brain complexity [7-11], cellular metabolism [12], endocrine and reproductive function [13]. The differences may be caused by genetic redundancy or altered biochemical pathways in mice [14,15]. Additionally, limited cognitive and behavioral tests are available for rodents, and these are not always applicable to studying neurodegenerative diseases such as AD, PD and HD. Therefore, due to the high number of physiologic [16], neurologic and genetic similarities [17-19] between humans and NHPs, the latter is considered one of the best models for understanding human physiology and diseases. Due to progressive neurodegeneration, deleterious alterations in behavior, and psychiatric status associated with neurodegenerative diseases such as AD, PD and HD in humans, the NHP is the only animal model providing accessibility to a wide range of testing methods and the possibility for high resolution brain imaging such as <u>f</u>unctional <u>m</u>agnetic <u>r</u>esonance <u>i</u>maging (fMRI). The creation of a transgenic NHP with genetic alteration that leads to human genetic diseases will further mimic patient conditions with impacts on understanding disease development, thereby paving the way for the development of cures [6,7,20-24].

Major difficulties in generating genetically modified NHP

The major difficulty in producing transgenic NHPs is low efficiency of the gene transfer method. Although the first transgenic mouse was born in 1974 by infection of a pre-implantation stage embryo using a competent retrovirus [25,26], the technique was not widely accepted due to biosafety concerns, suppression of transgene expression and high mosaicism rate [27-30]. On the other hand, pronuclear microinjection (PI) has dominated the transgenic field for more than two decades despite its relatively low efficiency [30-34], because there were no more efficient method. Despite concerted efforts to develop a new gene transfer method and techniques that could improve gene transfer efficiency [30,33,35], significant improvement has only been achieved recently. The development of replication defective retroviral and lentiviral vector systems [36-44], sperm mediated gene transfer (SMGT) [45-48], nuclear transplantation technology [49-53], and reliable transgenic reporters [54-57] have led transgenesis and animal biotechnology to a new era in which broad applications can now be achieved.

An efficient gene delivery method is the crucial factor in transgenic NHP production because, due to ethical concerns, the small number of available animals is the major limiting factor in the development process. We have developed the first transgenic NHP, named "AND*i*", by using a pseudotyped retroviral vector as a vehicle to deliver the gene of interest into an unfertilized oocyte at a relatively high rate of efficiency [22]. Indeed, the same method was first proven to be highly efficient in cattle before it was applied in NHPs [58]. Furthermore, rapid development of lentiviral vector technology has significantly impacted transgenic technology. A comparison of the two viral vector systems will be discussed in the following section.

Alternative strategies for generating transgenic NHP for biomedical research

A number of gene transfer methods has been described for the production of such transgenic animals such as rodents, livestock, primates and other species [22,44,45,58,59]; however, until recently no efficient and reliable method had been developed for producing the NHP. Vesicular stomatitis virus envelope glycoprotein G (VSV-G) pseudotyped retroviral vector (RV-pseudotype) is an efficient gene delivery system in tissue culture cells, embryonic stem (ES) cells, gametes and early preimplantation embryos. Transgenic pigs, cattle and NHPs have also been successfully produced by infecting unfertilized oocytes [22,58,60]. The unfertilized oocyte was targeted because of the disassembly of the nuclear membrane at metaphase; thus it became accessible for the viral preintegration complex to achieve integration. Compared to the RV, lentiviral vector (LV) capable of infecting both mitotic and non-mitotic cells, has led to optimism in transgenic and gene therapeutic technology, although with limitations. Three promising methods of creating genetically modified NHP models for biomedical research are retroviral vector, lentiviral vector, and cloning. One of the major differences between viral vector gene transfer and cloning is the transgene integration pattern in the resulting animals. Although high gene transfer efficiency has been demonstrated in RV and LV, site specific integration is not feasible; however, animal cloning using gene targeted donor cells has been proven as a feasible strategy. Thus, the method for creating transgenic animals should be carefully chosen dependent on the requirements of the animal models and limitations of available technology.

Retroviral vector vs lentiviral vector

Retrovirus and lentivirus belong to the Retroviridae viruses, which are double stranded (ds) RNA enveloped. The viral core is comprised of the dsRNA genome and enzymes such as reverse transcriptase for reverse transcription of the RNA genome to DNA followed by integration achieved by integrase [61]. Both vectors have been

Table 1: Autosomal dominant neurodegenerative diseases [97-98]

Disease	Mutant Gene	Mutation	Transgenic Mouse Model
FAD	APP	Mainly missense	Mutant APP and APP:PS1
FAD	PS1	Mainly missense	Mutant PS1
FAD	PS2	Mainly missense	Mutant PS2
FALS	SOD1	Mainly missense	Mutant SOD1
FTDP-17	Tau	Missense & splice	Mutant Tau
PD	α-synuclein	Missense	wt/mutant αsyn
Prion	PrP	Mainly missense	Mutant PrP
HD	huntingtin	Polyglutamine	huntingtin (expanded repeat)
SCA-1	ataxin-1	Polyglutamine	ataxin-1 (expanded repeat)
SCA-3	ataxin-3	Polyglutamine	ataxin-3 (expanded repeat)
DRPLA	atrophin-1	Polyglutamine	atrophin-1 (expanded repeat)

FAD: Familial Alzheimer's Disease FALS: Familial amyotrophic lateral sclerosis with Parkinsonism HD: Huntington's Disease DRPLA: Dentatorubral and pallidoluysian atrophy PD: Parkinson's Disease SCA: Spinocerebellar ataxias FTDP: Frontotemporal Dementia

successfully used to generate transgenic animals at a relatively high rate [22,44,58,60]. Transgenic pigs, cattle and NHPs have been produced by infecting unfertilized oocytes using an RV-pseudotype followed by *in vitro* fertilization, whereas transgenic mice and rats have been produced by infecting fertilized oocytes (zygotes) using the LV-pseudotype (Chan unpublished) [44]. Nevertheless, the capability of infecting non-mitotic cells has been overwhelmed by the potential application of LV in gene therapy, particularly for patients suffering from neurodegenerative diseases. Neurons are primarily non proliferative or at a low mitotic rate, which makes LV a superior delivery system over other gene transfer methods including RV. Thus, the LV is undeniably outstanding in gene therapy for neurologic disorders; however, whether LV is superior over RV in the production of transgenic animals such as the NHP remains unknown, and further investigation is necessary.

To date, the only successful method of producing transgenic NHPs is the infection of metaphase II (MII) oocytes using an RV-pseudotype [22]. Except for NT, all other gene transfer methods result in random integration of the transgene. Due to the presence of the endogenous homolog in the target cell genome, a recessive gene defect that requires mutation at both alleles will not be achieved by present gene transfer techniques. Therefore, it is necessary to develop a transgenic NHP model for disease caused by a single dominant gene disorder that would result from the gain of function of the mutant gene. Some inherited genetic disorders resulting from a single dominant gene defect make them good candidates for the creation of a transgenic NHP model (Table 1). A single dominant gene disorder has additional advantages over complex gene disorders. Complex diseases, often resulting in multiple gene defects, require long selection and breeding programs to establish animal colonies with stable genotypes and phenotypes for further study. Thus, due to the lengthy breeding time and limitation in present genetic manipulation techniques, an NHP with a long gestation period requiring years to reach reproductive age is not appropriate for complex genetic disorders.

Undoubtedly the crucial role of LV in gene therapy development, particularly relating to neurodegenerative diseases, has held great promise. However, it is still unknown whether LV is more advantageous than RV in the creation of transgenic animal models such as NHP. The major difference between LV and RV is the target cell. LV is capable of infecting the non-mitotic cell, whereas RV infects mitotic cells only because the presence of the nuclear envelope in non-mitotic cells precludes entry of the pre-integration complex into the nucleus followed by integration. Transgenic rats and mice have been generated by infecting zygotes using LV. Because LV is capable of infecting target cells at any stage, continuous infection during early embryonic development and a high mosaicism rate is expected. On the other hand, RV infecting primarily mitotic cells is more efficient when targeting MII oocytes than zygotes. A 100% transgenic rate was achieved when targeting MII oocytes compared to 25% when targeting zygotes [58]. This suggests the importance of infection time and its profound influence on the transgene integration pattern in the resulting animals. Nonetheless, the efficiency of generating transgenic animals by RV and LV-pseudotypes is relatively low compared to pronuclear microinjection. Thus, further investigation is necessary to determine the gene transfer efficiency, integration and expression pattern when a sufficient number of transgenic animals is available.

Cloning and ES cell

During the past few years, research in ES cells and cloning has flourished. Since the establishment of embryonic cell lines from mouse preimplantation embryos in 1981, the continuous effort to create identical transgenic animals using ES cells, embryonic cells and somatic cells by nuclear transfer finally has been rewarded. ES cell lines from NHP and human preimplantation embryos have been established [62-66].

Pluripotency of NHP and human ES cells has also been demonstrated by the derivation of cell types from various lineages including neurons, islet cells and cardiac muscle cells [6,64,65,67]. However, genetic modification of NHP and human ES cells remains challenging. Gene transfer in primate ES cells is relatively inefficient compared to that of mouse ES cells using conventional methods such as lipofectamine or electroporation. This could be due to the suboptimal culture condition and difficulty in single cell culture. Although success in transfecting NHP and human ES cells has been reported, gene transfer efficiency remains low and challenging for complex gene modification such as rare homologous recombination events [68,69]. Not until recently, has development of the lentiviral vector significantly improved gene transfer efficiency in cell types such as neurons with low mitotic rates or which are non-proliferative [70,71]. As a result, NHP and human ES cells expressing a green fluorescent protein gene (GFP) were established [72]. Development of the lentiviral vector, greatly improving gene transfer efficiency in NHP and human ES cells, is an important step in the development of gene and cell therapy using *in vitro* derived cell types. Nonetheless, one of the major limitations of RV and LV is the random integration event that allows only overexpression of the gene of interest. An alternative strategy is the application of RNA interference (RNAi) technology to achieve gene targeted knock-down of a specific gene product. By introducing a homologous dsRNA to the specifically targeted gene product, a null or hypomorphic phenotype resulted from the loss of the targeted endogenous mRNA [73,74]. Additionally, recently reported gene targeting by homologous recombination in the human ES cell has led to a new era of human ES cell application [69]. However, targeting efficiency remains low and limited to simple gene manipulation. Thus, alternative approaches such as RNAi and a tetracycline-regulated gene expression system should be considered [71]. Furthermore, due to the ethical limitations of human ES cells, some procedures such as cell transplantation are allowed to be performed in patients only at the end stage. Development and validation of the efficacy of medical intervention and cures is difficult without an appropriate animal model. Thus, an animal model such as NHP is important for the advancement of developing cures and preventive medication. However, genetic disorders are caused not only by single dominant gene defects, but also by recessive and multiple gene defects. Therefore, it will not be possible to generate an animal model by simply over expressing the mutant gene; it will be necessary to replace both endogenous alleles. Thus, gene targeting by RNAi or homologous recombination in ES cells followed by NT are the only alternatives in developing genetically modified NHP models for recessive gene disorders.

Although tremendous effort has been expended to clone NHPs using ES cells or somatic cells, no success has been reported [51,75]. Nonetheless, efforts will continue to develop cloning techniques in the NHP because of the foreseeable impact of NHP models with identical genes targeted in biomedical research – the creation of a herd of identical animals carrying a unique genotype and potentially an identical phenotype for pharmaceuticals, therapeutics and vaccine testing [21,76-79]; for the production of valuable proteins [80,81], and for the study of cell differentiation and potential applications of therapeutic cloning [82-88]. Although many applications of NT can be easily linked to NHP models, and the profound impact of stem cell research and gene targeted mice has been clearly demonstrated [6,88-93], the technical barriers for NT in the NHP hss not yet been overcome. To date, no cloned NHP has been produced using embryonic stem cells or somatic cells as the donor nucleus. There have been reports of low efficiency, high fetal loss, and deficiency in immune, respiratory and other systems in newborns resulting from NT using somatic nuclei in many species [94]. Criticism has been raised on current cloning methods in the NHP and further investigation should be performed before conclusive comments can be made. Other techniques such as NT using blastomeres from preimplantation embryos [51] and embryo splitting [21] have also been suggested as an alternative strategy to produce identical NHPs for immediate needs. However, the number of blastomeres is the major limitation and the production of gene targeted identical individuals is not feasible [21,95].

In addition to production of an identical NHP model, *in vitro* differentiation of ES cells is a potential source for replacement therapy in cellular degenerative diseases like AP, PD and HD. Cell types such as neurons, cardiac muscle and islets, have been successfully generated from ES cells *in vitro* under stringent culture conditions [65,67,88]. However, high purity ES cell derived cell types must be achieved before cell transplantation can proceed, undifferentiate ES cells are potentially neoplastic. Additional concerns include the function of the derived cell types, and potential immuno-rejection must be determined before any clinical application. Thus, a transgenic NHP model will be crucial for determining the neoplasticity,

function and immuno-rejection of the derived cell types following transplantation.

Prospect

Animal models play an important role in biomedical advancement. Appropriate use and design of an animal model that mimics physiologic and pathologic conditions in humans is the determining factor for advancing our knowledge. Undoubtedly, rodents are critical for the understanding of diseases and the development of cures but they are not without limitations. Although no single animal model perfectly mimics human diseases, comparative studies among a variety of animal models will broaden our view from several perspectives. Clearly, because of their high genetic and physiologic similarity to humans, NHPs are considered among the best models for humans, yet they can't be used in all cases. Rodents and livestock are superior to NHPs in availability, handling and cost. Therefore, NHPs should only be considered when others are inadequate in mimicking such human conditions as the lack of true neurodegeneration. Progress in gene and cell therapy, the advancement of stem cell technology and the need for efficacious and reliable vaccines have driven the development of NHP models that are not only similar in physiology, but also carry similar genetic defects that lead to diseases such as AD, PD and HD. Therefore, the need of NHP models in complex diseases such as neurodegenerative disorders is foreseeable due to the lack of true representation in rodents. Transgenic and gene targeted NHPs are important for the development of cures; our goal was well described by Brooksbank [96]: "We hope *disease models* will not only be of educational value but will also stimulate the use and development of models that are truly relevant to human disease, which will eventually catalyse the development of safe and efficacious therapeutics for human use."

Acknowledgement

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