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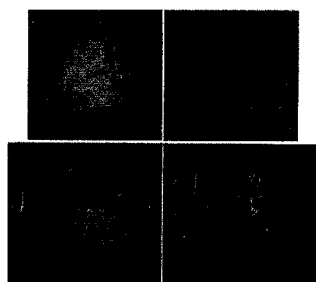
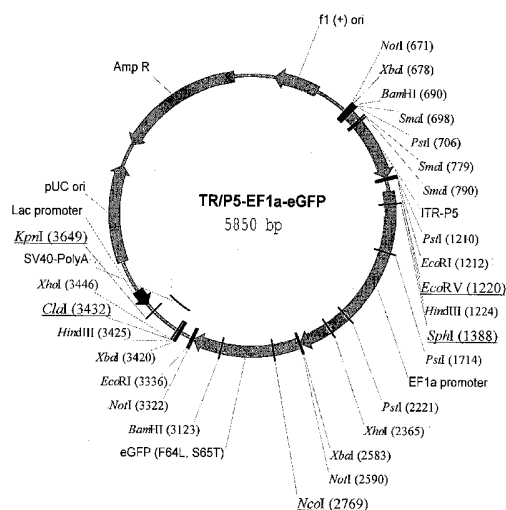
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(54) Title: A METHOD FOR PRODUCING NON-HUMAN TRANSGENIC PRIMATES

(57) Abstract: The invention relates to transgenic non-human primates. The invention further relates to methods of making chromosomally targeted transgenic non-human primates.



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A METHOD FOR PRODUCING NON-HUMAN TRANSGENIC PRIMATES

GOVERNMENT SUPPORT

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10 described herein, which was made in part with funds from the National Institute of Health,
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RELATED APPLICATIONS

This application claims priority from US Provisional Application No. 60/602,812,
15 entitled "A Method Of Producing Non-Human Transgenic Primates," filed August 19,
2004.

BACKGROUND OF THE INVENTION

Non-human primates are used extensively in biomedical research due to close
20 similarities to human physiology and disease pathophysiology. The ability to modify
animal genomes through transgenic technology has opened new avenues for medical
applications.

Currently, there are very few reliable methods of producing transgenic non-human
primates. One such method is pronuclear microinjection. Using pronuclear
25 microinjection methods, transgene integration into the genome occurs in only 1-3% of the
microinjected embryos (Ebert et al. (1993) Theriogenology, 39:121-135).

Mouse embryonic stem cells can be isolated, propagated in vivo, genetically
modified and, ultimately, can contribute to the germline of a host embryo (Evans et al.
(1981) Nature 292:154-156; Martin (1981) Proc Natl Acad Sci USA 78:7634-7638;
30 Bradley et al. (1984) Nature 309:255-256). Because of this, murine embryonic stem cells

have been extensively exploited in developmental and genetic studies (Mansour et al. (1988) Nature 336:348-352).

A need exists for methods of obtaining transgenic animals, such as non-human primates, for use in medical research.

BRIEF SUMMARY OF THE INVENTION

We now provide new methods for generating non-human primates. The new methods provide advantages over the previously known methods, including, for example, targeted introduction of the transgene into a particular, active site in the genome. There is currently no method for efficiently and safely making chromosomally targeted transgenic non-human primates.

According to one aspect, the methods of the invention include generating a transgenic non-human primate comprising introducing an AAV Rep protein and a genetic construct into a zygotic pronuclei to form a transgenic zygote. The genetic construct may comprise a transgene encoding a protein, polypeptide, or genetic transcript of interest operatively linked to a promoter; and a first AAV ITR or portion thereof and a second AAV ITR or portion thereof, wherein the first and second AAV ITRs flank the transgene.

According to certain embodiments, a portion of the genetic construct integrates into a chromosome of the pronuclei. In certain preferred embodiments, portions of the genetic construct integrate at the site of an AAV integration sequence or target. In certain other preferred embodiments, the AAV integration sequence is located on chromosome 19 of the non-human primate.

In certain embodiments, the product of the transgene alters the function of a gene, a protein, or a nucleic acid. In certain other embodiments, the product of the transgene causes a human disease or a condition with symptoms and affects comparable to that seen in affected human patients. In certain preferred embodiments, the product of the transgene causes a human dominant negative disease. In preferred embodiments, the

product of the transgene is a protein, an RNAi, an siRNA, a ribozyme, a tRNA, a mRNA, a ribosomal RNA, a snRNA, a 5SRNA, or an active portion of an RNP.

In certain preferred embodiments, the oocyte and or the sperm is from a non-human primate, for example, a rhesus monkey, African Green monkey, simian, gorilla, rhesus macaque, baboon, cynomolgus macaque, marmoset, chimpanzee, squirrel monkey, proboscis monkey, langur, tamarin, potto, dusky titi, orangutan, marmoset, capuchin, spider monkey, howler monkey, prosimians, pongo pygmaeus, pan paniseus, or other non-human primate.

The method of certain preferred embodiments may further include creating an embryonic stem cell line from the embryo.

The method of other preferred embodiments includes transferring the transgenic embryo to a host animal by mini-laparoscopy, laparoscopy, or cervical canulation. Other preferred embodiments include allowing the embryo to develop to term in the host animal.

According to another aspect, the invention provides methods that comprise generating transgenic non-human primates comprising recovering an oocyte from an adult menstrually cycling non-human primate, fertilizing the oocyte to produce a zygote having zygotic pronuclei, introducing an AAV Rep protein and a genetic construct into a zygotic pronuclei to form a transgenic zygote, culturing the transgenic zygote to form a blastocyst, and transferring the blastocyst into the uterus of a female non-human primate at a time appropriate for implantation.

According to certain embodiments, the transferred blastocyst is allowed to develop to term to produce a transgenic non-human primate. In certain preferred embodiments, the transgenic zygote, the blastocyst, or the transgenic non-human primate are sources of transplant material.

Other embodiments of the invention are disclosed *infra*.

BRIEF DESCRIPTION OF THE DRAWINGS

Figure 1 depicts the TR/P5-EF1a-eGFP plasmid.

Figure 2 depicts SEQ ID NO: 4, which is the nucleotide sequence of the TR/P5-EF1a-eGFP plasmid. The inverted terminal repeat (ITR) and P5 sequences of AAV2 are capitalized. The trs is double underlined and the Rep protein binding sequence (RBS) is single underlined.

Figure 3 depicts the Rep 68 nucleotide and amino acid sequences.

Figure 4 depicts two AAV-GFP transgenic rhesus monkey embryos that were injected with a DNA construct of the invention at the 1-cell zygote stage and photographed 8 days later at the 200 cell blastocyst stage. Left panels: DAPI staining to show all cells. Right panels: GFP antibody immunostaining (red fluorescence). Approximately 80% of the cells of the inner cell mass are GFP-positive suggesting that most fetal tissues from such blastocysts would be transgene positive.

DETAILED DESCRIPTION OF THE INVENTION

We now provide new methods for generating transgenic non-human primates. Further disclosed herein are methods for targeting a transgene to a specific chromosomal location. The invention provides methods of producing a transgenic non-human primate that both safely and efficiently targets integration and avoids the size limitation of conventional gene therapy vectors. The methods surprisingly utilize AAV Rep protein to target a transgene to specific, safe, and biologically active sites on non-human primate chromosomes to safely and efficiently produce viable non-human primate embryos for generating transgenic non-human primates and/or ES cells for a wide variety of medical applications.

As used herein, non-human primate, includes all primates, for example, rhesus monkey, African Green monkey, simian, gorilla, rhesus macaque, baboon, cynomolgus

macaque, marmoset, chimpanzee, squirrel monkey, proboscis monkey, langur, tamarin, potto, dusky titi, orangutan, marmoset, capuchin, spider monkey, howler monkey, prosimians, pongo pygmaeus, and pan paniscus.

Adeno-associated virus (AAV) is a single-stranded DNA virus known to reside latently in primates and its genome is composed of linear single-stranded 4.7 kb DNA (McCarty, D. M. et al., J. Virol. 65: 2936-2945, 1991; McCarty, D. M. et al., J. Virol. 68:4988-4997, 1995). During latency in humans, AAV type 2 (AAV2) preferentially integrates at a site on chromosome 19q13.3ter by targeting a sequence composed of an AAV Rep binding element (RBE), a spacer, and a nicking site. Unlike other viruses, AAV is naturally defective, requiring coinfection with a helper virus (e.g. adenovirus or herpes virus) to establish a productive infection. No human disease has been found to be associated with AAV infection (Blacklow et al., 1968). The host range of AAV is broad; unlike retroviruses, AAV can infect both quiescent and dividing cells in vitro and in vivo (Flotte et al., 1993; Kaplitt et al., 1994; Podsakoff et al., 1994; Russell et al., 1994) as well as cells originating from different species and tissue types in vitro (Lebkowski et al., 1988; McLaughlin et al., 1988). When infection occurs in the absence of a helper virus, wild-type AAV can integrate into the cellular genome as a provirus, until it is rescued by superinfection with adenovirus. (Handa et al., 1977; Cheung et al., 1980; Laughlin et al., 1986).

The DNA sequence of an African green monkey AAV integration site isolated from CV-1 cells has 98% homology to the analogous human site, including identical spacer and nicking sequences. However, the simian RBE is expanded, having five perfect directly repeated GAGC tetramers. The frequency of site-specific integration is twofold greater in Cos-7 cells than in HeLa cells. The simian RBE, identified in CV-1 cells, functions analogously to the human RBE. (Terry J. Amiss, Doug M. McCarty, Anna Skulimowski, and R. Jude Samulski, Identification and Characterization of an Adeno-Associated Virus Integration Site in CV-1 Cells from the African Green Monkey, Journal Of Virology, Feb. 2003, p. 1904-1915.).

Eight different but closely related adeno-associated virus (AAV) serotypes, which infect primates have been identified (Berns, K. I. 1996. Parvoviridae: the viruses and their replication, p. 2173-2197; B. N. Fields et al. (ed.), *Fields Virology*, 3rd ed., vol. 2. Raven Press, Philadelphia, Pa and Gao, G. P., M. R. Alvira, L. Wang, R. Calcedo, J. Johnston, and J. M. Wilson, 2002, Novel adeno-associated viruses from rhesus monkeys as vectors for human gene therapy, *Proc. Natl. Acad. Sci. USA* 99:11854-11859).

Portions of the AAV genome have the capability of integrating into the DNA of cells to which it is introduced. As used herein, "integrate," refers to portions of the genetic construct that become covalently bound to the genome of the zygote or oocyte, for example through the mechanism of action mediated by the AAV Rep protein and the AAV ITRs. For example, the AAV virus has been shown to integrate at 19q13.3-qter, in the human genome. The minimal elements for AAV integration are the inverted terminal repeat (ITR) sequences and a functional Rep 78/68 protein. The present invention incorporates the ITR sequences into a vector for integration, and supplies a purified Rep protein to facilitate the integration of the transgene into the host cell genome for sustained transgene expression. The genetic transcript may also integrate into other chromosomes if the chromosomes contain the AAV integration site.

The AAV genome is relatively simple, containing two open reading frames (ORFs) flanked by short inverted terminal repeats (ITRs). "ITR," as used herein, refers to an inverted terminal repeat, for example, a DNA sequence that is repeated at either end of the virus in an inverted form. The ITRs contain, inter alia, cis-acting sequences for virus replication, rescue, packaging and integration. The integration function of the ITR permits the AAV genome to integrate into a cellular chromosome after infection (Samulski et al., 1989).

The nonstructural or replication (Rep) and the capsid (Cap) proteins are encoded by the 5' and 3' ORFs, respectively. Four related proteins are expressed from the rep gene; Rep78 and Rep68 are transcribed from the p5 promoter while a downstream promoter, p19, directs the expression of Rep52 and Rep40. The larger Rep proteins

(Rep78/68) are involved in AAV replication as well as regulation of viral gene expression (for review, see Muzyczka, 1992).

The predictability of insertion site reduces the danger of random insertional events into the cellular genome that may activate or inactivate host genes or interrupt coding sequences, consequences that limit the use of vectors whose integration is random, e.g., retroviruses. The Rep protein mediates the integration of the genetic construct containing the AAV ITRs and the transgene. The use of AAV elements is advantageous for predictable integration. Because it has not been associated with human or non-human primate diseases, thus obviating many of the concerns that have been raised with virus-derived gene therapy vectors are obviated.

An "isolated" or "purified" protein or biologically active portion thereof is substantially free of cellular material or other contaminating proteins from the cell or tissue source from which the AAV Rep protein is derived, or substantially free from chemical precursors or other chemicals when chemically synthesized. The language "substantially free of cellular material" includes preparations of AAV Rep protein in which the protein is separated from cellular components of the cells from which it is isolated or recombinantly produced. In one embodiment, the language "substantially free of cellular material" includes preparations of AAV Rep protein having less than about 30% (by dry weight) of non-AAV Rep protein (also referred to herein as a "contaminating protein"), more preferably less than about 20% of non-AAV Rep protein, still more preferably less than about 10% of non-AAV Rep B protein, and most preferably less than about 5% non-AAV Rep protein. When the AAV Rep protein or biologically active portion thereof is recombinantly produced, it is also preferably substantially free of culture medium, i.e., culture medium represents less than about 20%, more preferably less than about 10%, and most preferably less than about 5% of the volume of the protein preparation. The production of proteins from cloned genes by genetic engineering is well known. See, e.g. U.S. Pat. No. 4,761,371 to Bell et al, which is incorporated herein by reference in its entirety.

The language “substantially free of chemical precursors or other chemicals” includes preparations of AAV Rep protein in which the protein is separated from chemical precursors or other chemicals, which are involved in the synthesis of the protein. In one embodiment, the language “substantially free of chemical precursors or other chemicals” includes preparations of AAV Rep protein having less than about 30% (by dry weight) of chemical precursors or non-AAV Rep chemicals, more preferably less than about 20% chemical precursors or non-AAV Rep chemicals, still more preferably less than about 10% chemical precursors or non-AAV Rep chemicals, and most preferably less than about 5% chemical precursors or non-AAV Rep chemicals.

As used herein, a “biologically active portion” or an “active portion” of a protein or DNA sequence or RNA sequence includes the portions thereof that are necessary and sufficient for biological activity. For example and active portion of an AAV Rep protein includes a fragment of a AAV Rep protein which participates in an interaction between a AAV Rep molecule and a non-AAV Rep molecule (e.g., assisting in the integration of a genetic construct of the invention into the genome of a zygotic pronuclei). Biologically active portions of a AAV Rep protein include peptides comprising amino acid sequences sufficiently homologous or identical to or derived from the AAV Rep amino acid sequences, e.g., the amino acid sequences described herein, which include sufficient amino acid residues to exhibit at least one activity of AAV Rep. Typically, biologically active portions comprise a domain or motif with at least one activity of the AAV Rep protein, e.g., the ability to assist the genetic construct of the invention to integrate into the zygotic genome.

Purified Rep protein may be provided as described in Cell, vol. 61, pp 447-457 (1990), or may be chemically synthesized and optionally purified. Figure 3 depicts the AAV Rep 68 nucleotide and amino acid sequences.

“Portion of the genetic construct integrates into a chromosome” refers to the portion of the genetic construct that will become covalently bound to the genome of the oocyte or zygote upon introduction of the genetic construct into the cell. The integration is mediated by the AAV ITRs flanking the transgene and the AAV Rep protein. Portions

of the genetic construct that may be integrated into the genome include the transgene and the AAV ITRs. The promoter, if present, may also be integrated into the chromosome. Figures 1 and 2 show an example of a genetic construct of the invention. Portions that integrate are from about 1 nt to about 145 nt.

“A portion thereof,” as used herein, refers to any portion of the AAV ITR sequence that would be sufficient to promote integration of the transgene. An example of a first or a second AAV ITR is SEQ ID NO: 1 or portion thereof. Another example of a first or a second second AAV ITR is SEQ ID NO: 2, and or an operative portion thereof. An alternate example of a first or a second AAV ITR is SEQ ID NO: 3.

SEQ ID. NO: 1: ttggccactc cctctctgcg cgctcgctcg ctactgagg ccgggcgacc

SEQ ID. NO: 2: aaaggctgcc cgacgcccg gctttgccg ggcggcctca gtgagcgagc

SEQ ID. NO: 3 gagcgcgag agagggagtg gccactcca tcactagggg ttct

As used herein, the term “oocyte” refers to a female gamete cell and includes primary oocytes, secondary oocytes and mature, unfertilized ova. An oocyte is a large cell having a large nucleus (i.e., the germinal vesicle) surrounded by ooplasm. The ooplasm contains non-nuclear cytoplasmic contents including mRNA, ribosomes, mitochondria, yolk proteins, etc. “Developmental competence” refers to the ability of an oocyte to undergo embryonic development or parthenogenetic activation and develop at least to the morula or to the blastocyst stage in terms of embryo development. Development to the morula blastocyst stage is seen as a reliable indicator of the developmental competence of an oocyte. The terms “unfertilized egg” or “unfertilized oocyte” as used herein, refers to any female gamete cell that has not been fertilized and these terms encompass both pre-maturation and pre-fertilization oocytes.

The term “zygote” as used herein, refers to a fertilized oocyte that has not yet undergone the first cleavage step in the development of an embryo (i.e., it is at the single-cell stage).

The term “transgenic animal” refers to any animal, preferably a non-human primate, in which one or more of the cells of the animal contain heterologous nucleic acid

introduced by way of intervention, (e.g., human), such as by transgenic techniques disclosed herein. The nucleic acid may be introduced into the cell, directly or indirectly, for example, by microinjection or by infection with a recombinant virus. The term genetic manipulation does not include classical cross-breeding, or in vitro fertilization, but rather is directed to the introduction of a recombinant DNA molecule. This molecule may, for example, be integrated within a chromosome. The term "transgenic," should not be taken to be limited to referring to animals containing in their genome or germ line one or more genes from another species, although many transgenic animals will contain such a gene or genes. Rather, the term refers more broadly to any animal whose germ line or genome has been the subject of technical intervention by recombinant DNA technology. So, for example, an animal in whose germ line an endogenous gene has been deleted, duplicated, activated or modified is a transgenic animal for the purposes of this invention as much as an animal to whose genome or germ line an exogenous DNA sequence has been added.

Transgenic non-human primates of the invention may be generated to mimic human diseases or to contain human disease causing genes or genetic reagents that reduce, increase, obliterate, up-regulate, down-regulate, or otherwise alter normal gene function. The transgenic primate allows non-human primate models of human disease to be made to study disease pathology and to develop therapies in a species close to humans. For example, dominant negative genes, recessive genes, and genes involved in multigenic diseases. Transgenic non-human primates with genes that alter organ/tissue rejection in humans may be a useful source of transplant material.

The "transgene" may contain a transgenic sequence or a native or wild-type DNA sequence. The transgene may become part of the genome of the non-human primate, which develops in whole or in part from the zygote. In certain aspects of the invention, the transgene is integrated into the chromosomal genome, for example, into chromosome 19. A transgenic sequence can be partly or entirely species-heterologous, i.e., the transgenic sequence, or a portion thereof, can be from a species, which is different from the cell into which it is introduced.

A transgenic sequence can be partly or entirely species-homologous, i.e., the transgenic sequence, or a portion thereof, can be from the same species as is the cell into which it is introduced. If a transgenic sequence is homologous (in the sequence sense or in the species-homologous sense) to an endogenous gene of the cell into which it is introduced, then the transgenic sequence, preferably, has one or more of the following characteristics: it is designed for insertion, or is inserted, into the cell's genome in such a way as to alter the sequence of the genome of the cell into which it is inserted (e.g., it is inserted at a location which differs from that of the endogenous gene or its insertion results in a change in the sequence of the endogenous gene); it includes a mutation, e.g., a mutation which results in misexpression of the transgenic sequence; by virtue of its insertion. A transgenic sequence can include one or more transcriptional regulatory sequences and any other nucleic acid sequences, such as introns, that may be necessary for a desired level or pattern of expression of a selected nucleic acid, all operably linked to the selected nucleic acid or gene. The transgenic sequence can include an enhancer sequence and or sequences that allow for secretion.

As used herein, the term "stably maintained" refers to characteristics of transgenic non-human primates that maintain at least one of their transgenic elements (i.e., the element that is desired) through multiple generations. For example, it is intended that the term encompass the characteristics of transgenic non-human primates that are capable of passing the transgene to their offspring, such that the offspring are capable of maintaining the expression and/or transcription of the transgene. It is not intended that the term be limited to any particular organism or any specific recombinant element. The term "stable transfection" or "stably transfected" refers to the introduction and integration of foreign DNA into the genome of the cell. The term "stable transfectant" refers to a cell that has stably integrated foreign DNA into the genomic DNA.

As used herein, the terms "transgene encoding," "nucleic acid molecule encoding," "DNA sequence encoding," and "DNA encoding" refer to the order or sequence of deoxyribonucleotides along a strand of deoxyribonucleic acid. The order of these deoxyribonucleotides may, for example, determine the order of amino acids along

the polypeptide (protein) chain. The DNA sequence thus may code for the amino acid sequence.

Standard recombinant DNA techniques may be employed to construct the vectors and genetic constructs of the present invention (see, e.g., *Current Protocols in Molecular Biology*, Ausubel, F. et al., eds, Wiley and Sons, New York 1995). Such methods include the utilization of compatible restriction sites at the borders of the adenovirus associated genes and the ITR sequences or DNA linker sequences which contain restriction sites, as well as other methods known to those skilled in the art. The presence of known restriction sites in the AAV genome may be used to derive subgenomic fragments for insertion into the genetic constructs. Reference for adenovirus associated DNA sequence information is given in standard genetic/sequence databases. Plasmids routinely employed in molecular biology, e.g., pBR322 (New England Biolabs, Beverly, Mass.), pRep9 (Invitrogen, San Diego, Calif.) or pBS (Stratagene, La Jolla, Calif.), may be used as the basis for the plasmid into which adenovirus genes and the AAV ITR may be inserted.

As used herein, the “product of the transgene” may include a protein, an RNAi, an siRNA, a ribozyme, a tRNA, an mRNA, a ribosomal RNA, a snRNA, a 5SRNA, or an active portion of an RNP.

For example, proteins may include a hormone, an immunoglobulin, a plasma protein, or an enzyme. The transgenic sequence can encode any protein whose expression in the transgenic non-human primate is desired including, alpha-1 proteinase inhibitor, alkaline phosphatase, angiogenin, extracellular superoxide dismutase, fibrogen, glucocerebrosidase, glutamate decarboxylase, human serum albumin, myelin basic protein, proinsulin, soluble CD4, lactoferrin, lactoglobulin, lysozyme, lactoalbumin, erythropoietin, tissue plasminogen activator, human growth factor, antithrombin III, insulin, prolactin, alpha 1-antitrypsin, and the like.

A polypeptide may be any polypeptide of interest, for example, a polypeptide forming a catalytic domain of an enzyme, an antibody (e.g., a single chain antibody), a binding domain, an activating domain, a protein motif, or the like.

Peptides include, neuro-active peptides such as neuropeptide Y, and enkephalins. Peptides also include, for example, lipotropins, cartiotropins, and G-protein ligands. peptide antigens, and the like. A product of the transgene may alter the function of a gene, a protein, or a nucleic acid. For example, the product of the transgene may bind to a gene to prevent the gene from being expressed. The product may bind to a protein to prevent the protein from functioning normally or bind to a nucleic acid to prevent it from being translated. Other examples include, inducers of gene expression, protein/RNA stability factors, nucleases and proteases.

The product of the transgene may also, for example, cause a human disease or symptoms characteristic of a human condition or comparable to that seen in affected human patients. For example, the gene may cause a human dominant negative disease, which may include, known autosomal dominant forms of macular degeneration, retinitis pigmentosa, cone and cone/rod dystrophies, Leber's Congenital Amaurosis and optic atrophy, Emery-Dreifuss muscular dystrophy, spinocerebellar ataxia, Ehlers-Danlos syndrome, parkinson disease, renal tubular acidosis, diabetes insipidus, keratitis-ichthyosis-deafness syndrome, Marfan syndrome, and the like. The transgene may alternately cause diseases wherein the expression of a mutant form of a protein causes the disease. For example, Creutzfeldt-Jakob disease. The transgene could also cure a disease manifested because of the lack of a protein, for example, Fragile X Syndrome. More generally, the methods of the invention could be used to generate analogous non-human primate models for any autosomal dominant human disease in which the mutant cDNA has been cloned. By inserting ribozymes, siRNA or similar reagents in the AAV plasmid DNA, the methods disclosed herein may generate non-human primate models of recessive human diseases, for example, retinal diseases. Transferring genes that initiate or mimic pathogenic processes may also generate non-human primate models for human diseases of known or unknown genetic etiology. In general any genetically based disease could be modeled in nonhuman primates by delivering the cooresponding mutant gene, for example lysosomal storage diseases, any genetically based predisposition to disease, for example certain mutations in BCRAI, any predisposition to genetically based pathology, such as poor wound healing, vascular insufficiency or athlersclerosis (APOE).

The terms “vector” and “genetic construct” are used interchangeably herein and refer to a recombinant molecule containing a desired coding sequence and appropriate nucleic acid sequences necessary for the expression of the operably linked coding sequence in a particular host organism. Nucleic acid sequences necessary for expression in prokaryotes usually include a promoter, an operator (optional), and a ribosome binding site, and other sequences. Eukaryotic cells are known to utilize promoters, enhancers, and termination and polyadenylation signals. The term “genetic cassette” as used herein refers to a fragment or segment of nucleic acid containing a particular grouping of genetic elements. The cassette can be removed and inserted into a vector or plasmid as a single unit. An example of a genetic construct of the invention is shown in Figures 1 and 2.

The terms “in operable combination,” “in operable order,” and “operably linked,” as used herein refer to the linkage of nucleic acid sequences in such a manner that a nucleic acid molecule capable of directing the transcription of a given gene and/or the synthesis of a desired protein molecule is produced. The term also refers to the linkage of amino acid sequences in such a manner so that a functional protein is produced.

In certain embodiments, the design of the genetic construct places the gene of interest between the AAV ITR sequences. The AAV ITR sequences have cis-acting functions that facilitate integration of the construct into a cell following its introduction into the cell.

As used herein, the term “protein of interest” refers to any protein for which expression is desired. For example, the term encompasses any recombinant forms of a protein that is desired. The term “gene of interest” refers to any gene that is desired. In particularly preferred embodiments, the gene of interest encodes at least a portion of a protein of interest.

As used herein, the term “primer” refers to an oligonucleotide, whether occurring naturally as in a purified restriction digest or produced synthetically, which is capable of acting as a point of initiation of synthesis when placed under conditions in which synthesis of a primer extension product which is complementary to a nucleic acid strand is induced, (i.e., in the presence of nucleotides and an inducing agent such as DNA

polymerase and at a suitable temperature and pH). The primer is preferably single stranded for maximum efficiency in amplification, but may alternatively be double stranded. If double stranded, the primer is first treated to separate its strands before being used to prepare extension products. The primer must be sufficiently long to prime the synthesis of extension products in the presence of the inducing agent. The exact lengths of the primers will depend on many factors, including temperature, source of primer and the use of the method.

As used herein, the term "polymerase chain reaction" (PCR) refers to the methods of U.S. Pat. Nos. 4,683,195, 4,683,202, and 4,965,188, all of which are hereby incorporated by reference, directed to methods for increasing the concentration of a segment of a target sequence in a mixture of genomic DNA without cloning or purification. As used herein, the terms "PCR product" and "amplification product" refer to the resultant mixture of compounds after two or more cycles of the PCR steps of denaturation, annealing and extension are complete. These terms encompass the case where there has been amplification of one or more segments of one or more target sequences.

As used herein, the terms "restriction endonucleases" and "restriction enzymes" refer to bacterial enzymes, each of which cut double-stranded DNA at or near a specific nucleotide sequence.

As used herein, the term "recombinant DNA molecule" as used herein refers to a DNA molecule, which is comprised of segments of DNA joined together by means of molecular biological techniques.

As used herein, a nucleic acid sequence, even if internal to a larger oligonucleotide, also may be said to have 5' and 3' ends. In either a linear or circular DNA molecule, discrete elements are referred to as being "upstream" or 5' of the "downstream" or 3' elements. This terminology reflects the fact that transcription proceeds in a 5' to 3' fashion along the DNA strand. The promoter and enhancer elements which direct transcription of a linked gene are generally located 5' or upstream of the coding region. However, enhancer elements can exert their effect even when

located 3' of the promoter element and the coding region. Transcription termination and polyadenylation signals are located 3' or downstream of the coding region.

As used herein, an oligonucleotide having a nucleotide sequence encoding a gene refers to a DNA sequence comprising the coding region of a gene or in other words the DNA sequence, which encodes a gene product. The coding region may be present in either a cDNA or genomic DNA form. Suitable control elements such as enhancers/promoters, splice junctions, polyadenylation signals, etc., may be placed in close proximity to the coding region of the gene if needed to permit proper initiation of transcription and/or correct processing of the primary RNA transcript. Alternatively, the coding region utilized in the vectors of the present invention may contain endogenous enhancers/promoters, splice junctions, intervening sequences, polyadenylation signals, etc., or a combination of both endogenous and exogenous control elements.

As used herein, the term "promoter/enhancer" denotes a segment of DNA which contains sequences capable of providing both promoter and enhancer functions (i.e., the functions provided by a promoter element and an enhancer element, see above for a discussion of these functions). Promoters and enhancers consist of short arrays of DNA sequences that interact specifically with cellular proteins involved in transcription (Maniatis et al., Science 236:1237, 1987).

The enhancer/promoter may be "endogenous" or "exogenous" or "heterologous." An "endogenous" enhancer/promoter is one that is naturally linked with a given gene in the genome. An "exogenous" or "heterologous" enhancer/promoter is one that is placed in juxtaposition to a gene by means of genetic manipulation (i.e., molecular biological techniques) such that transcription of the gene is directed by the linked enhancer/promoter.

Promoters and enhancers may bind to specific factors, which increase the rate of activity from the promoter or enhancer. The term "factor" refers to a protein or group of proteins necessary for the transcription or replication of a DNA sequence. For example, SV40 T antigen is a replication factor necessary for the replication of DNA sequences containing the SV40 origin of replication. For example, transcription factors are proteins

that bind to regulatory elements such as promoters and enhancers and facilitate the initiation of transcription of a gene. The selection of a particular promoter and enhancer depends on what cell type is to be used to express the protein of interest. Some eukaryotic promoters and enhancers have a broad host range while others are functional in a limited subset of cell types (Voss et al., Trends Biochem. Sci., 11:287, 1986; and Maniatis et al., *supra.*, 1987).

It is often desirable to express a product of a transgene, for example, a protein, in a specific tissue or fluid, e.g., the milk, eye, or neural tissue of a transgenic animal. The heterologous protein can be recovered from the tissue or fluid in which it is expressed. For example, it is often desirable to express the heterologous protein in milk. Examples of tissue specific promoters include the following: a neural-specific promoter, e.g., nestin, Wnt-1, Pax-1; Engrailed-1, Engrailed-2, Sonic hedgehog; a liver-specific promoter, e.g., albumin, alpha-1 antitrypsin; a muscle-specific promoter, e.g., myogenin, actin, MyoD, myosin; an oocyte specific promoter, e.g., ZP1, ZP2, ZP3; a testes-specific promoter, e.g., protamin, fertilin, synaptonemal complex protein-1; a blood-specific promoter, e.g., globulin, GATA-1, porphobilinogen deaminase; a lung-specific promoter, e.g., surfactant protein C; a skin- or wool-specific promoter, e.g., keratin, elastin; endothelium-specific promoters, e.g., Tie-1, Tie-2; a bone-specific promoter, e.g., BMP; AAV P5, rod opsin, cone opsin, RPE specific promoters such as VMD2 or RPE65, or any other organ, tissue or cell specific promoter, or any combination of promoter elements. General promoters can be used for expression in several tissues. Examples of general promoters include beta-actin, ROSA-21, PGK, FOS, c-myc, Jun-A, Jun-B, CMV, chicken beta-actin, E1alpha, or any combination of promoter elements.

The presence of "splicing signals" in a transgene often results in higher levels of expression of the transcript. Splicing signals mediate the removal of introns from the primary RNA transcript and consist of a splice donor and acceptor site (See e.g., Sambrook, J. et al., Molecular Cloning: A Laboratory Manual, 2nd ed., Cold Spring Harbor Laboratory Press, New York (1989), pp. 16.7-16.8). A commonly used splice donor and acceptor site is the splice junction from the 16S RNA of SV40.

Efficient expression of DNA sequences in eukaryotic cells requires expression of signals directing the efficient termination and polyadenylation of the resulting transcript. Transcription termination signals are generally found downstream of the polyadenylation signal and are a few hundred nucleotides in length. The term "poly A site" or "poly A sequence" as used herein denotes a DNA sequence which directs both the termination and polyadenylation of the nascent RNA transcript. Efficient polyadenylation of the transgene transcript is desirable as transcripts lacking a poly A tail are unstable and are rapidly degraded. The poly A signal utilized in a genetic construct may be "heterologous" or "endogenous." An endogenous poly A signal is one that is found naturally at the 3' end of the coding region of a given gene in the genome. A heterologous poly A signal is one which is isolated from one gene and placed 3' of another gene. A commonly used heterologous poly A signal is the SV40 poly A signal. The SV40 poly A signal is contained on a 237 bp Bam HI/Bcl I restriction fragment and directs both termination and polyadenylation (Sambrook, J., *supra*, at 16.6-16.7).

Oocytes for use in the invention include oocytes at any state of maturity that will allow fertilization, preferably, oocytes in metaphase II stage of meiotic cell division, e.g., oocytes arrested in metaphase II, a telophase stage of meiotic cell division, e.g., telophase I or telophase II. Oocytes in metaphase II contain one polar body, whereas oocytes in telophase can be identified by the absence of a germinal vesicle and polar body or based on the presence of a protrusion of the plasma membrane from the second polar body up to the formation of a second polar body. In addition, oocytes in metaphase II can be distinguished from oocytes in telophase II based on biochemical and/or developmental distinctions. For example, oocytes in metaphase II can be in an arrested state, whereas oocytes in telophase are in an activated state. Preferably, the oocyte is a non-human primate.

Oocytes can be obtained or recovered at various times at a various stages of development or maturation during a non-human primates reproductive cycle. For example, at given times during the reproductive cycle, a significant percentage of the oocytes, e.g., about 55%, 60%, 65%, 70%, 75%, 80% or more, are oocytes in prophase or telophase I. Such oocytes at various stages of the cell cycle can be obtained or recovered

from the non-human primate and then induced in vitro to enter a particular stage of meiosis.

Oocytes can also be collected or recovered from a female non-human primate during superovulation. Briefly, oocytes can be recovered surgically by inserting a needle into each ovarian follicle and aspirating the follicular content. Alternately, oocytes that have been ovulated can be recovered by flushing the oviduct of the female donor. In this case, the female donor has either ovulated during a natural cycle or has been subjected to a modified superovulation protocol. Fertilized oocytes are also useful and can be obtained or recovered from the oviducts of mated non-human primates. Such protocols are well known in the art and one of skill in the art, having the benefit of this disclosure would know how to effect superovulation in a female non-human primate or recover oocytes from mated females. Methods of inducing superovulation in non-human primates and the collection of oocytes is described in the examples herein.

According to certain aspects, the oocyte is from a non-human primate, for example, a rhesus monkey, African Green monkey, simian, gorilla, rhesus macaque, baboon, cynomolgus macaque, marmoset, chimpanzee, squirrel monkey, proboscis monkey, langur, tamarin, potto, dusky titi, orangutan, marmoset, capuchin, spider monkey, howler monkey, prosimians, pongo pygmaeus, pan paniseus, or the like.

The method includes contacting the oocyte with sperm under conditions that permit the fertilization of the oocyte to produce an embryo. Fertilizing the oocyte to produce a zygote having zygotic pronuclei may be done by intracytoplasmic sperm injection, sperm incubation, or the like. These techniques are described in Ouhibi et al.

In preferred embodiments, the genetic construct is preferably introduced into a single-cell zygote. Such introduction may be achieved by pronuclear injection or microinjection (Wang, et al. *Molecular Reproduction and Development* (2002) 63:437-443), cytoplasmic injection or microinjection (Page, et al. *Transgenic Res* (1995) 4(6):353-360), retroviral infection (e.g., Lebkowski, et al. *Mol Cell Biol* (1988) 8(10):3988-3996), or electroporation ("Molecular Cloning: A Laboratory Manual. Second Edition" by Sambrook, et al. Cold Spring Harbor Laboratory: 1989).

Introduction may also be by chemical assistance, for example, by lysosomal vesical packaging or other similar technique. For injection or microinjection and electroporation protocols, the introduced DNA may comprise linear or circular DNA, as prepared from the vectors or constructs of the invention. This introduction of the genetic construct and the AAV Rep protein should not interfere with early embryo development and should result in transgene expression. According to further methods, the zygote is allowed to further develop into, for example, a pre-implantation embryo suitable for implantation into a recipient female for fetal development. The genetic construct may be introduced, for example, into the male pronuclei, the female pronuclei, or both the male and female pronuclei.

Other references for introduction of transgenes into embryonic cells are known in the art. See, for example, "Transgenic Animal Technology: A Laboratory Handbook," C. A. Pinkert, editor, Academic Press, 2002, 2nd edition, 618 pp.; "Mouse Genetics and Transgenics: A Practical Approach," I. J. Jackson and C. M. Abbott, editors, Oxford University Press, 2000, 299 pp.; "Transgenesis Techniques: Principles and Protocols," A. R. Clarke, editor, Humana Press, 2001, 351 pp., Briskin et al. (1991) *Proc. Natl. Acad. Sci. USA*, 88:1736-1740; Pfeifer et al. (2002), *Proc. Natl. Acad. Sci. USA*, 99:2140-2145; Houdebine and Chourrout (1991) *Experientia*, 47:891-897, Carver, et al., *Bio/Technology* 11:1263-1270, 1993; Carver et al., *Cytotechnology* 9:77-84, 1992; Clark et al., *Bio/Technology* 7:487-492, 1989; Simons et al., *Bio/Technology* 6:179-183, 1988; Swanson et al., *Bio/Technology* 10:557-559, 1992; Velander et al., *Proc. Natl. Acad. Sci. USA* 89:12003-12007, 1992; Hammer et al., *Nature* 315:680-683, 1985; Krimpenfort et al., *Bio/Technology* 9:844-847, 1991; Ebert et al., *Bio/Technology* 9:835-838, 1991; Simons et al., *Nature* 328:530-532, 1987; Pittius et al., *Proc. Natl. Acad. Sci. USA* 85:5874-5878, 1988; Greenberg et al., *Proc. Natl. Acad. Sci. USA* 88:8327-8331, 1991; Whitelaw et al., *Transg. Res.* 1:3-13, 1991; Gordon et al., *Bio/Technology* 5:1183-1187, 1987; Grosveld et al., *Cell* 51:975-985, 1987; Brinster et al., *Proc. Natl. Acad. Sci. USA* 88:478-482, 1991; Brinster et al., *Proc. Natl. Acad. Sci. USA* 85:836-840, 1988; Brinster et al., *Proc. Natl. Acad. Sci. USA* 82:4438-4442, 1985; Al-Shawi et al., *Mol. Cell. Biol.* 10(3):1192-1198, 1990; Van Der Putten et al., *Proc. Natl. Acad. Sci. USA* 82:6148-6152,

1985; Thompson et al., Cell 56:313-321, 1989; Gordon et al., Science 214:1244-1246, 1981; and Hogan et al., Manipulating the Mouse Embryo: A Laboratory Manual (Cold Spring Harbor Laboratory, 2002), which are each incorporated herein by reference in their entirety.

According to certain embodiments, once the genetic construct and the AAV Rep protein are introduced into the oocyte, zygote, or embryo; the oocyte, zygote or embryo may be optionally cultured in vitro. Embryos may be cultured in CMRL 1066 supplemented with serum with or without coculture on buffalo rat liver cell monolayers (CMRL/BRL) or on human placental cells. Preferably, embryos may also be cultured in HECM-9 in the absence of coculture. Culture in HECM-9 produces faster development than that seen in CMRL/BRL with compact morulae present on day 3 post fertilization by ICSI, cavitating blastocysts on day 5 and expanded blastocysts on day 7. These rates are approximately 1 day advanced over CMRL/BRL.

In a preferred embodiment, the method of the present invention further comprises, transferring the oocyte, zygote, blastocyst, or embryo into a hormonally synchronized non-human recipient animal (i.e., a female animal at the correct stage of the menstrual cycle to support embryo implantation and development or a female animal hormonally synchronized to stimulate early pregnancy). Methods of transfer include, embryo placement into the oviduct by laparoscopy or mini-laparotomy, and a non-surgical, transcervical approach of uterine deposition. Other acceptable methods of transfer include, cervical cannulation, and the like. In another preferred embodiment, the method comprises the step of allowing the transferred embryo/pregnancy to develop to term. Developing to term includes developing until the transgenic embryo would be viable outside of the uterus. In still another preferred embodiment, at least one transgenic offspring is identified from the offspring allowed to develop to term.

The selection of embryos for transfer is normally based on developmental progression, presence of the appropriate number of nucleated blastomeres, absence of fragmentation and general appearance. Usually only the highest quality embryos are transferred, and assessment of the quality of the embryo is well within the skill in the art,

(criteria include, e.g., growth, cell appearance, morphology, etc.). The ability to freeze embryos and conduct transfers when recipients are available is highly convenient because it supports the shipment of embryos to other facilities.

In a preferred embodiment, the method further includes mating the transgenic non-human primate that develops from the transgenic embryo with a second non-human primate. The second non-human primate can be a normal non-human primate, a second non-human primate which develops from a transgenic embryo or is descended from a non-human primate which developed from a transgenic embryo or a second non-human primate developed from a transgenic embryo, or descended from a non-human primate which developed from a transgenic embryo, which was formed from genetic material from the same animal, an animal of the same genotype, or same cell line, which supplied the genetic material for the first non-human primate. In a preferred embodiment, a first transgenic non-human primate that develops from the transgenic embryo can be mated with a second transgenic non-human primate which developed from a transgenic embryo and which contains a different transgene than the first transgenic non-human primate.

In a preferred embodiment, the transgenic non-human primate is a male non-human primate. In other preferred embodiments the transgenic non-human primate is a female non-human primate.

The methods of the invention may also apply to any mammalian species that contains a natural or engineered chromosomal target for AAV Rep. For example, a transgenic mouse containing a natural or engineered chromosomal target for AAV Rep may be made according to techniques well known in the art. The methods disclosed herein would then be applicable to the transgenic mouse and one of skill in the art would be able to adapt the teachings herein to produce chromosomally targeted transgenic mice.

Certain embodiments of the invention include treating a human or non-human primate in need of an organ transplant by transplanting an organ from a transgenic non-human primate created according to the methods of the invention. The transgenic non-human primates of the invention may be sources of transplant material, for example, a kidney, liver, heart, lung, pancreas, skin, eye and the like, may be harvested from a

transgenic non-human primate produced according to the methods of the invention and transplanted into a human or non-human primate in need thereof. Harvesting and transplanting techniques are well known in the art.

According to other embodiments, the transgenic non-human primate oocyte, blastocyst, embryo, or offspring may be used as a model for a human disease.

In certain embodiments, the cells of the transgenic oocyte, zygote, blastocyst, or embryo are used to establish embryonic stem (ES) cell lines. Stem cells are defined as cells that have extensive proliferation potential, differentiate into several cell lineages, and repopulate tissues upon transplantation. The quintessential stem cell is the embryonic stem (ES) cell, as it has unlimited self-renewal and multipotent differentiation potential (Thomson, J. et al. 1995; Thomson, J. A. et al. 1998; Shambloot, M. et al. 1998; Williams, R. L. et al. 1988; Orkin, S. 1998; Reubinoff, B. E., et al. 2000). These cells are derived from the inner cell mass of the blastocyst (Thomson, J. et al. 1995; Thomson, J. A. et al. 1998; Martin, G. R. 1981), or can be derived from the primordial germ cells from a post-implantation embryo (embryonal germ cells or EG cells). ES and EG cells have been derived from mouse, and more recently also from non-human primates and humans. When introduced into mouse blastocysts, ES cells can contribute to all tissues of the mouse (animal) (Orkin, S. 1998). Murine ES cells are therefore pluripotent. When transplanted in post-natal animals, ES and EG cells generate teratomas, which again demonstrates their multipotency. ES (and EG) cells can be identified by positive staining with the antibodies to stage-specific embryonic antigens (SSEA) 1 and 4.

Embodiments of the invention include the use of the ES cell lines derived from the transgenic zygote, embryo, blastocyst or non-human primate to treat human and non-human primate diseases. Methods include implanting ES cells into an organ, for example, the brain, liver, heart, kidney, pancreas, skin, and the like, and allowing the cells to develop into the organ tissue. For example, the ES cell lines may be implanted into the brain of a human suffering from Parkinson's, or into the pancreas of a diabetic patient, and the like to treat the condition. In addition, embryonic stem cells transduced with

disease-causing gene mutations as provided herein, can provide an *in vitro* system to investigate disease pathogenesis and to test potential therapeutic strategies.

“Detecting expression of the transgene,” includes examining the cell or cells of the transgenic zygote, embryo, blastocyst, fetus, or transgenic non-human primate cells for the integration of the transgene and/or the expression of the transgene. The integration of the transgene may be detected, for example, by Southern blot or Polymerase chain reaction (PCR) may be performed with primer sets that cover the transgene. Expression of the transgene may be examined in transgenic non-human primates, for example, in their hair, blood, umbilical cord, placenta, cultured lymphocytes, buccal epithelial cells, and urogenital cells passed in urine. Expression may also be examined by extracting total RNA for reverse transcription followed by PCR amplification (RT-PCR) with primer sets specific for the transgene.

EXAMPLES

The following non-limiting examples are illustrative of the invention.

EXAMPLE 1

Ovarian stimulation, recovery of rhesus macaque oocytes, fertilization by intracytoplasmic sperm injection, pronuclear microinjection, and embryo culture

For controlled ovarian stimulation and oocyte recovery cycling rhesus macaque females were subjected to follicular stimulation using twice-daily intramuscular injections of recombinant human FSH as well as concurrent treatment with Antide, a GnRH antagonist, for 8-9 days. Unless indicated otherwise, all reagents were from Sigma-Aldrich Co. (St. Louis, MO) and all hormones and Antide were from Ares Advanced Technologies Inc. (Norwell, MA). Females received recombinant human LH on days 7-9 and recombinant HCG on day 10. Cumulus-oocyte complexes were collected from anesthetized animals by laparoscopic follicular aspiration (28-29 hrs post HCG) and placed in Hepes-buffered TALP (modified Tyrode solution with albumin, lactate and pyruvate) medium containing 0.3% BSA (TH3) at 37° C. Oocytes, stripped of cumulus cells by mechanical pipetting after brief exposure (<1 min) to hyaluronidase (0.5 mg/ml),

were placed in chemically defined, protein-free HECM-9 medium (Hamster Embryo Culture Medium) at 37° C in 5% CO₂, 5% O₂ and 90% N₂ until further use.

Mature metaphase II (MII) stage oocytes were fertilized by intracytoplasmic sperm injection. Briefly, sperm were diluted with 10% polyvinylpyrrolidone (1:4; Irvine Scientific, Santa Ana, CA) and a 5µl drop was placed in a micromanipulation chamber. A 30µl drop of TH3 was placed in the same micromanipulation chamber next to the sperm droplet and both were covered with paraffin oil (Zander IVF, Vero Beach, FL). The micromanipulation chamber was mounted on an inverted microscope equipped with Hoffman optics and micromanipulators. An individual sperm was immobilized, aspirated into an ICSI pipette (Humagen, Charlottesville, VA) and injected into the cytoplasm of a MII oocyte, away from the polar body. After ICSI, injected oocytes were placed in 4-well dishes (Nalge Nunc International Co., Naperville, IL) containing protein-free HECM-9 medium and cultured overnight at 37° C in 5% CO₂, 5% O₂ and 90% N₂. Cultures were maintained under paraffin oil. Pronuclear stage embryos were selected approximately 12-16 hours after ICSI and placed in the micromanipulation chamber. DNA injection buffer consisted of 18mM Hepes, 80 mM KCl, 20 mM MgCl₂, 10% glycerol, 2ng/µl TR/P5, EF1α-GFP vector, 10ng/µl Rep 68 protein and 0.5 mM DTT. Approximately, 6-8 pl (1% of zygote volume) of DNA buffer was injected directly into both male and female pronuclei. Injected zygotes were placed into HECM-9 medium and cultured at 37° C in 5% CO₂, 5% O₂ and 90% N₂. Embryos at the 8-16 cell stage were transferred to fresh plates of HECM-9 medium supplemented with 5% fetal bovine serum (FBS; HyClone, Logan, UT) and cultured for a maximum of 7 days with medium change every other day. During the culture period, embryos were periodically scored based on morphological criteria and monitored for GFP expression under epifluorescence microscope. Figure 4 depicts two AAV-GFP transgenic rhesus embryos that were injected with a DNA construct of the invention at the 1-cell stage and photographed 8 days later at the 200 cell blastocyst stage. Left panels: DAPI staining to show all cells. Right panels: GFP antibody immunostaining (red fluorescence). Approximately 80% of the cells of the inner cell mass are GFP-positive suggesting that most fetal tissues from such blastocysts would be transgene positive.

EXAMPLE 2

DNA and Rep 68 protein

The TR/P5-EF1 α -eGFP plasmid, of the sequence shown in Figure 2, is injected into a zygote as described above and contains AAV inverted terminal repeats (TR) flanking the P5 region of AAV2, followed by elongation factor 1 alpha (EF1- α) promoter and an enhanced green fluorescence protein (eGFP) cDNA sequence and a SV40 viral polyadenylation (SV40-PolyA) signal sequence. The plasmid was constructed using pBluescript II (Stratagene) as a backbone. TR and P5 sequences of AAV2 were inserted into PstI site of pBluescript II. The EF1- α promoter and eGFP sequences were cloned into the HindIII site. The SV40-PolyA sequence was cloned in between XhoI and KpnI sites. The plasmid was sequenced and purified by double CsCl density gradient purification. The plasmid map is shown in Figure 1.

His-tagged Rep68 fusion protein was purified from transfected SG13009 cells (Qiagen) by passing the cellular protein extract over nickel-nitrilotriacetic columns (Qiagen), followed by elution with a 0.1 to 0.5 M imidazol gradient. Eluted protein was analyzed by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) and silver staining (Bio-Rad). Protein concentration was determined by standard bovine serum albumin (BSA) assay. A sequence of Rep 68 is represented in Figure 3.

EXAMPLE 3

Integration Assay

Individual monkey blastocytes are lysed in PCR buffer containing 100ng/ml proteinase K at 50°C for 30 min, and the proteinase K was inactivated at 95°C for 10min. AAVS1 primer 5'-CGGGGAGGATCCGCTCAGAGGACA-3' and AAV TR primer 5'-CGGCCTCAGTGAGCGAGCGAGC-3' are used along with DNA (equal to 30 cells) extracted from monkey individual blastocytes in PCR assay. The temperature cycles are 95°C for 5 min, followed by 35 cycles of 95°C for 1 min, 67°C 1 min, and 72°C for 2.5 min. If necessary a second PCR internal to the first PCR product (nested PCR) will be

carried out using different set of AAVS1 primers and primers derived for TR/P5-GFP plasmid.

EXAMPLE 4

AAV plasmid DNA containing the GFP cDNA was injected into pronuclei of 10 rhesus monkey zygotes together with purified Rep protein. Viable blastocysts developed in 3 of 9 plasmid-treated embryos (1 lysed during injection) compared to 6 of 16 viable blastocysts for the no treatment controls. This suggests little, if any, effect of plasmid AAV DNA injection on embryo viability. GFP expression was detected by direct epifluorescence at early cleavage stages and later confirmed by immunocytochemistry (figure 4). Expression was mosaic, with approximately 80% of viable blastomeres exhibiting strong GFP fluorescence at the 200-cell blastocyst stage.

Such mosaic GFP expression was detected in 2 of 3 viable blastocysts and included cells of both the inner cell mass and trophoblast. Given the number of cell divisions between the zygote and blastocyst stages, GFP expression is likely derived from integrated plasmid DNA copies. Sequence analysis for specific integration event is currently underway.

EXAMPLE 5

Ovarian stimulation, oocyte recovery and fertilization by ICSI

Cycling females were subjected to follicular stimulation using twice-daily intramuscular injections of recombinant human FSH as well as concurrent treatment with Antide, a GnRH antagonist, for 8-9 days. Females received recombinant human LH on days 7-9 and recombinant hCG on day 10. Cumulus-oocyte complexes were collected from anesthetized animals by laparoscopic follicular aspiration (27-29 hr post hCG) and placed in HEPES-buffered (TALP) containing 0.3% bovine serum albumin (BSA) (TH3) at 37°C. Tubes containing follicular aspirates were placed in a portable incubator (Minitube, Verona, WI) at 37° C for transport to the laboratory. Aspirates were sifted through a cell

strainer (Becton-Dickinson, Franklin Lakes, N.J.; Falcon, 70 μ m mesh size). Hyaluronidase (0.5 mg/ml; Sigma Chemical Co., St. Louis, MO; in TH3) was added directly to the tubes containing aspirates followed by incubation at 37° C (30 sec) before the contents were gently agitated with a serological pipette to disaggregate cumulus and granulosa cell masses and poured on the strainer. Oocytes were retained in the mesh, while blood, cumulus and granulosa cells were sifted through the filter. The strainer was immediately backwashed with TH3 and the medium containing oocytes was collected. Residual cumulus cells were removed with a small bore pipette (approximately 125 μ m in inner diameter) before recovered oocytes were examined for determination of developmental stage germinal vesicle (GV), metaphase I (MI) or metaphase II (MII) and quality (granularity, shape and color of the cytoplasm). Oocytes were placed in chemically defined, protein-free HECM-9 medium checked at 37°C in 5% CO₂, 5% O₂ and 90% N₂ covered with paraffin oil (Ovoil, Zander IVF, Vero Beach, FL).

Semen was collected by penile electroejaculation and allowed to liquefy at 27-32° C for 10-15 min. The liquid portion was harvested from the coagulum into a 15 ml conical centrifuge tube (Fisher Scientific, Tustin, CA) and washed twice by centrifugation at 130-150 x g for 5 minutes and resuspension in 5ml TH3. Motility and concentration were evaluated microscopically and only samples with an initial motility in excess of 70% with a strong forward progression were utilized. For sperm cryopreservation, the washed sperm pellet was resuspended in 0.25 ml of TES-TRIS buffer containing 3% glycerol, 30% egg yolk, 20% skim milk, 0.06 M glucose and equilibrated at 4°C for 1 h (Sanchez-Partida et al BOR 663:1092-1097, 2000). This sperm suspension was frozen in 20-50 μ l drops by placing individual drops for 10 minutes in small pits carved in the surface of dry ice. The goal was to create drops of 1-2 million washed sperm each, ideally in a 20 :1 volume. Frozen drops (up to 10) were then transferred with precooled forceps to precooled cryovials (Nalge Nunc International Co., Naperville, IL) and the vials were placed on a cane before plunging into liquid nitrogen for storage. For thawing, a single pellet, retrieved from liquid nitrogen, was placed in a dry test tube, suspended in a 37°C water bath for 40 sec and then washed in 5 ml of TH3 as described above. Individual

sperm, either fresh or after cryostorage were selected for ICSI on the basis of normal morphology and progressive motility. Embryos were cultured employing HECM-9.

EXAMPLE 6

Fertilization by ICSI

After ICSI, injected oocytes are placed in 4-well dishes containing protein free HECM-9 medium (0.7ml) and covered with paraffin oil (0.3ml; Ovoil). For extended culture, embryos at the 8-cell stage are transferred to fresh plates of HECM-9 medium supplemented with 5% fetal bovine serum and cultured for a maximum of 7 days with medium change every other day.

Embryo transfer is undertaken in recipients chosen on the basis of general health and physical condition, usually a record of previous pregnancy and live birth, and a history of normal ovarian cycles. Beginning 8 days after menses detection during a spontaneous menstrual cycle, blood samples are collected daily from the saphenous vein for determination of estradiol by radioimmunoassay. The LH surge is estimated to occur prior to the precipitous decline in serum estradiol, typically to levels below 100 pg/ml. The day when serum estradiol peaked is considered the day before ovulation (day-1). This peak occurred on average 11 days post menses with a range from 8 to 17 days. Two to 6 days after the estradiol peak, fresh or frozen-thawed embryos, typically 2/recipient are transferred surgically to the oviduct ipsilateral to the ovary bearing the ovulatory stigma in anesthetized recipients. A non-surgical, trans-cervical approach to embryo transfer may also be used.

Monkeys are anesthetized with isoflurane gas vaporized in 100% oxygen, and undergo comprehensive physiologic monitoring throughout the surgery, including electrocardiogram (ECG), peripheral oxygen saturation, and end-expired carbon dioxide. Orotracheal intubation and mechanical ventilation to maintain expired CO₂ at less than 50 mm Hg. After sterile skin preparation and draping, the abdomen is insufflated with CO₂ at 15 mm Hg pressure and the viewing telescope is inserted via a small supraumbilical incision, with accessory ports placed in the paralumbar region. The monkey is placed in

the Trendelenburg position, allowing the viscera to migrate in a cephalad direction exposing the reproductive organs. After insertion of the telescope, the ovaries are examined with a self retaining micro retractor inserted at a high paramedian position. The transfer is conducted into the oviduct with an ovulation site on the associated ovary. The fimbria is grasped with a Patton retractor (Cook, Ob/Gyn, Spencer, IN) and placed in traction. The guide cannula is introduced into the oviduct. Typically, two ICSI or IVF embryos are transferred. To this end, embryos are removed from culture medium and transferred to a dish containing TH3 medium. The Patton polyurethane transfer catheter (Cook OB/GYN) connected to a 1 ml syringe is filled with about 0.01-0.02 ml of TH3 medium avoiding air bubbles. Embryos are carefully loaded near the catheter tip with a total volume not exceeding 0.03 ml. The catheter is then inserted transabdominally and advanced through the fimbrian into the oviduct to a distance of 1-3 cm where the embryos are deposited. Following transfer, the catheter is removed and carefully examined and rinsed to ensure that all embryos had been expelled. In the event of a retained embryo, a second transfer is attempted. The insufflation is reduced and the incisions closed with interrupted absorbable suture in an intradermal pattern.

Pregnancy detection and monitoring

To detect pregnancy, estradiol and progesterone profiles are monitored every third day after ET for 25 days at which time the existence of a clinical pregnancy is confirmed by fetal cardiac activity as determined by ultrasonography. Confirmed pregnancies are monitored periodically throughout gestation by ultrasound. For the timed mated breeding (TMB) colony, females are palpated 30 days post pairing. All positives and questionable 30-day palpations are repalpated at 60 days. Subsequently, pregnancy is monitored by weight gain and evidence of "menses" or by ultrasound if deemed clinically necessary.

Birth weights, gestational age and growth rates

Gestational age is measured from the middle day of pairing (pairing is usually for 3 days) for the TMB colony and from the day of ovulation for ART related pregnancies. Computerized records of vital statistics are maintained by ONPRC. Gestation day (GD) 165 ± 10 is considered as the normal gestational period for rhesus macaques. Delivery

before 155 days is considered pre-term and before 140 days, premature. Pregnancy loss in <140 days is considered a spontaneous abortion (SAB) while loss after 140 days is considered a stillborn (SB).

The disclosures of each and every patent, patent application and publication cited herein are hereby incorporated herein by reference in their entirety.

While the invention has been disclosed with reference to specific embodiments, it is apparent that other embodiments and variations of the invention may be devised by others skilled in the art without departing from the true spirit and scope of the invention. The appended claims are intended to be construed to include all such embodiments and equivalent variations.

What is claimed is:

1. A method of generating transgenic non-human primates comprising:
introducing an AAV Rep protein and a genetic construct into a zygotic pronuclei to form a transgenic zygote, wherein the genetic construct comprises a transgene encoding a protein, polypeptide, or genetic transcript of interest; and a first AAV ITR or portion thereof and a second AAV ITR or portion thereof, wherein the first and second AAV ITRs flank the transgene.
2. The method of claim 1, wherein a portion of the genetic construct integrates into a chromosome of the pronuclei.
3. The method of claim 2, wherein the portion of the genetic construct that integrates into the chromosome includes the transgene and the promoter.
4. The method of claim 2, wherein the portion of the genetic construct that integrates into the chromosome includes the transgene and the first and second AAV ITR.
5. The method of claim 2, wherein the chromosome is chromosome 19.
6. The method of claim 1, further comprising establishing an ES cell line from the transgenic zygote.
7. The method of claim 1, wherein a product of the transgene causes of a condition similar to a human disease.
8. The method of claim 7, wherein the disease is a dominant negative disease, a recessive disease, or a multigenic disease.

9. The method of claim 8, wherein the dominant negative disease is Emery-Dreifuss muscular dystrophy, spinocerebellar ataxia, Ehlers-Danlos syndrome, parkinson disease, renal tubular acidosis, diabetes insipidus, keratitis-ichthyosis-deafness syndrome, or Marfan syndrome.
10. The method of claim 1, wherein a product of the transgene alters the function of a gene, a protein, or a nucleic acid.
11. The method of claim 1, wherein the introducing is by injection, electroporation, physical shock, or chemical assistance.
12. The method of claim 10, wherein the product of the transgene is a protein, an RNAi, an siRNA, a ribozyme, a tRNA, a mRNA, a ribosomal RNA, a snRNA, a 5SRNA, or an active portion of an RNP.
13. The method of claim 1, further comprising a promoter operatively linked to the transgene.
14. The method of claim 13, wherein the promoter is an AAV promoter P5, beta-actin, ROSA-21, PGK, FOS, c-myc, Jun-A, Jun-B, CMV, chicken beta-actin, or E1alpha.
15. The method of claim 13, wherein the promoter integrates into the chromosome.
16. The method of claim 1, wherein the first AAV ITR is SEQ ID NO. 1 or operative portion or variant thereof.
17. The method of claim 1, wherein the second AAV ITR is SEQ ID NO. 2 or an operative portion or variant thereof.
18. The method of claim 1, wherein the zygotic pronuclei are formed from the fertilization of an oocyte with a sperm.

19. The method of claim 18, wherein the oocyte is from a rhesus monkey, African Green monkey, simian, gorilla, rhesus macaque, baboon, cynomolgus macaque, marmoset, chimpanzee, squirrel monkey, proboscis monkey, langur, tamarin, potto, dusky titi, orangutan, marmoset, capuchin, spider monkey, howler monkey, prosimians, pongo pygmaeus, or pan paniseus.

20. The method of claim 18, wherein the sperm is from a rhesus monkey, African Green monkey, simian, gorilla, rhesus macaque, baboon, cynomolgus macaque, marmoset, chimpanzee, squirrel monkey, proboscis monkey, langur, tamarin, potto, dusky titi, orangutan, marmoset, capuchin, spider monkey, howler monkey, prosimians, pongo pygmaeus, or pan paniseus.

21. The method of claim 1, further comprising recovering an oocyte from a cycling or non-cycling non-human primate.

22. The method of claim 21, further comprising fertilizing the oocyte to produce a zygote having zygotic pronuclei.

23. The method of claim 22, wherein the fertilizing is by intracytoplasmic sperm injection, sperm incubation, or by mating a female and male non-human primate.

24. The method of claim 1, wherein the AAV Rep protein and the genetic construct are injected into the male pronuclei.

25. The method of claim 1, wherein the AAV Rep protein and the genetic construct are injected into the female pronuclei.

26. The method of claim 1, wherein the genetic transcript of interest encodes a ribozyme, an siRNA, an RNAi, an siRNA, a ribozyme, a tRNA, a mRNA, a ribosomal RNA, a snRNA, a 5SRNA, or any RNA portion of an RNP.

27. The method of claim 1, further comprising culturing the transgenic zygote to form an embryo.
28. The method of claim 27, further comprising creating an embryonic stem cell line from the embryo.
29. The method of claim 1, further comprising transferring the transgenic zygote pronuclei into the uterus of a female non-human primate.
30. The method of claim 1, further comprising culturing the transgenic zygote to form a blastocyst.
31. The method of claim 30, further comprising creating an embryonic stem cell line from the blastocyst.
32. The method of claim 30, further comprising transferring the blastocyst into the uterus of a pseudo-pregnant female non-human primate.
33. The method of claim 32, wherein the transferring is by mini-laparoscopy, laparoscopy, or cervical canulation.
34. The method of claim 32, further comprising allowing the blastocyst to develop to term to form a transgenic non-human primate.
35. The method of claim 34, further comprising breeding the transgenic non-human primate
36. The method of claim 35, wherein the transgene is stably maintained in the offspring.

37. The method of claim 1, further comprising detecting expression of the transgene.
38. The method of claim 1, wherein the genetic construct comprises SEQ ID 4.
39. A method of generating transgenic non-human primates comprising:
recovering an oocyte from a cycling non-human primate;
fertilizing the oocyte to produce a zygote having zygotic pronuclei;
introducing an AAV Rep protein and a genetic construct into a zygotic pronuclei
to form a transgenic zygote;
culturing the transgenic zygote to form a blastocyst; and
transferring the blastocyst into the uterus of a pseudo-pregnant female non-human primate.
40. The method of claim 39, wherein the transferred blastocyst is allowed to develop to term to produce a transgenic non-human primate.
41. The method of claim 40, wherein the transgenic zygote, the blastocyst, or the transgenic non-human primate are sources of transplant material.
42. The method of claim 39, further comprising establishing an ES cell line from the transgenic zygote or the blastocyst.

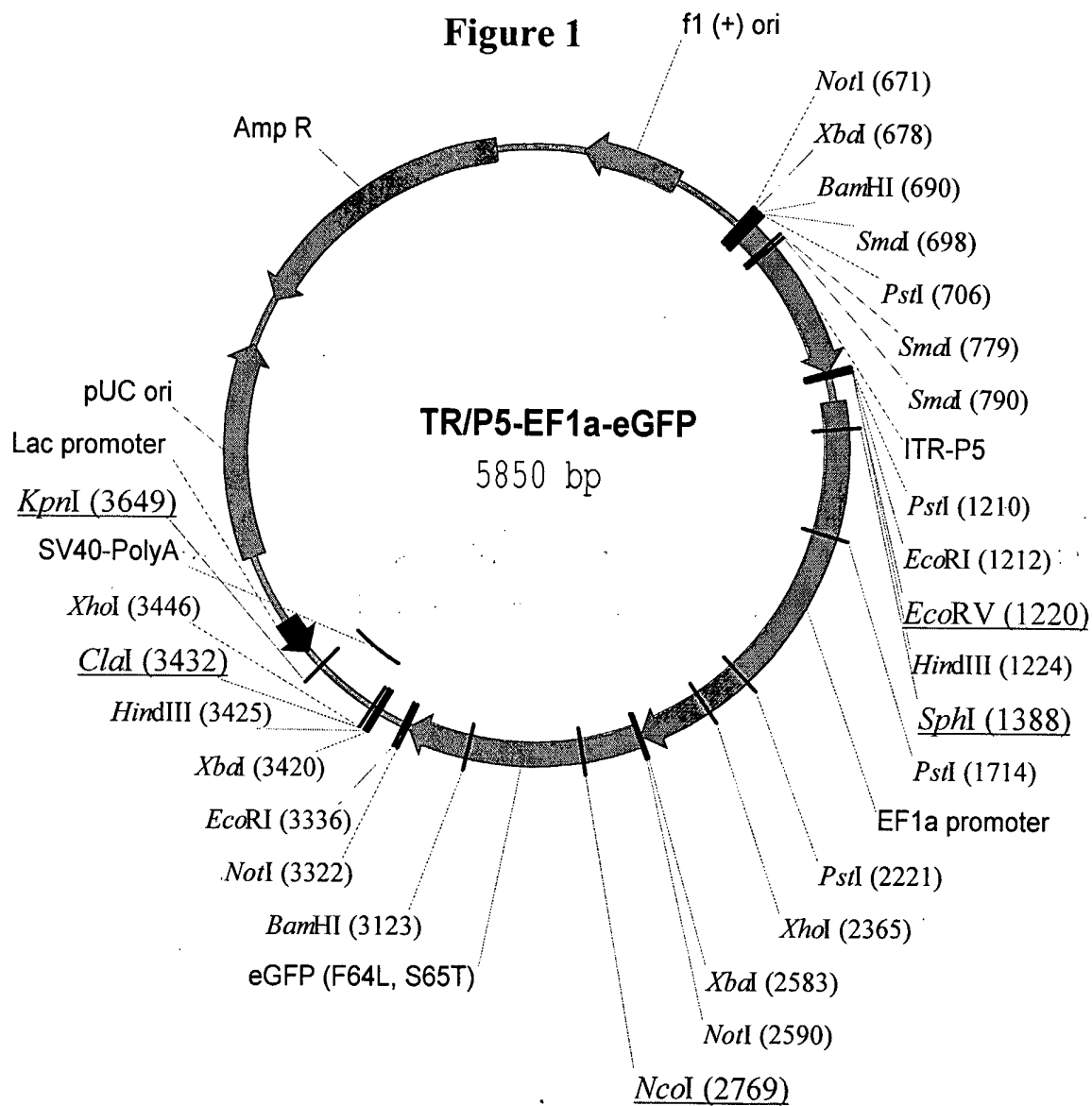
Figure 1

Figure 2

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Figure 2 (CONT.)

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Figure 3

Rep 68 Nucleotide and AA Sequence

```

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Figure 3 (CONT.)

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Rep 68 nucleotide and protein sequence (accession AAC03774)

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cgcggaagcttcgatcaactacgcattggctcgaggacactctctc
Rep68 nucleotide sequence

Figure 4

