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Generation of Transgenic Monkeys with Human Inherited Genetic Disease

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Abstract

Modeling human diseases using nonhuman primates including chimpanzee, rhesus, cynomolgus, marmoset and squirrel monkeys has been reported in the past decades. Due to the high similarity between nonhuman primates and humans, including genome constitution, cognitive behavioral functions, anatomical structure, metabolic, reproductive, and brain functions; nonhuman primates have played an important role in understanding physiological functions of the human body, clarifying the underlying mechanism of human diseases, and the development of novel treatments for human diseases. However, nonhuman primate research has been restricted to cognitive, behavioral, biochemical and pharmacological approaches of human diseases due to the limitation of gene transfer technology in nonhuman primates. The recent advancement in transgenic technology that has led to the generation of the first transgenic monkey in 2001 and a transgenic monkey model of Huntington's disease (HD) in 2008 has changed that focus. The creation of transgenic HD monkeys that replicate key pathological features of human HD patients further suggests the crucial role of nonhuman primates in the future development of biomedicine. These successes have opened the door to genetic manipulation in nonhuman primates and a new era in modeling human inherited genetic disorders. We focused on the procedures in creating transgenic Huntington's disease monkeys, but our work can be applied to transgenesis in other nonhuman primate species.

Keywords

Transgenesis; Nonhuman primates; Human inherited genetic diseases; Huntington's disease

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1. Introduction

The successful development of transgenic animal models for human diseases has led to remarkable breakthroughs that have impacted significantly on the diagnosis, treatment and intervention in human diseases [1,2]. They have further clarified our understanding of disease mechanisms, as well as the onset and course of disease pathology. Compared to rodents, nonhuman primates such as rhesus macaque share high degrees of physiologic, neurologic and genetic similarity with humans [1,2]. The high similarity in brain anatomy allows non-invasive, high resolution imaging of the brain structures and functions by functional magnetic resonance imaging (fMRI) and positron emission topography (PET). Furthermore, appropriate cognitive and behavioral tests applicable for studying neurodegenerative diseases and the physiologic decline of aging in monkey are available [2-9]. Thus, the monkey is considered one of the best models for understanding human physiology and disease [1,2,6,8,10]. The creation of a transgenic monkey in 2001 [10] demonstrated that the monkey genome could be genetically modified efficiently. This remarkable event set the stage for the creation of a transgenic nonhuman primate with genetic alterations that mimic patient conditions, both physiologically and genetically [2,6,8-12]. The recent development of transgenic Huntington's disease (HD) monkeys further suggests the potential role of transgenic monkeys in modeling human inherited genetic diseases [2]. HD monkeys revealed unique cellular changes and pathogenesis that were highly comparable to that of HD patients [2,13]. The HD monkeys also developed key clinical HD features including dystonia, chorea and seizure, which were comparable to human HD patients that no other animal models were able to replicate. In addition to understanding the underlying cause of disease mechanism, a transgenic monkey can be a powerful model to develop biomarkers for monitoring disease progression, determine therapeutic efficacy of new medications and establish new therapeutic approaches such as gene and cell therapy using a patient's own stem cells. The success in generating a transgenic HD monkey model has demonstrated the feasibility of producing genetically modified monkeys and the potential application of transgenic monkeys in modeling human inherited genetic disorders.

An efficient gene delivery method is the most important factor in the production of transgenic monkeys, because a minimal number of animals should be used to reduce ethical concerns. Although different methods, including sperm mediated gene transfer (SMGT), pronuclear microinjection and somatic cell nuclear transplantation (SCNT), have been successfully used for generating transgenic animals, their efficiency remains far from satisfactory [14]. Although retroviral gene transfer was first described in the early 1970's [15], the application of viral vector in the generation of transgenic animals was not seriously considered until the development of replication incompetent retroviruses and the highly efficient production of transgenic cattle by targeting metaphase II arrested oocytes [16]. These latest advancements has led to the generation of the first transgenic monkey in 2001 [10] and the first transgenic monkey model of human disease in 2008 [2]. A similar strategy has also been used to generate different transgenic species including rodents, farm animals and nonhuman primates [2, 17-21]. Lentiviral gene transfer has shown to be a powerful tool in genetic modification of animal genomes and is one of the most efficient methods in creating transgenic animals, especially for species that are limited in source and difficult to achieve efficient gene transfer by other methods.

We have focused on the procedures of lentiviral transgenesis for generating transgenic Huntington's disease monkeys and hope to broaden the application of this lentiviral transgenesis to species with limited access.

2. Construction of lentiviral vectors

A lentiviral-based vector “pFUW” [F: human immunodeficiency virus–1 flap element (HIV-flap), U:ubiquitin promoter, W: woodchuck hepatitis virus post-transcriptional regulatory element (WRPE)][20] was used to construct mutant *htt* and *GFP* lentiviral vector (Figure 1). In brief, pFUW is a self-inactivating vector composed of WRPE, which was used to increase transcription level, RNA stability and minimize position effect. Additionally, an HIV-flap element was inserted between the 5' long terminal repeat (5'LTR) and the internal promoter to increase lentiviral titer. The enhancement of gene expression and attenuation of position effect by composing an F and W sequence in the lentiviral vector has been demonstrated in tissue culture, mice, rat and nonhuman primates [2,9,20,21]. The ubiquitin (ubi) promoter was used because of its high expression level without tissue specificity. Exon 1 of the huntingtin (*htt*) gene with 84 CAGs (*htt*-84Q) located at the 5' terminus of the *htt* gene was inserted into a lentiviral vector to create pLVU-*htt*-84Q, which was regulated by the ubiquitin promoter. For GFP lentiviral construct, the *htt*-84Q gene in the vector was replaced by the *GFP* gene to create pLVU-GFP(2).

****Remarks:** the number of CAG repeats in *htt* gene is extremely unstable. It is easy to obtain a truncated form of *htt* gene during the construction because of the shortening of CAG repeats.

3. Preparation of high titer VSV-G Pseudotyped lentiviruses

Vesicular stomatitis virus glycoprotein (VSVG) pseudotyped LVU-*htt*-84Q (VSVG-LVU-*htt*-84Q) and LVU-*GFP* (VSVG-LVU-*GFP*) were generated by co-transfection of pLVU-*htt*-84Q (or pLVU-*GFP*), pΔ8.9 (composed of the structural genes for virion assembly) and pVSVG (plasmid DNA expressing VSV-G envelope protein; Invitrogen, Inc.) into a 293FT packaging cell (Invitrogen, Inc.) at 60%-70% confluency. 293FT packaging cells were freshly passaged the day before transfection. The transfection ratio of plasmid DNA in a 15 cm Petri dish was “lentiviral vector (25μg): pΔ8.9 (18.75μg): pVSVG (12.5μg) (2:1.5:1)”. Beginning at 48-hours post transfection, culture medium was collected for three consecutive days at 24-hour intervals. Each daily collection was filtered through a 0.45μm filter (Milipore Inc.) to remove cell debris followed by ultracentrifugation. Supernatant was centrifuged at 25,000 × g for 90 minutes using a fixed angle or swing bucket rotors. After centrifugation, carefully remove the supernatant without disturbing the pellet, which can be very clear and difficult to see. The viral pellet was re-suspended in 50-100μl of phosphate buffered saline (PBS). Lentiviruses were then aliquoted in 5μl, titered and kept frozen at -80°C. Once thawed, viruses should not be reuse or re-freeze. All plastic ware and instruments that had contact with the lentiviruses were decontaminated with 10% bleach or autoclaved before disposal.

CaCl₂ transfection in 293FT cells for lentiviral vector production

Day 1:

Split 293FT cells into 15cm dish in regular culture medium, make sure that cells should be 60-70% confluent on the day of transfection, and cells are healthy.

Day 2:

1. mix plasmid
 - 25μg lentiviral vector
 - 18.75 μg pΔ8.9
 - 12.5 μg pVSVG
 - in 1.5 ml sterilized tube, add ddH₂O to 1250 μl

2. add 1250 μ l of 0.5M CaCl_2 to 1.5 ml tube in step 1, vortex to form DNA/ CaCl_2 solution
3. add 2500 μ l 2 $\times$ HeBS to 50ml steriled tube.
4. use pipettor to bubble the 2 $\times$ HeBS and add DNA/ CaCl_2 solution slowly, then vortex to form precipitate
5. leave the precipitates at RT for 30 min.
6. add the precipitates dropwise to 15 cm dish by rocking the plate back and forth
7. incubate the cells overnight at 37C in 5% CO_2 incubator
8. replace with fresh medium on the following day (Day 3)

*2 $\times$ HeBS: NaCl (0.28 M final), HEPES (0.05M final) and Na_2HPO_4 , anhydrous (1.5 mM final). **pH is important. 7.0 is optimal for precipitation. Below 6.96, precipitates will not form while above 7.05, coarse precipitates will be formed with low transfection efficiency

*Supernatant will be collected at 48 hours after the replacement of fresh medium on Day 2

*Concentrate by ultracentrifugation at $25,000 \times g$ for 90 min

****Remarks:** supernatant can be kept at 4°C until the last collection at 72 hours post-transfection followed by ultracentrifugation.

****Remarks:** the pellet should be completely soaked in the buffer, kept at 4°C overnight until the pellet is completely dissolved.

****Remarks:** retroviruses and lentiviruses are relatively stable at -80°C, but avoid repeatedly being frozen-thawed.

4. Titering LVU-*htt-84Q* and LVU-*GFP*

Titer represents the concentration of infectious virus particles. It was determined by the selection of antibiotic resistant colonies or expression of the protein of interest after infection by the tested lentiviruses. 2.5×10^5 293FT cells (or any cell lines of interest) were plated in a 24-well plate and used as target cells. The following day, target cells were co-cultured with serially diluted pseudotyped lentiviruses in the presence of 8 μ g/ml of polybrene (Hexadimethrine bromide: Sigma Inc.). A stock solution of polybrene can be prepared by dissolving 1mg of polybrene in 1ml sterile water with 10% DMSO; the stock solution is aliquoted and is stable at -20°C. For LVU-*htt-84Q*, immunostaining with mEM48 antibody (Chemicon Inc.) that specifically recognized mutant htt was used to determine the expression of mutant htt in 293FT cells that were successfully infected by LVU-*htt-84Q*. For LVU-*GFP*, epifluorescent microscopy was used to identify GFP expressed cells. To determine viral titer, the number of HD expressing cells (GFP positive cells) were multiplied by the dilution factor of each well. Viral titer can reach 10^8 - 10^9 cfu/ml after ultracentrifugation, about 1,000 folds higher than non-concentrated viruses.

****Remarks:** Polybrene is a polycation, which neutralizes the surface charge of the target cells' surface and the vector particles in order to enhance efficient viral adsorption [14,22-24].

****Remarks:** 293FT cells do not attach to culture dishes tightly; therefore, be sure to pipet the medium gently when manipulating these cells.

****Remarks:** viral titer of at least 1×10^9 cfu/ml is necessary for achieving high gene transfer efficiency while gene transfer rate is expected to reduce significantly if lower titer is used.

5. Selection of oocyte donor and surrogate females

For oocyte donors: adult healthy females with regular menstrual cycle at their reproductive age were the prime candidates. Females between the age of six-to-15 with body weight of six-to-10 kilograms were identified as oocyte donors. Once animals were assigned, at least two menstrual cycles were confirmed by vaginal bleed before hormone stimulation for superovulation. Vaginal bleed was monitored daily to establish individual menstrual profiles, while females with regular cycles were identified and enrolled in follicle stimulation schemes. For surrogate females, healthy adult females with regular reproductive cycles and prior pregnancies were the prime candidates. Females between the age of seven-to-15 with body weights of seven-to-10 kilograms were identified as surrogates. Once animals were assigned, vaginal bleed was monitored daily to establish individual menstrual profiles, while females with regular cycles were identified and enrolled in surrogate selection pool.

****Remarks:** overweight females tend to be less responsive to hormone treatment and difficult to use for laparoscopic oocyte retrieval.

****Remarks:** overweight females tend to be more difficult to use for embryo transfer.

6. Regimen of follicular stimulation and oocyte retrieval

6.1 Follicular stimulation

Female rhesus monkeys exhibiting regular menstrual cycles were induced with exogenous gonadotropins [25,26]. The expression of monkey endogenous gonadotropins was down regulated at the beginning of menses (Day one-to-two) by daily subcutaneous injections of Gonadotropin-releasing hormone (GnRH) antagonist (Antide; Ares Serono, 0.5mg/kg body weight) for six days and by twice daily injection of recombinant human follicle-stimulating hormone [r-FSH: Organon Inc. 30 IU, intramuscular injection (i.m.)] concomitantly. This was followed by the injection of r-FSH + recombinant human luteinizing hormone (r-hLH; Ares Serono; 30 IU each, i.m., twice daily) on the last three days. Ultrasonography was performed on day seven of the stimulation to confirm follicular responses. An i.m. injection of 1,000 IU recombinant human chorionic gonadotropin (r-hCG; Ares Serono,) was administered for ovulation induction when there were follicles at 3-4 mm in diameter. In general, r-hCG was administered at approximately 37 hours prior to oocyte retrieval for optimal maturation of metaphase II arrested oocytes.

6.2 Oocyte retrieval

Follicular aspiration was performed 37 hours post-hCG. Oocytes were aspirated from follicles using a needle suction device lined with Teflon tubing. Briefly, a 10 mm trocar was placed through the abdominal wall and a telescope was introduced. Ovaries were visualized by a monitor attached to the telescope. Two small skin incisions facilitated the insertion of 5 mm trocars bilaterally. Grasping forceps were introduced through each trocar to fixate the ovary at two points. Once stabilized, a 20 gauge stainless steel hypodermic needle connected with teflon tubing was attached to a vacuum regulator for oocyte aspiration. The tubing was first flushed with sterile Tyrode's Lactate-Pyruvate-Hepes medium (TALP-Hepes) [27], supplemented with 5 IU/ml of heparin (Sigma Inc.) in order to prevent blood clots in the tubing during aspiration. Follicles were aspirated with continuous vacuum pressure adjusted at approximately 40-60 mm Hg into a 15 ml conical tube containing 1 ml of TALP-Hepes supplemented with 5 IU/ml of heparin and maintained at 37°C.

6.3 Collection and evaluation of rhesus oocytes

Collection tubes with follicular fluid were diluted in TALP-Hepes supplemented with 2 mg/ml hyaluronidase (Sigma Inc.). Follicular fluid with buffer was filtered through a 70µm cell strainer (BD Inc.) to remove cell debris and blood clots while the follicular fluid was collected in a 60mm petri dish containing 5 ml of TALP-Hepes with hyaluronidase. Oocytes were picked up under a dissecting microscope, and cumulus cells were removed in TALP-Hepes with hyaluronidase (2 mg/ml) and then transferred to a dish containing fresh TALP-Hepes with no hyaluronidase. Oocytes were rinsed and then transferred to 50 µl pre-equilibrated maturation medium that contained Connaught Medical Research Laboratories medium 1066 (CMRL-1066; Invitrogen Inc.) supplemented with 10% heat-inactivated fetal bovine serum (HyClone Inc.), 40µg/ml Sodium pyruvate, 150µg/ml Glutamine, 550µg/ml Calcium lactate, 100ng/ml Estradiol and 3ug/ml of Progesterone under mineral oil (Sigma Inc.). Metaphase II-arrested oocytes, exhibiting a distinct perivitelline space and first polar body, were maintained in maturation medium before fertilization. Immature oocytes were matured in maturation medium for up to 24 hours.

****Remarks:** A thorough wash is critical in microdrop culture in order to reduce carryover medium.

7. Collection and preparation of rhesus monkey sperm for intracytoplasmic sperm injection (ICSI)

Rhesus males of proven fertility were trained to routinely produce semen samples by penile electroejaculation. Freshly collected samples were placed inside a biosafety cabinet at room temperature to allow sample to liquefy. After approximately 10 minutes, 0.5ml liquid portion of the semen was transferred into a 15ml disposable centrifuge tube followed by serial washes using 10 ml of TALP-hepes and centrifugation at 700 ×g for 10 minutes. After two washes, supernatant was carefully removed and the pellet was gently re-suspended in 1ml of TALP-hepes. The sperm sample was then counted and diluted to a concentration of 2×10^7 sperms/ml. Sperm suspension was incubated at room temperature in air for at least four to six hours prior to ICSI.

8. Perivitelline space (PVS) injection of lentiviruses

MII arrested oocytes were selected for PVS injection followed by ICSI in oocytes. VSVG-LVU-*htt-84Q* and VSVG-LVU-*GFP* pseudotyped viral solutions were loaded into the injection needle (tip of 100-to-200 µm in diameter; Sutter Inc) by a micropipet, mounted onto a micromanipulator (Narishigi Inc.), and connected to a 50ml glass syringe. After penetration through the zona-pellucida, lentiviruses with at least 1×10^9 CFU/ml was slowly infused into the PVS until the zona-pellucida was fully expanded (Figure 2). After microinjection, the oocytes were returned to the maturation medium at 37°C with 5%CO₂ and 90%N₂ until fertilization by ICSI.

****Remarks:** PVS injection should have no problem with further development unless the oolemma was damaged.

****Remarks:** we target MII oocyte for lentiviral gene transfer to reduce mosaic rate in the offspring because transgene integration prior to fertilization will ensure all progeny cells derived from the oocyte carry the identical transgene at the same locus.

****Remarks:** the ooplasm was distorted during injection and it will be recovered after injection. To reach optimal transgenic rate, slowly inject as much viral solution as possible.

****Remarks:** while it is difficult to lay out the projected success rate by a simple formula, the two key factors for achieving high transgenic rate are: viral titer of at least 1×10^9 CFU/ml (the higher the better) and the injection volume, which is related to the titer of the viruses. In theory, one viral particle per one picoliter of viral solution will be delivered at a titer of 1×10^9 CFU/ml.

9. Intracytoplasmic sperm injection (ICSI), *in vitro* culture (IVC), confirmation of fertilization and assisted hatching

9.1 Intracytoplasmic sperm injection (ICSI)

A holding pipet (O.D. 100 μ m; I.D. 20 μ m) and a microinjection needle (O.D. 7-8 μ m and I.D. 4-5 μ m), with a 50° beveled tip (Humagen Inc.) were mounted on an Olympus inverted microscope equipped with Relief Contrast optics. The holding and injection pipettes were filled with mineral oil, connected to a CellTram microinjector (Eppendorf Inc.) and held on a Narishigi micromanipulator. Injection was carried out in a 20 μ l drop of TALP-Hepes buffer covered with mineral oil on the cover of a 3.5 cm petri dish. Ejaculated sperm were diluted 1:100 in 7% polyvinylpyrrolidone (PVP; Humagen Inc.) to reduce motility and were placed in a separate drop on the manipulation dish. A single sperm was aspirated tail first from the sperm-PVP drop into the injection needle and transferred to the oocyte-containing drop. Oocytes were held by holding pipet with the polar body at the six o'clock or 12 o'clock position; followed by insertion of the injection needle with a sperm through the zona into the cytoplasm. The oolemma was then penetrated by gentle cytoplasmic aspiration and the sperm was expelled into the oocyte (Figure 3).

****Remarks:** it is important to immobilize the sperm by gently squashing the mid-piece of the sperm against the bottom of the dish using the injection pipet and by injecting a minimum amount of PVP-containing buffer into the oocyte.

9.2 *In vitro* culture

After ICSI, oocytes were washed twice in Hamster Embryo Culture Medium 9 (HECM-9) [28,29] before being transferred into a pre-equilibrated four-well plate with 500 μ l of HECM-9, covered with 300 μ l of mineral oil and incubated at 37°C with 5% CO₂ and 90% N₂ until the next morning. In brief, for the first 48 hours post-ICSI, embryos were cultured in HECM-9 supplemented with amino acids/pantethionate (AAP) stock [28,29] without FBS (HyClone Inc.). Fresh HECM-9/AAP supplemented with 10% FBS was replaced at 48 hours post-ICSI/ or at the four-eight cell stage, which was then cultured until the blastocyst stage.

****Remarks:** we transferred the zygotes to fresh HECM-9/AAP supplemented with 10% FBS every 48 hours.

9.3 Confirmation of fertilization

The detection of a second polar body and two pronuclei using an inverted microscope confirmed successful fertilization. Zygotes were then selected and returned to culture until four-eight cell stage for embryo transfer or continued *in vitro* culture in a freshly pre-equilibrated HECM-9/AAP/FBS culture medium at 37°C with 5% CO₂ and 90% N₂.

9.4 Assisted hatching

At the four-eight cell stage (48 hours post-ICSI), embryos with clear nuclei and equal blastomere size were identified and submitted for assisted hatching (Figure 4) to improve hatching rate and enhance implantation and pregnancy rate. Selected embryos were transferred into an environment similar to those described for PVS injection and ICSI. A blunt ended

micropipette (7 μ m I.D.) attached on a PIEZO-device (Primetech Ltd.) was used to puncture holes at the zona pellucida with the largest PVS. These embryos were then used for oviductal transfer.

10. Selection of surrogate females and embryo transfer

10.1 Selection of surrogate females for embryo transfer

To identify surrogate females for embryo transfer that synchronized with the embryonic stage, screening was performed by collecting daily blood samples beginning on day eight of the menstrual cycle (day one is the day of menses) and analyzed for serum progesterone and estrogen. When serum estrogen increases two-four times of the basal levels, the LH surge has occurred and ovulation usually follows within 12 to 24 hours. Timing of ovulation can be detected by a significant decrease of serum estrogen and an increase of serum progesterone greater than 1 ng/ml. Surgical embryo transfers were performed on day two following ovulation.

10.2 Embryo transfer by laparotomy

Surgical embryo transfers were performed by mid-ventral laparotomy. The oviduct was carefully inserted with a Tomcat catheter containing two four-to-eight cell stage embryos in TALP-Hepes buffer. Embryos were slowly expelled from the catheter into the oviduct with a minimal amount of medium while the catheter was slowly withdrawn. The catheter was then flushed with medium following removal to ensure that the embryos were successfully transferred.

11. Confirming and monitoring of pregnancy

To confirm implantation, blood samples were collected bi-weekly and analyzed for serum estrogen and progesterone concentrations. If hormone levels indicated a possible pregnancy, the pregnancy was confirmed by a transabdominal ultrasound on day 60 post-transfer. During ultrasound examination, measurements were taken of total fetal length, fetal cardiac activity and size of yolk sac. These were compared to similar measurements made from natural pregnancies. Ultrasound examination was performed once more, during the second trimester, to determine developmental normalcy.

12. Infant care and nursery

Singletons were born naturally while multiples were delivered by cesarean section at approximately 150 days of pregnancy to avoid potential complication. Babies were weighed, physical measurements such as head circumference were recorded and then the infants were kept and raised in the primate nursery. The infants were raised by a principal human caregiver who fed and handled them several times a day from the day they arrived in the primate nursery. The principal human caregiver spent approximately six hours daily, five days a week in the primate nursery with the infants. On evenings and weekends, other familiar human caregivers fed, handled and played with the infants two-three times a day for a total of two-four hours. In addition, at three months of age until approximately nine months of age, all infants received daily social interactions (three-four hours, five days/week) with other age- and sex-matched peers of the same cohort and in the presence of one to three of the familiar human caregivers. From three-four months, socialization with peers took place in a play pen/cage located in the primate nursery and containing toys and towels. From five-nine months, socialization with peers took place in a large enclosure containing perches and toys and located in the nursery. From 10 months to two years, socialization takes place in our large social enclosure located in a room in the housing facility.

13. Confirmation of transgenic status

Due to the fact that a newborn monkey is about 500 g, the amount of tissue that can be retrieved at the time of birth was very limited. Samples like cord blood, placenta, hair follicles and buccal swab were the only accessible tissues for analysis at this age, while whole blood, lymphocyte and an ear punch can only be collected at one-two months of age.

13.1 Genomic DNA extraction, PCR Analysis and determination of CAG repeat length

The monkey tissues, such as umbilical cord, placental tissues and peripheral tissues, were collected from each monkey for genomic DNA extraction. Genomic DNA was extracted using the Wizard® Genomic DNA Purification Kit (Promega Inc.), and DNA quality was determined by BioPhotometer (Eppendorf Inc.). To detect the foreign *htt* gene, ubiquitin-F forward primer (5'-GAGGCGTCAGTTTCTTTGGTC-3') and *htt*-R reverse primer (5'-GCTGGGTCACTCTGTCTCTG-3') were used to yield 818 bp products after amplification of genomic DNA from the HD tissue. However, variation in the size of PCR products resulted because of variable numbers of CAG repeats. Genomic DNA (100 ng) was first subjected to PCR with 1.65M Betaine (Sigma Inc.) at 96°C for five minutes; at 96°C for 45 seconds, 62°C for 45 seconds, 72°C for 150 seconds for 35 cycles and then 72°C for seven minutes. To determine the number of CAG repeats in HD monkeys, the PCR products were sequenced using HD exon 1 forward primer (5'-GGCGACCCTGGAAAAGCTGA-3'). To detect the *GFP* gene, GFP-F forward primer (5'-TTCAAGGACGACGGCAACTAC-3') and GFP-R reverse primer (5'-TAGTGGTTGTCGGGCAGCAG-3') were first used for amplification at 94°C for five minutes; then at 94°C for 30 seconds, 64°C for 30 seconds, 72°C for 20 seconds for 35 cycles and then 72°C for seven minutes, yielding a product of 302 bp. DNA from wild-type monkeys were used as the control.

13.2 Southern blotting analysis

Ten micrograms of genomic DNA was digested overnight using *EcoR* I, which was only cut once within the transgene. The digested genomic DNA was then separated by gel electrophoresis on a 0.8% agarose gel and transferred to the Hybond-N+ nylon membranes (Amersham, Inc.). In order to determine the number of integration events of the two viruses (LVU-*htt*-84Q or LVU-*GFP*) in HD monkeys, a subtraction approach was used because both constructs have identical lentiviral backbone and the short fragment of *htt*-84Q is not sufficient to distinguish between the mutant *htt* and endogenous *htt*. We first hybridized the membrane with a (³²P)-labeled probe that specifically binds to the *GFP* gene. Once the number of integration events of the LVU-*GFP* was determined, the membrane was stripped with high stringency buffer followed by hybridization with a (³²P)-labeled probe that recognizes a unique sequence of the lentiviral backbone to quantify all integration events. By subtracting the number of LVU-*GFP* integration events from the total number of integration events, the number of LVU-*htt*-84Q integration events could be determined. Integration sites were determined by exposing (³²P) hybridized member to the phosphor screen and then scanned by Typhoon phosphorimager (GE, Inc). Plasmid DNA (pLVU-*htt*-84Q and pLVU-*GFP*) digested with *EcoR* I was used as positive controls. Genomic DNA of semen donor, oocyte donor, and surrogate female were used as negative controls.

13.3 RNA extraction, reverse transcription (RT), RT-PCR and real-time quantitative PCR (Q-PCR) Analysis

The RNA of tissues from HD monkeys was extracted by using RNeasy Mini Kit (Qiagen Inc.). The extraction procedure was performed as recommended by the manufacturer. RNA quality was determined by BioPhotometer (Eppendorf Inc.). RT was performed using the High Capacity cDNA Reverse Transcription Kit (Applied Biosystems Inc.), and cDNA was used for RT-PCR and Q-PCR. To determine the RNA expression of mutant *htt* via RT-PCR, two

specific primers, HD Exon 1-F forward primer (5'- GTTTTTGGCTTTTTTGTAGACGA-3') and HD Exon 1-R reverse primer (5'-TCAGCTTTTCCAGGGTCGCC-3'), were first used for amplification at 94°C for five minutes; at 94°C for 30 seconds, 64°C for 30 seconds, 72°C for 10 seconds for 35 cycles and then 72°C for seven minutes, yielding a product of 79 bp. To quantify the relative RNA expression levels, Q-PCR was performed using the cDNA. 2× Power SYBR® Green PCR Master Mix (Applied Biosystems) was mixed with specific primers and cDNA, and first subjected to the iQ5 real-time PCR detection system (Bio-Rad Inc.) at 96°C for 12 minutes; then at 96°C for 15 seconds, and 60°C for 30 seconds for 50 cycles. 18S primers were used as an internal control to normalize expression levels. The sequences for the specific primers used for Q-PCR were the following:

HD Exon 1 Q-PCR -F: ATGGCGACCCTGGAAAAGCT

HD Exon 1 Q-PCR -R: TGCTGCTGGAAGGACTTGAG

18S F: CGGCTACCACATCCAAGGAA

18S R: CCTGTATTGTTATTTTTCGTCACCT

13.4 Western blotting

Total protein was extracted from different cell types and tissues by using the appropriate volume of 1× RadioImmuno Precipitation Assay buffer (RIPA buffer; 50mM Tris-HCl pH8.0, 150mM NaCl, 1mM EDTA pH 8.0, 1mM EGTA pH 8.0, 0.1% SDS, 0.5% deoxycholate, 1% Triton) with 1× protease inhibitor cocktail (Roche Inc.). The samples were lysed in a 1.5ml tube using a sonic dismembrator (Fisher Inc.) and then kept on ice for at least 30 minutes. The samples inside 1.5 ml tubes were then centrifuged at 13,000 rpm speed for 10 minutes, and the concentration of the supernatant was determined using the Bradford assay (Pierce Inc.). After measuring the protein concentration, equal amounts of protein extract with 1× loading dye (0.25 × Tris-HCl/SDS stacking gel buffer, pH 6.8, 2% SDS, 10% Glycerol, 0.1% 2-mercaptoethanol and 0.002% bromophenol blue) were boiled for 10 minutes before loading into a polyacrylamide gel. After electrophoresis, proteins were transferred onto a Polyvinylidene Fluoride (PVDF) membrane (Bio-Rad Inc.) using protein mini trans-blot cells (Bio-Rad Inc.) overnight at 4°C followed by blocking in 5% skimmed milk for two hours. The membrane was incubated with the primary antibodies, mouse monoclonal mEM48 (1:50 dilution; Chemicon Inc.), 1C2 (1:2000 dilution; Sigma Inc.) and γ -tubulin (1:2,000 dilution; Sigma Inc.), for at least two hours, followed by secondary peroxidase-conjugated antibodies (Jackson ImmunoResearch laboratories) for detecting proteins with an Amersham ECL kit (PerkinElmer Inc.).

****Remarks:** Misfolded mutant htt was trapped in the stacking gel, thus the stacking gel also needed to be transferred to PVDF membrane to detect the oligomeric htt at high molecular weight.

13.5 Immunohistochemistry (IHC) of Transgenic Monkey Tissues

Tissue biopsy and postmortem tissues of transgenic monkeys were fixed in 4% paraformaldehyde (Sigma Inc.) overnight, transferred to 30% sucrose, stored at 4°C, embedded in Optimal Cutting Temperature (OCT) medium (Sakura Inc.) and cut at 50 μ m, followed by DAB (3, 3'-diaminobezidine) Immunohistochemistry staining. Tissues embedded in OCT medium were cut at 50 μ m, and used for DAB Immunohistochemistry staining. For DAB Immunohistochemistry, sections were incubated with 0.3% hydrogen peroxide for 15 minutes, blocked for one hour at room temperature, and incubated with mEM48 (1:50) at 4°C overnight. After washing with DPBS, the brain sections were processed with avidin-biotin using the Vectastain Elite ABC kit (Vector Laboratories Inc.), and immediately stained with DAB (Vector Laboratories Inc.) for 30–40 seconds or as required. Tissues sections were mounted

on the slides with mounting media (Sigma Inc.), and images were examined by Olympus B×51 microscope and captured by MetaMorph software (Universal Imaging Inc.). For 1C2 (1:4000 dilution) staining, the tissues were treated with formic acids (88%) for 10 minutes before being subjected to the DAB immunohistochemistry staining.

****Remarks:** do not overfix tissues in paraformaldehyde, as it may lead to the disruption of epitopes.

****Remarks:** do not overexpose samples in DAB, as it may result in high background.

14. Conclusions

There is no perfect model of human disease. An animal model that is not only physiologically but also genetically comparable to human patients is a unique and valuable tool for understanding the underlying mechanism of human diseases and the development of novel treatments for those diseases. All animal models have their pros and cons; however, it is our obligation as researchers to identify the best and most appropriate animal model that fits a specific study. The idea of genetically modifying a higher primate that could better mimic human conditions is no longer in question, yet our efforts in perfecting nonhuman primate models continue. Unlike other animal species, additional challenges are expected in creating transgenic nonhuman primates. While success gene transfer with high efficiency is critical, a decent number of superb surrogate females (20-30) with prior pregnancy are important for achieving high pregnancy rate. Thus, the coordination between oocyte donors and surrogate females are key factors for the success, which is depending on a good team of animal care personnel. Base of our experience, transgenic rate can reach over 90% with high titer lentiviruses while establishing successful pregnancy become a critical step in the process. We have presented a protocol that has been successfully used to generate the first transgenic nonhuman primate model of Huntington's disease. This first step proved the principle by demonstrating that creating a transgenic monkey is an achievable goal. The next step is to perfect our current technology for modeling human disorders in nonhuman primates and develop new technologies that allow gene targeting in nonhuman primates, which will further broaden the applications of primate modeling of human diseases.

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pLVU-htt-84Q pLVU-GFP **Figure 1.**

DNA construct of pLVU-*htt-84Q* and pLVU-*GFP*. LTR: long terminal repeat; Ubi: ubiquitin promoter; *htt-84Q*: Exon 1 of huntingtin gene with 84 CAGs inserted at the 5' terminus; *GFP*: green fluorescent protein; Flap: HIV-flap element; WPRE: woodchuck hepatitis virus post-transcriptional regulatory element.

Figure 2.

Perivitelline space injection of lentiviruses. The MII arrested oocyte is held by a blunt ended holding pipet (left) and a pipet loaded with viral vector solution is placed on the opposite side (right) (A) followed by penetration through the zona pellucida into the PVS (B). Successful injection of the vector solution is indicated by the expansion of the PVS (B & C).

Figure 3.

Intracytoplasmic sperm injection (ICSI) in rhesus macaque oocyte. A single sperm is aspirated into a beveled injection pipet (A; Arrow). The injection needle is then penetrated through the zona pellucida and the oolemma of the oocyte (B) followed by expelling of the sperm into the ooplasm (C).

Figure 4.

Assisted hatching in rhesus macaque 4-cell embryo. Small holes at the zona pellucida of four-cell rhesus macaque embryo are created by a blunt ended pipet attached to a PIEZO device. A blunt ended pipet is held next to the zona pellucida with the largest PVS (A) and several holes are punctured at different positions of the zona pellucida (B & C).