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Germline transmission in transgenic Huntington's disease monkeys

Sean Moran^{1,2,†}, Tim Chi^{1,2,†}, Melinda S. Prucha^{1,2,†}, Kwang Sung Ahn^{1,2,†}, Fawn Connor-Stroud³, Sherrie Jean³, Kenneth Gould³, and Anthony W. S. Chan^{1,2,*}

¹Department of Human Genetics, Emory University School of Medicine, Atlanta, GA 30322

²Division of Neuropharmacology and Neurologic Disorders, Yerkes National Primate Research Center, Emory University, Atlanta, GA 30329

³Division of Animal Resources, Yerkes National Primate Research Center, Emory University, Atlanta, GA 30329

Abstract

Transgenic nonhuman primate models are increasingly popular model for neurological and neurodegenerative disease because their brain functions and neural anatomies closely resemble those of humans [1–6]. Transgenic Huntington's disease monkeys (HD monkeys) developed clinical features similar to those seen in HD patients, making the monkeys suitable for preclinical study of HD [6–12]. However, until HD monkey colonies can be readily expanded, their use in preclinical studies will be limited [1, 13, 14]. In the present study, we confirmed germline transmission of the mutant huntingtin (*mHTT*) transgene in both embryonic stem cells (ESCs) generated from three male HD monkey founders (F0), as well as in second-generation offspring (F1) produced via artificial insemination by using intrauterine insemination (IUI) technique. A total of five offspring were produced from fifteen females that were inseminated by IUI using semen collected from the three HD founders (5/15; 33%). Thus far, sperm collected from HD founder (rHD8) has led to two F1 transgenic HD monkeys with germline transmission rate at 100% (2/2). *mHTT* expression was confirmed by quantitative real-time PCR (qPCR) using skin fibroblasts from the F1 HD monkeys, as well as induced pluripotent stem cells (iPSCs) established from one of the F1 HD monkeys (rHD8-2). Here we report the stable germline transmission and

*Correspondence to: Anthony W. S. Chan, Yerkes National Primate Research Center, Rm 2212, Neuroscience Research Bldg., 954 Gatewood Rd. N.E., Atlanta, GA 30329, USA, awchan@emory.edu.

†These authors contributed equally

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Author Contributions

SM: preparation of semen for *in vitro* fertilization, artificial insemination, development and assistance in artificial insemination, infant and general animal care, preparation of samples for molecular analyses, sperm analysis and preparation of manuscript. TC: preparation of semen for *in vitro* fertilization and artificial insemination, development and assistance in artificial insemination, infant and general animal care and preparation of samples for molecular analyses. MSP: genotyping, gene expression analysis and preparation of manuscript. KA: Derivation and characterization of ESCs and iPSCs. FCS, SJ and KG: development and performance of the artificial insemination technique. ASWC: experimental design and coordination, data analysis, development of and assistance with artificial insemination, wrote and prepared manuscript and final approval.

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expression of the *mHTT* transgene in HD monkeys, which suggest possible expansion of HD monkey colonies for preclinical and biomedical researches.

1. Introduction

The rationale for developing the HD monkey was to create a large preclinical animal model of HD that could pass the *mHTT* transgene down through generations, such that offspring with predictable phenotypes could facilitate basic and preclinical research in HD, which remains a terminal disease today [1, 13, 14]. Thus, with such a large animal model we could develop and test novel treatments using clinical assessment tools and methods similar to those used in humans [1, 13, 14]. A cohort of three male HD monkeys (rHD6, rHD7 and rHD8; rHDs6–8) carrying the *mHTT* transgene containing exons 1–10 of the human *HTT* gene with an expanded polyglutamine (polyQ) tract (69–72Q) in exon 1 driven by the human *HTT* promoter were used as founders for the production of F1 HD monkeys. We have been conducting an ongoing longitudinal study to monitor disease onset and progression in the HD monkeys since their birth using a variety of clinical assessment tools, including non-invasive imaging, cognitive behavioral assessments and molecular profiling studies [7, 8]. While our model shows great promise as a preclinical large animal model for HD, the practicality of using the HD monkeys in a clinical research setting depends largely upon whether the *mHTT* transgene can be transmitted through the germline, thereby making the cohort of HD monkeys readily expandable and available to researchers. Unlike rodents and small primate species, such as the marmoset, rhesus macaques reach sexual maturity at approximately 4–5 years of age, with a subsequent seasonal reproductive cycle peaking between fall and late spring [1, 15]. Because of this, the inheritability of the *mHTT* transgene has been assessed only recently with F0 HD monkeys reaching pubertal age.

2. Materials and Methods

2.1 Animal models

HD monkeys were generated by lentiviral-mediated transgenesis as previously described [6]. rHD6, rHD7 and rHD8 are male rhesus macaques that carry exons 1–10 of the *HTT* gene with expanded polyglutamine repeats (66–74Q, 66–72Q and 71–74Q), under the control of the human *HTT* promoter [6, 8, 15]. All monkeys received the same treatments and procedures designed for the longitudinal study, including MRI scans, cognitive behavioral assessments and scheduled blood draws. All monkeys were housed in the same room and were nursery-raised at the center.

2.2 Semen collection and sperm preparation for artificial insemination [6, 15]

Ejaculates were collected in the morning. All male monkeys were chair trained for semen collection by penile electroejaculation. Monkeys were sedated with a light dose of ketamine (2–3 mg/kg body weight) by intramuscular injection. The ejaculates were kept at room temperature (RT) for 20 min to liquefy. The liquid portion of semen was transferred into 15 mL conical tubes and washed with TALP-HEPES medium supplemented with 4 mg/mL bovine serum albumin (BSA), followed by centrifugation at $112 \times g$ at RT for 5 min. Sperm concentration, motility and viability were determined and recorded.

2.3 Sperm analysis

Sperm from rHD8 was used for determining viability by using Hoechst 33342 (Molecular Probe), propidium iodine (PI; Sigma) and fluorescein isothiocyanate-peanut agglutinin (PNA; Sigma) staining. Fresh sperm samples were diluted to a concentration of $1.2 \times 10^7/\text{ml}$ in a black Eppendorf tube to block light. $0.5 \mu\text{M}$ Hoechst was added and incubated in a 37°C water bath for 5 min, followed by the addition of PI ($2.0 \mu\text{M}$) for 5 min. Finally, PNA ($4.8 \mu\text{M}$) was added to the sample and incubated for 20 min. Sperm samples were then placed on a microscope slide coated with 0.1% poly-D-lysine (Sigma) and covered with a 22 mm^2 coverslip. At least 2000 total sperm were counted per sample based on Hoechst-positive stained sperm. Membrane damage and acrosome damage stained positive with PI and PNA, respectively.

2.4 Production of transgenic HD monkey embryos and establishment of embryonic stem cells

MII arrested oocytes were retrieved from hormone-stimulated female rhesus macaques and used for *in vitro* fertilization by intracytoplasmic sperm injection (ICSI) using HD monkey sperm followed by *in vitro* culture [16]. Expanded blastocysts were selected for inner cell mass (ICM) isolation using an XYClone laser (Hamilton Throne, Inc.). The isolated ICMs were cultured *in vitro* and attached onto mouse fetal fibroblasts (MFFs) to form an outgrowth. Outgrowths with prominent stem cell morphology were mechanically passaged and continued in culture [11]. Monkey ESCs were cultured in medium composed of knockout-Dulbecco's modified Eagle's medium (KO-DMEM) supplemented with 20% Knock-out Serum Replacement (KSR; Invitrogen), 1 mM glutamine, 1% nonessential amino acids and supplemented with 4 ng/ml of human basic fibroblast growth factor (bFGF; Chemicon).

2.5 Preparation of surrogate female monkeys for artificial insemination

The menstrual cycles of adult females with prior pregnancies were monitored daily, and females were trained for conscious bleeding. Blood samples were used to determine ovulation time based on the levels of estradiol (E2) and progesterone (P4). Artificial insemination was performed either on the day of E2 peak or the day before/after E2 peak or on both days. A total of fifteen females were used for artificial insemination by intrauterine insemination (IUI).

2.6 Percutaneous ultrasound-guided intrauterine artificial insemination (IUI)

Female rhesus macaques were anesthetized with 3–5 mg/kg of ketamine and placed on a surgical bed in left lateral recumbency. The caudal right abdominal wall was prepared aseptically, and rectal palpation was used to stabilize the uterus in a fixed position up against the right abdominal wall. A 12-gauge sharp-tipped needle was introduced into the abdominal wall sterilely under ultrasound guidance, then directed caudally through the uterine wall. The tip of the needle was gently advanced into the lumen of the uterus. Micro medical tubing, 0.58 mm I.D. $\times$ 0.99 mm O.D. (Scientific Commodities Inc., Lake Havasu City, AZ), was advanced through the needle until it was visualized on the ultrasound image. Approximately 0.5 inch of tubing was advanced into the uterine lumen. The semen was then

slowly infused using a 1.0-ml syringe. The needle was removed from the uterine lumen while the tubing was held in place. Finally, the tubing was removed. The animal was then placed in ventral recumbency and electro-stimulation using a rectal probe was performed to enhance uterine contraction. Animals were returned to their home cage immediately after IUI and were monitored every 15 min until fully awake and recovered.

2.7 Infant care and sample collection for molecular analyses

Post-delivery, infants were surrogate/nursery-reared in the primate nursery of the Yerkes National Primate Research Center (Atlanta, GA) according to procedures developed by Sackett and colleagues (2002) [17] that allow normal growth as well as the development of species-specific social skills. These procedures included daily social interactions with peers, intensive human contact and cognitive testing that began in the first weeks of life and continued through adulthood (for additional details on rearing conditions, see Goursaud and Bachevalier, 2007) [18]. Diet consisted of infant Similac formula (SMA with iron) supplemented with banana pellets starting at 3–4 weeks old (190 mg, P.J. Noyes, Cleveland, OH). Starting around 4 months of age, they were fed jumbo primate chow (Lab Diet #5037, PMI Nutrition International Inc., Brentwood, MO) and fresh fruit daily.

The day after delivery, peripheral blood (peripheral blood cells), buccal swab (buccal cells) and ear punch (skin cells) were collected for genotype, the establishment of skin primary culture and transgene expression analyses. All experimental procedures were approved by the Institutional Animal Care and Use Committee of Emory University (Atlanta, GA) and conformed to the NIH Guide for the Care and Use of Laboratory Animals.

2.8 Derivation of induced pluripotent stem cells (iPSCs) [9]

Skin fibroblasts from F1 HD monkeys were isolated and cultured as described previously [9]. To derive iPSCs, skin fibroblasts were infected by retrovirus expressing rhesus macaque Oct4, SOX2 and Klf4. At approximately 2–3 weeks post-retroviral transfection, a primate ES cell-like colony was selected based on morphology and mechanically passaged onto MFF feeder cells with primate ES media. HD monkey iPSCs were then cultured and characterized as described for HD-ESCs.

2.9 Immunostaining of stem cell markers [9–11, 19]

ESC or iPSC cultures were fixed in 4% paraformaldehyde (PFA), permeabilized by 1% Triton-X (excluded for cell surface markers) and blocked with 2% BSA and 130 mM glycine in phosphate buffer saline (PBS). After overnight incubation with primary antibodies Oct4 (Santa Cruz Biotechnology Cat# sc-5279 Conc: 1:500), SSEA-4 (Chemicon Cat# MB4303 Conc: 1:250) and TRA-1-60 (Chemicon MAB4360 1:200) followed by thorough washes, a secondary antibody conjugated with Alexa Red (Molecular Probe Cat# A21203 Conc: 1:1000) was used for detection of the primary antibodies. DNA was counterstained with Hoechst 33342 (5 µg/ml Molecular probe Conc 1:10,000). The specimen was examined using an epifluorescent microscope. Alkaline phosphatase assay was performed following the manufacturer's instructions (Vector Lab Cat# SK-5300).

2.10 In vitro neural differentiation to neuronal lineage

rHD8-ESC3 and rHD8-2-iPSC were manually dissected into small clumps and cultured in suspension for seven days for the formation of embryoid bodies (EBs), which were then allowed to attach onto a polyornithine/laminin coated plate. EBs were cultured in N1 medium (KO-DMEM (Invitrogen) supplemented with minimum essential amino acid (Invitrogen), 200mM of L-glutamine (Invitrogen) and N2 supplement (Invitrogen)) for seven days. After seven days culture, N1 medium was replaced by N2 medium (N1 medium supplemented with 20 ng/ml bFGF) for 14 days and followed by N3 medium (KO-DMEM supplemented with 1% FBS (Hyclone) and B27 supplement (Invitrogen)) for seven days to allow differentiation into mature neurons. The resulted neural cells was confirmed by the expression of Nestin (1:1000; Millipore), neuron specific β III tubulin (1:300; Millipore) and glial fibrillary acidic protein (GFAP; 1:500; Chemicon) by using specific antibodies [9, 11, 20]. Hoechst 33342 (5 μ g/ml Molecular probe Conc 1:10,000)

2.11 Genomic DNA extraction and PCR genotyping

Genomic DNA (gDNA; used for sequencing and genotype experiments) was extracted from various sample types, including whole blood, sperm, lymphocytes, skin, buccal swabs and other cell lines, using a number of commercially available kits following each manufacturer's recommended protocol (see Table 1). Total RNA (used for quantitative real-time PCR) was extracted from fibroblasts and iPSC samples using Trizol (Invitrogen) and subsequently column-purified using a Qiagen RNeasy Mini Kit according to the manufacturer's protocol for RNA cleanup with on-column DNase digestion. All protocols were performed according to each of the manufacturer's protocols. DNA and RNA integrity was verified by spectrophotometry and agarose gel electrophoresis prior to use in genotyping by quantitative real-time polymerase chain reaction (qPCR) experiments.

2.12 PCR genotyping and sequencing of the transgenic polyQ region in HD monkeys

50 ng of gDNA purified from available tissues (see above) was used as a template for PCR amplification of a 1,080-bp region of the *mHTT* construct. Each 20- μ l PCR reaction was set up using Denville Choice-Taq Blue Polymerase according to the manufacturer's protocol, with each reaction supplemented with 5.5 M betaine. Each reaction contained 0.4 μ M each of the forward (HTT Pro F6: 5'-TCA CCC CAT TAC AGT CTC ACC-3') and reverse (HD Exon 4/5-R: 5'-CCG AGG GGC ACC ATT CTT TTT-3') primers. Each reaction was then subjected to amplification in a thermal cycler using the following cycling parameters: initial denaturation at 96°C for 5 min, followed by 35 amplification cycles (45 s – 96°C, 45 s – 56°C, 72°C – 2 min 30 s), and final extension at 72°C for 7 min. Following PCR, 10 μ l of PCR product was subsequently visualized on 1 $\times$ TAE/agarose/ethidium bromide gels by electrophoresis. The presence of a band at 1,080 bp confirmed the presence of the transgene construct in samples. For polyQ sequencing experiments, PCR products from whole blood samples were electrophoresed on a 1.5% agarose gel, and target bands were gel purified, cloned into the pGEM-T easy vector (Promega) and subsequently sequenced at GENEWIZ Corporation with T7 and SP6 primers. Sequencing results were analyzed visually for approximation of polyQ expansion numbers.

2.13 Reverse transcription and quantitative PCR (qPCR)

Following purification from fibroblast/iPSC cell pellets, 500 ng DNA-free total RNA was reverse transcribed in a total volume of 20 μ L using MultiScribe Reverse Transcriptase (Applied Biosystems) as outlined in the manufacturer's protocol. Primers used for real-time PCR were designed using Primer3Express [21] and are listed below. Each qPCR reaction consisted of 1 $\times$ SsoAdvancedTM SYBR[®] Green Supermix (Bio-Rad) with 0.4 μ M each primer (Table 2), and thermal cycling was performed according to the manufacturer's protocol. All *HTT* exon 1 data (primers amplified endogenous and transgenic *HTT*) were analyzed by normalization to both ubiquitin C (*Ubc*; housekeeping gene) and/or *HTT* exon 26 (primers specifically amplified endogenous *Htt* only).

2.14 Statistical methods

Sperm viability was analyzed between control and HD monkey sperm samples. Statistical analysis was performed using One-way ANOVA by a blind statistician using the statistical software program JMP. Three replicas were used for analysis. Any value of $P < 0.05$ was considered as significantly different.

3. Results

3.1 Derivation and genotyping of transgenic HD monkeys germ line and embryonic stem cells

Germline transmission of the *mHTT* transgene in HD monkeys was assessed primarily by performing polymerase chain reaction (PCR) genotyping using primers that specifically amplified a region (including the polyQ expansion in exon 1) of the *mHTT* transgene (Fig. 1a). To confirm the presence of transgene in germ cells, semen was collected from each of the three F0 founders (rHDs6–8). Genotype results confirmed the presence of the *mHTT* transgene in HD monkey sperm from each of the F0 HD monkeys (Fig. 1b). PCR-amplified products were then subcloned and sequenced to determine the number of glutamine (Q) repeats present in the first exon of the transgene in each animal. PolyQ counts were similar amongst the founders, ranging from 66–74Q (Fig. 1c).

In order to carefully assess germline transmissibility of the *mHTT* transgene, we used both *in vitro* and *in vivo* methodologies. Spermatozoa collected from each of the HD monkeys were used for intracytoplasmic sperm injection (ICSI), producing embryos from which we then derived embryonic stem cells (ESCs) (Fig. 1d; Supplemental Fig. 1 and 2). A total of 15, 14 and 8 blastocyst stage embryos derived from rHD6, rHD7 and rHD8, respectively, were used for the derivation of ESC lines. From these blastocysts, 6/15 (40%), 1/14 (7%) and 4/8 (50%) ESC lines were established from rHD6, rHD7 and rHD8 respectively. Genotype assessment confirmed transgene presence in ESCs derived from embryos produced from the gametes of rHD7 and rHD8, but not rHD6. Two (of four) rHD8-derived ESC lines (2/4; 50%), as well as the single ESC line derived from rHD7 (1/1; 100%), were positive for the *mHTT* transgene (Fig. 1d). ESC line derived from embryo fertilized by rHD8, rHD8-ESC3, presented distinct primate stem cell morphology with large nucleus and cobblestone morphology (Supplemental Fig. 1). rHD8-ESC3 also express stem cell markers which include OCT4, SSEA4, TRA-1-60 and alkaline phosphatase (Supplement Fig. 2). The

transgene was not detectable in ESC lines derived from rHD6. Because the quantity of established ESC lines available for our analysis was limited, we have yet to evaluate the rate of successful germline transmission of the transgene. One possible approach is to develop methods for sperm sorting and assess transgene transmission using individual sperm so that a more precise transmission pattern can be determined.

3.2 Analysis of HD monkey sperm and ultrasound guided intrauterine insemination

Following confirmation of *in vitro* capacity, we pursued an *in vivo* assessment of germline transmission in HD monkeys. Sperm viability was first assessed using semen samples collected from HD monkey founders. Membrane integrity was evaluated by staining sperm with propidium iodide (PI; Red), while acrosome damage was examined by staining with fluorescently labeled peanut (*Arachis hypogaea*) agglutinin (PNA; Green); sperm nuclei were stained using Hoechst (Blue; Fig. 2a). We performed PI+ and PNA+ sperm counts and observe similar sperm membrane and acrosome viability between fresh rHD8 and WT monkeys (Fig. 2b; n=3).

3.3 Production and genotyping of second generation HD monkeys

Artificial insemination was then performed to determine whether there was germline transmission of the transgene through the HD monkey spermatozoa to F1 offspring. We developed a modified ultrasound-guided intrauterine insemination (IUI) method for the production of F1 HD monkeys (Fig. 2c and 2d). Female WT monkey surrogates were inseminated by IUI using fresh semen collected from F0 HD monkey founders. Two offspring from each rHD6 and rHD8, and one from rHD7 were produced by IUI with success rate at approximately 33% (5/15). Among the five offspring, two female offspring of rHD8 (rHD8-1 and rHD8-2; Fig. 3) were confirmed to be transgenic by genotyping of blood, skin and buccal cells (Fig. 3a). Sequencing results from the polyQ region of the *mHTT* transgene revealed that rHD8-2 had polyQ repeat numbers close to rHD8, with approximately 72–74Q; however, rHD8-1 had fewer polyQ repeats, ranging from 53–61Q (Fig. 3d). F1 offspring were successfully generated from the gametes of F0 HD monkeys, showing that viable cohort expansion is possible in HD monkeys.

3.4 Derivation of induced pluripotent stem cells from second generation HD monkeys

We established primary cultures of skin fibroblasts from rHD8-1 and rHD8-2, which were confirmed positive for the *mHTT* transgene (Fig. 4a). Furthermore, expression of the *mHTT* transcript was measured and positively detected by qPCR at low level (Fig. 4b). Transcript levels were slightly elevated in these cells compared to those derived from WT monkey. The skin fibroblasts from rHD8-2 were then used for reprogramming and derivation of induced pluripotent stem cells (iPSCs), rHD8-2-iPSC (Supplemental Fig. 1 and 3). There was a slight elevation of *mHTT* transgene expression at a level similar to the skin fibroblasts of rHD8-2 (Fig. 4b and 4c). Based on our prior study in HD monkey ESCs and iPSCs, we expected increased expression of the *mHTT* transgene when differentiating to neural cells [9–11, 20].

3.5 In vitro neural differentiation of rHD8-ESC3 and rHD8-2-iPSC

To assess differentiation potential of ESCs and iPSC derived from HD monkeys, we performed *in vitro* neural differentiation because neuronal cell types are the most interesting cell types for HD research. We use a stage-wise *in vitro* neural differentiation method to guide the differentiation of rHD8-ESC3 and rHD8-2-iPSC to neurons [9, 11, 22]. After four weeks of *in vitro* neural differentiation, neural cells derived from rHD8-ESC3 and rHD8-2-iPSC were confirmed by the expression of neural specific markers including β III-tubulin, nestin and GFAP (Figure 5). This study demonstrated the differentiation capability of HD monkey derived ESCs and iPSCs.

4. Discussion

Development of a transgenic nonhuman primate model has proved a challenging task that requires the efficient and innovative use of genetic techniques; not only this, the innate physiological parameters of the primate itself pose a major obstacle since the rhesus macaque does not reach reproductive maturity until 4–5 years of age with seasonal breeding cycles [1, 7, 16]. Although genetically modified primate models like the marmoset have certain physiological advantages, such as their smaller size and non-seasonal breeding cycles, with a younger onset of puberty [5, 23], these models are not suitable as a model for human diseases such as those involve fine motor skills. Rhesus macaques may prove critical for modeling diseases where there is disruption of fine motor skills, locomotion and cognitive functions that worsens with disease progression, as happens in HD [1, 7]. While there is no perfect animal model for any human disease, it is vital to find ones that can address specific scientific and clinical questions [1, 13, 14, 24]. Recent success in the development of gene-targeted monkeys by clustered regularly interspaced short palindromic repeats (CRISPR) and transcription activator-like effector nuclease (TALEN) technologies sparked interest in developing nonhuman primate models for both healthy and disease conditions in humans [2, 3]. Here we demonstrate the successful germline transmission of the *mHTT* transgene in HD monkeys via production of transgenic cell lines and F1 HD monkey offspring.

Based on polyQ analysis by sequencing, different in the number of CAG repeats was not unexpected because of the well-known nature on the instability of the polyglutamine tract [25–27]. However, this observation was particularly important in developing a germline transgenic model because germline expansion in polyglutamine tract is one of the major factors that promote disease progression and severity [28–31]. The establishment of pluripotent stem cells such as iPSCs from F1 HD monkeys not only allow us to confirm whether successful germline transmission was achieved but also led to future opportunity in assessing personal medicine in HD monkey model [9, 20, 22]. HD monkey iPSCs can be used to derive neural cell for studying HD pathogenesis in a dish but also as a unique platform for drug discovery research as well as the development of cell replacement therapy [9, 11, 20, 22]. We have demonstrated that rHD8-ESC3 and rHD8-2-iPSC derived from F1 HD monkey are pluripotent and can be *in vitro* differentiated into neural cells based on the expression of neural specific markers (Figure 5).

Although genotyping analysis on sperm from all HD founders suggested the presence of the *mHTT* transgene (Fig 1b), only rHD8 has successfully produced F1 HD monkeys. On the other hand, variations in transmission rate were clearly demonstrated in ESC lines generated from HD monkey derived embryos (Fig 1d). While continue assessment in germline transmission rate of each founder is important for future production of F1 HD monkeys for preclinical applications, alternative method other than producing live offspring such as single sperm genotyping will facilitate the precise assessment of transmission rate in each founder as well as study on germline expansion of polyglytamine repeats. Here we presented the successful production of F1 HD monkeys. Germline transmissibility argues that the use of HD monkeys in preclinical research is indeed feasible. Ultimately, the development of HD monkeys is an important proof of principle that modeling human inherited disease in nonhuman primates is possible, holding out great promise for the utility of preclinical large animal models of human diseases.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgements

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Highlights

- Germline transmission of transgenic Huntington's disease monkey, the first transgenic monkey model of human inherited neurodegenerative disease
- Derivation of embryonic stem cells and induced pluripotent stem cells from second generation transgenic Huntington's disease monkey
- Production of second generation transgenic Huntington's disease monkeys by ultrasound guided intrauterine insemination

Fig. 1. Germline transmission of *mHTT* transgene in F0 HD monkeys
Genotyping confirmation was performed by PCR amplification of genomic DNA (gDNA) with primers specific to a region containing the polyQ expansion of the *mHTT* transgenic construct. Primer produced a 1,080-bp amplicon spanning the promoter – exon 1 junction of the transgene (a). Genomic DNA (gDNA) was extracted from sperm samples collected from each HD monkey founder (rHD6, rHD7 and rHD8) and visualized by gel electrophoresis (b). PCR products were cloned and sequenced. PolyQ lengths were calculated and in the range of 66Q–74Q among HD monkey founders (c). ESC lines were derived from embryos produced *in vitro* using sperm from founder HD monkeys. Genotyping results confirmed *mHTT* transgene was transmitted to ESC lines derived from rHD7 and rHD8 (d).

Fig. 2. Assessment of HD monkey sperm viability and IUI method

Gamete integrity was assessed in sperm samples collected from the F0 HD monkey rHD8 by fluorescent staining. Nuclei are stained with Hoechst (blue), acrosome damage is marked by staining with PNA (green), and membrane damage is marked by PI (red) staining (a). Percentage of sperm labelled with PNA and PI is illustrated to demonstrate viability of rHD8 sperm (b). Ultrasound-guided intrauterine insemination (IUI) was performed to impregnate female surrogate WT monkeys with sperm collected from HD monkey founders. Ultrasound images were captured showing the uterine lining both (c) pre- and (d) post-

procedure. The arrow highlights the uterine lumen before and after the deposition of sperm solution confirmed by the flash of light (d).

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Fig. 3. Germline transmission of *mHTT* transgene to F1 HD monkey offspring

The *mHTT* transgene was passed on to two female F1 offspring (a; rHD8-1 and rHD8-2), as confirmed by PCR genotyping using gDNA extracted from buccal cells (BC), skin biopsies (S) and whole blood samples (Blood) (a). Photographs of rHD8-1 (b) and rHD8-2 (c). PolyQ length was determined by sequencing of PCR product (d).

Fig. 4. Transgene expression in fibroblasts and iPSCs derived from F1 HD monkeys
Primary fibroblasts were cultured from F1 HD monkey offspring and confirmed transgenic by PCR analysis (a). qPCR analysis confirmed detection of *mHTT* transcript in both fibroblasts (b) and fibroblast-derived iPSCs (c) from F1 HD monkey offspring.

Fig 5. In vitro neural differentiation of ESC and iPSC derived from HD monkey
Immunostaining of in vitro neural differentiated rHD8-ESC3 and rHD8-2-iPSC with antibodies which recognize neural specific proteins, β III-tubulin, nestin and GFAP. Hoechst DNA staining in blue. Scale bar = 20 μ m

Table 1

Commercial kits and methods

Sample Type	Genomic DNA Kit	Manufacturer
Whole Blood	Maxwell® 16 Blood DNA Purification Kit	Promega
Lymphocytes (from buffy coat)	Maxwell® 16 Blood DNA Purification Kit	Promega
Skin Biopsy	Maxwell® 16 Mouse Tail DNA Purification Kit	Promega
Buccal Swab	QIAamp® DNA Mini Kit	QIAGEN
Sperm	QIAamp® DNA Mini Kit	QIAGEN
Fibroblasts, ESCs and iPSCs	Wizard® Genomic DNA Purification Kit	Promega

Table 2

List of primers

Gene of Interest	Forward (5'→3')	Reverse (5'→3')	Amplicon Size	[Final] _{reaction}
<i>Ubc</i> (rhesus) Acc. No. XM_001102090.2	5'-CCA CTC TGC ACT TGG TCC TG-3'	5'-CCA GTT GGG AAT GCA ACA ACT TTA-3'	113 bp	0.4 μM
<i>Htt</i> , exon 1 (rhesus) XM_001086119.2	5'-GCG ACC CTG GAA AAG CTG AT-3'	5'- CTG CTG CTG CTG GAA GGA CT-3'	60 bp	0.4 μM
<i>Htt</i> , exon 26 (rhesus) XM_001086119.2	5'-ACC CTG CTC TCG TCA GCT TGG-3'	5'-AGC AAG TTT CCG GCC AAA AT-3'	60 bp	0.4 μM