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Germline transmission in transgenic Huntington's disease monkeys

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ABSTRACT

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

1. Introduction

The rationale for developing the Huntington's disease (HD) monkey was to create a large preclinical animal model of HD that could pass the *mutant huntingtin* (*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 [1–3]. 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 [1–3]. A cohort of three male HD monkeys (rHD6, rHD7 and rHD8; rHDs6–8) carrying the *mHTT* transgene containing exons 1 to 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 [4,5]. We have been conducting an ongoing longitudinal study to monitor disease onset and progression in the HD monkeys by using a variety of clinical assessment tools, including noninvasive

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imaging, cognitive behavioral assessments, and molecular profiling studies [4,5]. Although 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 on 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 to 5 years of age, with a subsequent seasonal reproductive cycle peaking between fall and late spring [1,6]. 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

Huntington's disease monkeys were generated by lentiviral-mediated transgenesis as previously described [7]. rHD6, rHD7, and rHD8 are male rhesus macaques that carry exons 1 to 10 of the *HTT* gene with expanded polyQ repeats (66–74Q, 66–72Q, and 71–74Q), under the control of the human HTT promoter [5–7]. All monkeys received the same treatments and procedures designed for the longitudinal study, including magnetic resonance imaging 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

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 for 20 minutes to liquefy. The liquid portion of semen was transferred into 15-mL conical tubes and washed with Tyrode's albumin lactate pyruvate–HEPES medium supplemented with 4 mg/mL BSA, followed by centrifugation at 112 × *g* at room temperature for 5 minutes. Sperm concentration, motility, and viability were determined and recorded [6,7].

2.3. Sperm analysis

Sperm from rHD8 was used for determining viability by using Hoechst 33342 (Molecular Probes), propidium iodide (PI; Sigma), and fluorescein isothiocyanate–peanut agglutinin (PNA; Sigma) staining. Fresh sperm samples were diluted to a concentration of 1.2×10^7/mL in a black Eppendorf tube to block light. Hoechst (0.5 μM) was added and incubated in a 37 °C water bath for 5 minutes, followed by the addition of PI (2.0 μM) for 5 minutes. Finally, PNA (4.8 μM) was added to the sample and incubated for 20 minutes. 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 on the basis of 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

Meta phase II–arrested oocytes were retrieved from hormone-stimulated female rhesus macaques and used for IVF by intracytoplasmic sperm injection using HD monkey sperm followed by IVC [8]. Expanded blastocysts were selected for inner cell mass isolation using an XYClone laser (Hamilton Throne, Inc.). The isolated inner cell masses were cultured *in vitro* and attached onto mouse fetal fibroblasts to form an outgrowth. Outgrowths with prominent stem cell morphology were mechanically passaged and continued in culture [9]. Monkey embryonic stem cells (ESCs) were cultured in medium composed of knockout Dulbecco's modified Eagle's medium (KO-DMEM) supplemented with 20% Knockout Serum Replacement (KSR; Invitrogen), 1-mM glutamine, 1% nonessential amino acids and supplemented with 4 ng/mL of human basic fibroblast growth factor (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 on the basis of levels of estradiol (E2) and progesterone. Artificial insemination was performed on the day of E2 peak, the day before or after E2 peak, or on both days. A total of 15 females were used for artificial insemination by intrauterine insemination (IUI).

2.6. Percutaneous ultrasound-guided intrauterine artificial insemination

Female rhesus macaques were anesthetized with 5-10 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-ga sharp-tipped needle was introduced into the abdominal wall sterilely under ultrasound guidance and 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. × 0.99 mm O.D. (Scientific Commodities Inc., Lake Havasu City, AZ, USA), 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, whereas the tubing was held in place. Finally, the tubing was removed. The animal was then placed in ventral recumbency, and electrostimulation using a rectal probe was performed to enhance uterine contraction. Animals were returned to