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Key Words
Huntington’s disease · Pig model · Mutant huntingtin · Spermatozoa · Testes · Degeneration

Abstract
Background: Huntington’s disease is induced by CAG expansion in a single gene coding the huntingtin protein. The mutated huntingtin (mtHtt) primarily causes degeneration of neurons in the brain, but it also affects peripheral tissues, including testes. Objective: We studied sperm and testes of transgenic boars expressing the N-terminal region of human mtHtt. Methods: In this study, measures of reproductive parameters and electron microscopy (EM) images of spermatozoa and testes of transgenic (TgHD) and wild-type (WT) boars of F1 (24–48 months old) and F2 (12–36 months old) generations were compared. In addition, immunofluorescence, immunohistochemistry, Western blot, hormonal analysis and whole-genome sequencing were done in order to elucidate the effects of mtHtt. Results: Evidence for fertility failure of both TgHD generations was observed at the age of 13 months. Reproductive parameters declined and progressively worsened with age. EM revealed numerous pathological features in sperm tails and in testicular epithelium from 24- and 36-month-old TgHD boars. Moreover, immunohistochemistry confirmed significantly lower proliferation activity of spermatogonia in transgenic testes. mtHtt was highly expressed in spermatozoa and testes of TgHD boars and localized in all cells of seminiferous tubules. Levels of fertility-related hormones did not differ in TgHD and WT siblings. Genome analysis confirmed that insertion of the lentiviral construct did not interrupt any coding sequence in the pig genome. Conclusions: The sperm and testicular degeneration of TgHD boars is caused by gain-of-function of the highly expressed mtHtt.

Monika Macakova and Bozena Bohuslavova contributed equally to this work.
**Introduction**

Huntington’s disease (HD) is a neurodegenerative disorder caused by the expansion of CAG repeat in the gene encoding the huntingtin protein (Htt), which is expressed in most tissues. The onset of the disease is usually in the mid-thirties. The main target is the central nervous system, but it has an impact on the whole body. There is no available curative treatment to date. Even the pathogenesis of the disease is not well understood. Nevertheless, it is well known that mutated Htt (mtHtt) forms cytoplasmic and nuclear aggregates, particularly in the cerebral cortex, striatum and lateral hypothalamus [1]. Many rodent models of HD that express either truncated or full-length human mutant Htt display differences in the onset and severity of phenotypes. Rodent models have collectively provided valuable information related to target validation and drug therapy [2–4]. However, large animal models are expected to simulate the disease more faithfully and moreover enable the usage of medical techniques and equipment applicable for human patients [5].

Minipigs represent a desirable model for longitudinal safety studies and preclinical drug trials to fill the gap between rodent models and patients [6, 7]. The advantage of minipigs is their resemblance with the human brain as well as with the whole body in terms of size, anatomy and physiology. There is a 96% homology between porcine and human huntingtin genes and proteins [8] that provides further impetus to use the minipig as a model of HD. Therefore, a transgenic minipig model was generated using microinjection of a lentiviral vector encoding the N-terminal (1–548 aa) of human Htt containing 145 CAG/CAA repeats under the control of the human HTT promoter [9]. The mtHtt gene with 124 glutamines was incorporated into chromosome 1 (1q24–q25), and the expression of mtHtt was detected in numerous peripheral tissues. Successful germ line transmission occurred through 4 successive generations inheriting the mutation in Mendelian ratio [9].

Even though the neurological phenotype of HD patients is the most prominent, the first sign of phenotype development in TgHD boars of F1 generation was reproductive failure, starting at the age of 13 months [9]. Interestingly, among all organs, the testes display the most comparable gene expression pattern to the brain [10]. In accordance with this finding, the expression of mtHtt in R6/2 and YAC128 mouse models of HD results in atrophy of the brain and testes [11–13]. Closer examination of the testes in YAC128 mice revealed disorganized seminiferous epithelium and a reduced number of germ cells. YAC72 mice expressing mtHtt but lacking endogenous Htt (YAC72+/−) revealed an even more severe phenotype resulting in infertility with aspermia and massive apoptotic cell death in the testes [14]. Also, a detailed testes examination in HD patients documented testicular abnormalities as well as reduced numbers of germ cells and abnormal morphology of seminiferous tubules [13].

The question arises whether the defect in testes is caused by the presence of mtHtt in testes or by a defect in neurons responsible for hormonal changes. In R6/2 mice, a secondary effect due to the decreased level of gonadotropin-releasing hormone (GnRH)-immunoreactive neurons was suggested. Only 10% of GnRH neurons remained in R6/2 mice by 9 weeks of age, while testicular atrophy and infertility were detected at 12 weeks of age together with a decrease of testosterone levels in serum and testes [11]. Nonetheless, the direct effect of mtHtt was not considered. On top of that, a previous paper showed testicular atrophy in R6/2 mice by 4 weeks of age [15], a week prior to the start of GnRH neuronal loss. In the YAC128 mouse model, testicular degeneration developed between 9 and 12 months of age, but even at 12 months, there is no evidence for decreased testosterone levels in urinary and plasma samples or loss of GnRH neurons in the hypothalamus [13].

In this paper, we followed reproductive parameters of TgHD and WT minipig boars from F1 and F2 generations in order to describe their sperm and testicular pathology phenotype. Furthermore, we investigated whether the phenotype is caused by the primary effect of mtHtt. We ruled out hormonal changes or interruption of any coding sequence during insertion of the lentiviral construct. Here we show evidence for morphological and functional defects in sperm and testes over two generations of TgHD minipigs that accrue before the neurology defects and hormonal changes, suggesting a direct toxic consequence of the expressed N-terminal mtHtt.

**Materials and Methods**

**Animals**

Transgenic minipigs with the N-terminal part of human mtHtt [9] were studied. Transgenic boars (n = 17) and their wild-type male controls (n = 13) were used in experiments. All components of this study were carried out in accordance with the Animal Care and Use Committee of the Institute of Animal Physiology and Genetics and were conducted according to current Czech regulations and guidelines for animal welfare and with approval by the State Veterinary Administration of the Czech Republic.
For an overview of the animals used in experiments see supplementary material SM 1 (for all online suppl. material, see www.karger.com/go/10.1159/000443665).

Spermatozoa Collection, Measurement of Sperm Parameters and in vitro Fertilization Test

Semen was collected from boars of F1 (age 24–48 months, n = 4) and F2 (age 12–36 months, n = 8) generations. All samples were evaluated using a sperm cell analyzer (Microptic, Spain) immediately after collection. The number of spermatozoa per ejaculate and the motility and progressivity of the spermatozoa were assessed. In vitro fertilization tests were done as previously described [9].

Preparation of Testicular Tissue

Testicular tissue was obtained from boars of F2 generation aged 24 (n = 2) and 36 months (n = 4). Animals were perfused under deep anesthesia with cold PBS. The tissue of the right testis was fixed in 4% paraformaldehyde followed by cryoprotection in 30% sucrose in 0.1 M PBS and used for immunohistochemistry and electron microscopy (EM). The tissue of the left testis was used for SDS-PAGE and Western blot.

Electron Microscopy

Small blocks of testicular tissue and ejaculate samples were fixed in 300 mM glutaraldehyde (Sigma-Aldrich) in 100 mM cacodylate buffer for 2 h at room temperature (RT), washed in the same buffer and postfixed in 40 mM osmium tetroxide (Polysciences) in 100 mM cacodylate buffer for 1 h at RT. Samples of testicular tissue were embedded in araldite resin (Durcupan ACM; Sigma-Aldrich) after rinsing in cacodylate buffer and dehydration in ethanol. Ejaculate samples were embedded in agar blocks, dehydrated in ethanol and embedded in araldite resin (Durcupan ACM; Sigma-Aldrich).

For immunohistochemical analyses, samples of ejaculate were washed in PBS and fixed in 4% paraformaldehyde with 0.1% glutaraldehyde in PBS. The samples were embedded in agar blocks, dehydrated in ethanol and embedded in LR white resin (Sigma-Aldrich). Samples were incubated with mouse anti-polyglutamine monocronal primary antibody (MAB1574; Millipore; 1:50) over-night at 4 °C. Then the sections were rinsed in PBS and incubated with anti-mouse IgG-Gold antibody (10 nm gold particles; G7652; Jackson ImmunoResearch; 1:50) over-night at 4 °C followed by incubation with an avidin-peroxidase complex (Vector ABC Elite) and 3,3'-diaminobenzidine tetrahydrochloride (DAB; Sigma) chromogen. Sections were dehydrated in graded ethanol, cleared in xylene and then coverslipped using DPX. Slides were digitalized using a scanning microscope (Olympus BX) and images were edited using VS-120 software. Statistical analyses were performed using GraphPad Prism 5.0 software (one-way ANOVA with Duncan’s post hoc test). PCNA-positive and Ki67-positive cells were counted in 20–30 seminiferous tubules per animal.

SDS-PAGE and Western Blot

Testes were homogenized in liquid nitrogen using a mortar. Spermatozoa and homogenized testes were lysed in RIPA buffer (150 mM NaCl, 1% NP-40, 0.5% deoxycholate, 0.1% SDS, 50 mM Tris–HCl pH 8, inhibitors of phosphatases and proteases), sonicated, and centrifuged at 10,000 g for 10 min at 4 °C. Samples (20 µg of total protein) were loaded onto 3–8% Tris-acetate gel (EA03755; LifeTech) and run at 150 V. Gel was transferred onto nitrocellulose membrane (IB301001; LifeTech) at 250 mA. Membranes were blocked in 5% skimmed milk, and probed overnight with anti-Htt antibody (EPR5526; Abcam; 1:30,000 or AB1; Sigma Aldrich; 1:1000), or anti-polyQ antibody (3B5H10; Sigma Aldrich; 1:3000), tubulin staining was used as loading control (anti-tubulin; Sigma Aldrich; 1:10,000). Secondary antibody conjugated with HRP (anti-mouse, 711-035-152; Jackson ImmunoResearch; 1:10,000) or anti-rabbit (711-035-152; Jackson ImmunoResearch; 1:10,000) was used. Light reaction was induced by ECL (RPN2232; GE Healthcare) and the signal was captured on CL-Xposure films (34091; Thermo Scientific).

Hormonal Assay

Blood samples were collected 5 times from age-matched TgHD (n = 15) and WT (n = 8) boars (aged 7–30 months). Samples were allowed to clot for 60 min at RT, and centrifuged twice (1,500 g, 10 min, 4 °C). Serum levels of testosterone, luteinizing hormone (LH) and inhibin-a were determined by commercial ELISA kits (CSB-E06796p, CSB-E06791p, CSB-E12870p, CSB-EL-011718PI; CUSABIO, Wuhan, China). All measurements were performed in duplicate and according to the manufacturers’ protocols. Statistical analysis was done using the Kolmogorov-Smirnov normality test followed by the unpaired t test.

Jumping Library Whole-Genome Sequencing

Customized sequencing libraries were constructed based on published protocols [16] and sequenced with paired-end 50-bp reads on an Illumina HiSeq2500. Library barcodes were demulti-
Fig. 1. Sperm parameters. a Sperm count in the semen samples of TgHD boars from F1 generation collected at the age of 25–36 and 37–48 months was significantly decreased in comparison with control animals. b Number of sperms in ejaculate of F2 TgHD boars collected at the age of 13–24 and 25–36 months was also lower in comparison with their WT littermates. Motility of the sperms was decreased in both F1 (c) and F2 (d) generations in all tested ages. Also, progressivity of the sperms was lower in TgHD animals from F1 (e) and F2 (f) generations. Data were analyzed using the Kolmogorov-Smirnov normality test followed by the Mann-Whitney U test. p < 0.05 was considered significant.
plexed with CASAVA v1.7. Read quality was assessed with FastQC v0.11.2 (http://www.bioinformatics.babraham.ac.uk). Quality and adapter trimming was performed with TrimGalore v0.3.7 (http://www.bioinformatics.babraham.ac.uk). Reads were aligned to a modified version of *Sus scrofa* reference genome assembly Sscrofa10.2.74 (GCA_000003025.4; http://www.ensembl.org/Sus_scrofa) that included the full pHIV1-HD548aa-145Q vector sequence. Reads were aligned with BWA-backtrack v0.7.10-r789 [17]. Duplicates were marked with Picard Tools MarkDuplicates v0.1.111 (http://picard.sourceforge.net). All alignment manipulations, including sorting and indexing, was performed with sambamba v0.4.6 [18]. Alignment quality was assessed using Picard Tools, Samtools v1.0 and BamTools v2.2.2 [19, 20]. All chimeric read pairs mapping from endogenous reference sequences to the transgene or vector backbone sequences were isolated and clustered using our published algorithms BamStat and ReadPairCluster [21–23]. An independent algorithm, DELLY, was used to corroborate integration sites detected by principal methods [24]. Actual sequences of the integration junctions were determined by PCR and Sanger sequencing.

**Results**

**Sperm Pathology of TgHD Boars**

We showed altered reproduction parameters in 2 TgHD boars of F1 generation starting at the age of 13 months [9] as a potential HD phenotype in our porcine model. However, detailed analysis of a larger cohort of animals was needed to investigate the basis for the decline in fertility. We provided evidence on sperm reproductive parameters of TgHD and WT boars from F1 (24–36 months old) and F2 (12–36 months old) generations. Animals in the compared groups did not yet vary in weight, size or their motor movements.

Semen of TgHD and WT animals was collected and characterized using a sperm cell analyzer. Sperm count, motility and progressivity were evaluated. All parameters measured in semen samples of TgHD boars significantly decreased at around 13 months in both generations (fig. 1) and persisted at a low level with increasing age. In vitro fertilization tests showed a continuous decreased ability of TgHD sperms to penetrate the oocytes (fig. 2).

**Fig. 2.** Sperm penetration ability. **a** The ability to penetrate into oocytes is decreased in sperms of F1 boars at the age of 23–30 months, and also at the age of 34–41 months. The most striking difference in the penetration ability between WT and TgHD F2 boars was at the age of 23–30 months. **b** Such alterations were observed as well in animals from F2 generation who reached the age of 11–18 months (p = 0.0608). Each penetration test was repeated at least 5 times. Data were analyzed using the Kolmogorov-Smirnov normality test followed by the Mann-Whitney U test. p < 0.05 was considered significant.

EM analysis of semen samples revealed altered morphology of spermatozoa between TgHD and WT boars. Structural anomalies of spermatozoa were much more numerous in TgHD samples. These abnormalities were more pronounced in the F2 generation. Nearly all the spermatozoa of TgHD animals of F2 generation had a cytoplasmic droplet (most often proximal; fig. 3a). Severe structural alterations in TgHD spermatozoa were localized mainly in the connecting piece and midpiece of the tail. Abnormalities were manifested as deformation of the mitochondrial sheath in the tail midpiece and also other tail structures. Common findings were folded or coiled tails, and sometimes a double or triple axoneme with fused mitochondrial sheaths (fig. 3b, d). Deformity of the
nucleus associated with incomplete chromatin condensation and abnormal acrosome occurred occasionally (fig. 3c). Instability of acrosomes was in some cases manifested by a precocious acrosomal reaction. Proximal cytoplasmic droplets were often associated with disorganized mitochondrial sheaths (fig. 3e). In the F2 generation, there was a total absence of residual bodies in the ejaculate.

**Testicular Pathology of TgHD Boars**

After having demonstrated the sperm pathology of TgHD boars, we sacrificed one pair of 24-month-old and 2 pairs of 36-month-old animals from the F2 generation to perform the morphological analysis of testes.

At the age of 24 months, degenerative changes in seminiferous epithelium were more frequent in the TgHD boar in comparison with the wild-type one. Apoptosis was seen in supporting Sertoli cells as well as cells of sper-
matogenic lineage. Degeneration of Sertoli cells was characterized by increased density and vacuolation of cytoplasm, dilatation of endoplasmic reticulum, structural alterations of the nuclei and swollen mitochondria with defects of their internal structure (fig. 4a). Degeneration of spermatogonia was manifested by cell shrinkage, increased chromatin condensation in the nucleus, dilatation of endoplasmic reticulum and swollen mitochondria with defects of their internal structure (fig. 4b). In addition, other cells of the spermatogenic lineage were gradually degenerated (fig. 4c). Strongly reduced epithelium of the seminiferous tubules, restricted to the Sertoli cells, spermatogonia and the sparsely occurring spermatocytes and spermatids were occasionally observed. Multinucleated spermatogenic cells including spermatogonia (fig. 4d) were frequently recognized. The thick basal lamina was made up of several layers (2–3 laminae densae).

At the age of 36 months, the rate of degenerative changes in the testicular samples of 2 transgenic individuals was different. The changes were less pronounced in boar K104 than in boar K63 and resembled those of a TgHD boar at the age 24 months. Morphology of seminiferous tubules in boar K63 showed a significant reduction of spermatogenesis. In some of the tubules, spermatogenesis was preserved (but only to a limited extent; (fig. 5a); other tubules contained only Sertoli cells (fig. 5b). In the tubules with preserved spermatogenesis, the degenerative changes were detected in both Sertoli cells (fig. 5d) and spermatogenic elements, including the spermatogonia. Degenerative changes exhibited characteristics typical for early or advanced apoptosis – the increased density of cytoplasm associated with its vacuolation, swollen mitochondria, dilated endoplasmic reticulum and clumps of heterochromatin in the nucleus. The basal lamina was thick and made up of several layers (2–3 laminae densae).

![Fig. 4. Seminiferous epithelium of TgHD boar at the age of 24 months. Se = Sertoli cell undergoing apoptosis; N = nuclear structure; Sp = spermatocyte; L = lipid droplet; Sg = spermatogonia. a Sertoli cell undergoing apoptosis – increasing cytoplasm density, vacuolation, altered nuclear structure. Note spermatocyte and large lipid droplet. b Spermatogonia undergoing apoptosis. Note basal lamina (arrow). c Degenerated spermatocytes (arrows). Note large lipid droplet. d Spermatogonia with 3 nuclei. Note basal lamina (arrow).]
often formed concentrically arranged cisternae (fig. 5c). Structures, known as Charcot-Böttcher crystals, were often observed in these cells (fig. 5e). The basal lamina was made up of several layers (up to 6 laminae densae) and was strongly undulated (fig. 5e), probably as a result of tubules diameter reduction in the absence of spermatogenic elements.

At both ages, 24 and 36 months, the lamina propria of seminiferous tubules was made up of 1–2 layers of myofibroblasts and occasionally occurring fibroblasts (fig. 5f). No differences were found in comparison with the control animals of the same age. Numerous Leydig cells occupying extended areas adjacent to the tubules were present in the interstitium. Their number, location and morphology were identical in both TgHD and WT animals, regardless of age (fig. 5f).

An immunohistochemical cell proliferation assay detecting Ki67 and PCNA expression was performed in order to determine the mitotic activity of spermatogonia. Ki67 protein is expressed only in the nuclei of spermatogen-
Fig. 6. Proliferative analysis. PCNA (a–c) and Ki67 (d–f) staining revealed decreased number of spermatocytes (c) and spermatogonia (f) in seminiferous epithelium of the TgHD boar (K63) at the age of 36 months. g, h The number of spermatogenic cells of the TgHD boar (K63) was significantly decreased compared with the WT boar (p < 0.05). Seminiferous tubules of the 24-month-old (b) and 36-month-old (e) transgenic boars showed similar staining of spermatogenic cells to WT (a, d). h The exception is testicular tissue from TgHD boar K104, in which spermatogonia (Ki67-positive cells) were determined in a significantly reduced number of seminiferous tubules compared with WT. Scale bars = 50 μm (20 μm in insets). Results were plotted as mean ± SD of positive cells per seminiferous tubule. p < 0.05 was considered significant. * p = 0.0151; *** p < 0.0001.
gonia, while PCNA occurs also in the nuclei of primary spermatocytes in normal seminiferous epithelium [25] – 90.86% (726/799) of seminiferous tubules lacked PCNA-positive cells and 90.08% (745/827) of seminiferous tubules contained no Ki67-positive cell in the testis of the 36-month-old TgHD boar (K63; fig. 6 a–f). Spermatogonia serve as stem cells in the process of differentiation to spermatozoa, and their proliferative activity secures normal spermatogenesis. That means that impaired spermatogenesis involved 90% of seminiferous tubules. These results confirmed impaired spermatogenesis observed by EM (as indicated above). The remaining 10% of seminiferous tubules contained spermatogonia and spermatocytes stained by anti-PCNA and anti-Ki67 antibodies, but their number was significantly reduced in comparison with the seminiferous tubules of a WT boar. A significant decrease in the quantity of spermatogonia was also observed in the testis of the other TgHD boar at the age of 36 months (n = 2, K104 and K63). No difference was found in the testes of the 24-month-old TgHD boar (K8; fig. 6g, h).

Sperm and Testicular Degeneration: The Direct Effect of mtHtt
Supposing that sperm pathology is caused by the toxic effect of mtHtt, experiments showing localization and expression of mtHtt were done. The presence of the polyglutamine-containing proteins was observed in structures in the spermatozoa tail of TgHD boars using 10-nm gold particles examined under EM (fig. 3f). This finding was confirmed by a dotted immunohistochemical signal detected along the whole spermatozoa tail in all tested ages of F1- and F2-generation TgHD boars (but not in WT controls) using 3B5H10 (anti-N-terminal fragment of 171 aa of human Htt with 65Q) antibody (fig. 7a). Western blot analysis using EPR5526 (anti-N-terminal fragment of Htt) confirmed highly abundant mtHtt and a slightly lower level of endogenous Htt in spermatozoa (fig. 7b).
dition, anti-polyQ antibody (3B5H10) as well as anti-huntingtin antibody (EPR5526) revealed a fragmented form of mtHtt, which was reported to cause cellular toxicity [26]. Sperm samples from all tested ages in both generations (F1, F2) were analyzed, and no significant change in expression between samples was detected (only representative data from two pairs of samples are shown; fig. 7).

Consequently, we looked at Htt localization in TgHD and WT seminiferous tubules from testes of 24- and 36-month-old boars. The signal was widely spread in spermatogenic and Sertoli cells in normal as well as in the atrophic seminiferous tubules (fig. 8a). Western blot analysis showed a high expression of mtHtt form compared with endogenous Htt. The huntingtin antibody also revealed fragmented forms of mtHtt that may contribute to toxicity of the transgene (fig. 8b).

In order to eliminate the neuronal effect on the testicular phenotype, levels of fertility-related hormones were measured in the blood serum of two age groups: 7- to 17-month-old TgHD (n = 14) and WT (n = 9) boars, and 15–30-month-old TgHD (n = 11) and WT (n = 6) boars. Levels of testosterone, LH and inhibin-α were analyzed (see online suppl. material SM 2). No significant difference was observed between TgHD and WT animals.

**Mapping the HIV1-HD-548aaHTT-145Q Transgene Integration**

To detect any and all sites of vector integration into the pig genome in the transgenic lineage, we performed long-insert jumping library whole-genome sequencing of transgenic animals from F0 (founder female) and F2 gen-

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**Fig. 8.** Expression of Htt in testes. M = Myoid cells. a Total huntingtin protein was visualized immunohistochemically with EPR5526 antibody in sections of WT and transgenic (TgHD) porcine testis. EPR5526 (red; color in online version only) was detected in seminiferous epithelium demarcated by seminiferous tubule basement membrane (dashed line) and lumen (asterisk). Myoid cells were negatively stained. Cell nuclei were counterstained with DAPI (blue). Prominent Sertoli cell nuclei (arrowheads) present in atrophic seminiferous tubule (TgHD atrophic) documented the loss of germ cells. Blank, WT testis section stained only with A647-conjugated secondary antibody. Scale bar = 20 μm. b Western blot analysis of testis; representative sample shown. Anti-AB1 antibody detected endogenous Htt, transgenic mtHtt and fragments.
erations in comparison with a negative control. This method, which involves sequencing the ends of genomic DNA fragments after circularization and size reduction, has previously been shown to be an effective platform for vector integration site discovery in transgenic sheep and mice [21]. Whole-genome sequencing of jumping libraries prepared from random fragments (mean size 3.6 kb) resulted in an average of 65.5× coverage of mapped inserts across each base in the haploid genome for all 3 animals. The paired-end reads were examined for the signature of vector integration into the genome: chimeric fragments consisting of pig genomic sequence on one end and a portion of the introduced vector/transgene on the other. No integrations were detected in the negative control genome, while a single identical vector integration was detected in each of the F0 and F2 generations of TgHD animals. As suggested previously from FISH analysis, the integration occurred into chromosome 1q [9]. The jumping library analysis revealed that 5.3 kb of the HIV1-HD-548aaHTT-145Q vector DNA integrated as expected via the HIV LTR sequences and harbored an intact HD-548aaHTT145Q expression cassette. Sanger sequencing of the junctions with genomic DNA showed that the integration was in reverse orientation relative to the genomic sequence, between chr1 228,641,631 and 228,641,637, with loss of the 5 intervening bases of genomic DNA. This integration does not directly disrupt any annotated gene, and no further breakpoint or integration complexity or other genomic rearrangement was apparent at the resolution of the jumping library sequencing.

Discussion

In this study we described sperm and testicular degeneration, which is a result of the presence of mtHtt protein in the testes of transgenic minipig boars expressing the N-terminal part of human mtHtt. Cohorts of TgHD and their WT controls of F1 and F2 generations were directly compared. Previous studies on HD rodent models showed male sterility that was assumed to be due to a reduction of spermatozoa [15]. We confirmed this phenotype in a large animal model of HD, the minipig. Additionally, we reported both the reduction of spermatozoa and also their function measured by motility, progressivity and in vitro penetration assay. Spermatozoa of TgHD boars had severe problems to penetrate the minipig oocytes. The difference in values of sperm parameters was evident at 13 months and worsened with age. A comparison of animals from F1 and F2 generations of the same age (25–36 months) showed slightly worse values of all observed parameters in the F2 generation. There was also a wider variability of sperm parameters in the F2 generation, probably caused by a larger cohort of animals in the F2 group. Furthermore, EM analysis revealed deformation of the mitochondrial sheath in the tail midpiece of TgHD spermatozoa. Folded or coiled tails and a double or triple axoneme with fused mitochondrial sheaths were also observed. This can be caused by a failure of the disjunction of excess cytoplasm, which results in the presence of cytoplasmic droplets. This phenomenon, together with spermatozoa motility dysfunction, can be related to a decrease of mitochondrial energetic metabolism and functional impairment of respiratory chain complex II (unpublished observations). Occasionally, nucleus deformation associated with incomplete chromatin condensation and abnormal acrosome occurred in transgenic spermatozoa (but not in WT controls). These abnormalities were more pronounced in the F2 generation. Moreover, nearly all the spermatozoa of TgHD animals of the F2 generation contained a cytoplasmic droplet, and their ejaculate lacked residual bodies.

The testicular degeneration reported here is in agreement with observations in mice (R6/2 and Yac72) [14, 15], as well as in postmortem samples from humans [13]. Multinucleated spermatogenic cells were frequently present in the seminiferous epithelium of 24- and 36-month-old TgHD boars. The spermatogonia were shrunk and had dilated endoplasmic reticulum, swollen mitochondria and condensed chromatin in the nucleus. Spermatocytes and spermatids were observed occasionally. Reduced numbers of developing spermatocytes and spermatids were also observed in HD patients [13] and YAC128 mice [27]. Some tubules contained only Sertoli cells. Sertoli cells were characterized by increased density and vacuolization of the cytoplasm, dilatation of endoplasmic reticulum, structural alterations of the nuclei and swollen mitochondria. These are features typical for early or advanced apoptosis. Moreover, proliferative analysis of seminiferous tubules with elongated spermatozoa showed fewer cells expressing the proliferative markers PCNA and Ki67 in TgHD animals. The apoptotic nature of the cell death in a large number of degenerating spermatids with diffuse cytoplasmic vacuolization, condensed nuclei and electron-dense cytoplasm which were phagocytized and degraded by Sertoli cells was also observed in a mouse model lacking endogenous huntingtin YAC72+/− [14]. Similar-
ly, seminiferous tubules of YAC128 were disrupted by large vacuoles [27]. In addition, the seminiferous tubule wall was thickened in HD patients [13]. In the TgHD minipig, the thick basal lamina was made up of several layers (2–3 laminae densae) compared with the WT minipig. At the age of 36 months, spermatogenesis was more affected in comparison with the age of 24 months. Sertoli cells contained extreme amounts of endoplasmic reticulum and structures known as Charcot-Böttcher crystals. The basal lamina was made up of several layers (up to 6 laminae densae) and was strongly undulated, resulting probably from a reduction of the diameter of the tubules in the absence of spermatogenic elements. The rate of degenerative changes in the testicular samples of the 2 transgenic boars at the age of 36 months was different. The changes were less pronounced in boar K104 than in boar K63 and resembled those of the TgHD boar at the age 24 months. Boar K63 also showed more change in sperm parameters, including atrophy of seminiferous epithelium and impaired spermatogenesis. The difference in severity of pathology between age-matched boars might be a consequence of variation in the progression of the disease and genetic background of individual minipigs. The age of onset of HD depends on CAG length (around 70%), but also on other factors like polymorphism, modifier genes, etc. (30%) [28, 29]. We suggest that polymorphisms of proteins interacting with huntingtin could contribute to different degrees of testicular degeneration between transgenic boars of the same age.

After demonstrating testicular abnormalities we intended to clarify the reason for the pathological phenotype. We focused on the design of the transgenic minipig model. The number of CAG repeats was chosen in order to expect an earlier phenotype. This number of repeats imitates juvenile HD in patients. It has an earlier onset and faster progress, but the disease has the same characteristics as the adult form. Mixed CAG/CAA repeats, instead of just CAG, were used to increase the stability of the insert. This has been used in several rodent models (YAC128, BACHD mice) that also showed the phenotype [30, 31]. Therefore, the design was not a problem. We also checked whether the insertion of the lentiviral construct did not interrupt any coding sequence in the pig genome. Since the result was negative, the question was whether the defect in the testes was due to the expression of mtHtt or as a result of changed levels of fertility-related hormones.

There is evidence that the expression of mutant Htt leads to selective cellular dysfunction and degeneration [32]. The most affected cells are neurons. However, we also provided data for spermatozoa degeneration and testicular dysfunction in a minipig model of HD. We showed a high and stable expression of endogenous Htt as well as the transgenic N-truncated mutant form of human Htt in spermatozoa as well as in spermatogenic and Sertoli cells, and also in the atrophic seminiferous tubules of TgHD testes. In addition, we detected fragments of mtHtt in spermatozoa as well as in testes. It has been published that smaller fragments of mtHtt cause cellular toxicity and induce apoptosis [33, 34]. Furthermore, mutant N-terminal Htt fragments were also detected in tissues from HD patients and mouse models in the presymptomatic stage, suggesting their role in the progression of HD [35–37]. These facts also support our statement that the N-terminal part of human mtHtt causes testicular pathology in transgenic minipig boars.

Although testicular degeneration in HD is well described in mouse models (R6/2 and YAC128), it is not clear whether this phenotype is independent or a consequence of alterations of the hypothalamic-pituitary-gonadal axis (GnRH). A significant loss of GnRH neurons starting from 5 weeks of age followed by decreasing levels of plasma testosterone at 12 weeks of age was found in R6/2 mice [11], while testicular atrophy without concomitant loss of GnRH neurons was described in a YAC128 mouse model [13]. An analysis of testosterone levels in YAC128 mice did not reveal any significant difference compared with controls, even when testicular atrophy was already present [13]. Furthermore, testosterone treatment had no effect on the peripheral phenotype of HD, e.g. body weight loss or motor function in R6/2 mice [11]. An analysis of complete neuroendocrine status in HD patients showed no significant difference in the plasma levels of LH, FSH and testosterone between all male HD patients and controls [38]. However, Markianos et al. [39] observed significantly lower testosterone and LH levels in HD patients compared with healthy controls. These conflicting results suggested a detailed hormonal analysis of our porcine model. We observed no significant difference in the levels of fertility-related hormones between TgHD and control boars, and no changes in libido were observed during regular collection of semen. Moreover, in the interstitium, the number, location and morphology of the Leydig cells were identical regardless of the age or genotype of the animal, and no differences were found in the lamina propria of seminiferous tubules of the TgHD boars in both ages compared with WT controls. Similarly, unaffected Leydig cells were observed between degenerating tubules of stromal interstitial tissue in YAC72/−
mice [14]. Our results support the idea that testicular degeneration and fertility defects are related to mtHtt expression in testes and not to peripheral hormonal changes.

In conclusion, we demonstrated a failure in sperm parameters and extensive testicular pathology in a minipig model of HD. We showed that insertion of the lentiviral construct did not interrupt any coding sequence in the pig genome and suggest that the testicular defect was caused by the presence of mtHtt and its fragmented cytotoxic form in testicular tissue, since hormonal changes were not measured between TgHD boars and their WT controls.

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Macakova et al.
Testicular Pathology in TgHD Minipigs

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