Huntington’s and myotonic dystrophy hESCs: down-regulated trinucleotide repeat instability and mismatch repair machinery expression upon differentiation

Anna Seriola1,†, Claudia Spits1,†, Jodie P. Simard3, Pierre Hilven1, Patrick Haentjens2,4, Christopher E. Pearson3,5,* and Karen Sermon1,*

1Research Group Reproduction and Genetics (REGE) and 2Laboratory for Experimental Surgery, VUB, Laarbeeklaan 101, Brussels, 1090, Belgium; 3Program of Genetics & Genome Biology, The Hospital for Sick Children, 101 College Street, Rm 15-312 East Tower, Toronto, ON, Canada M5G 1L7; 4Centre for Outcomes Research, UZ Brussel, Laarbeeklaan 101, Brussels, 1090, Belgium and 5Department of Molecular Genetics, University of Toronto, Toronto, ON, Canada M5G 1L7

Received August 24, 2010; Revised and Accepted October 6, 2010

INTRODUCTION

Various diseases are caused by the genetic expansion of trinucleotide repeats (TNR), including Huntington’s disease (HD [MIM 143100]) and myotonic dystrophy type I (DM1 [MIM 160900]). HD is caused by an expansion of a CAG repeat tract in the coding region of the Huntingtin gene (HTT) to more than 35–40 repeats, but rarely above 60 repeats (1). The expansion gives rise to a toxic-gain-of-function protein with an expanded polyglutamine tract, which subsequently leads to neural cell death. The severity of disease symptoms, age of onset and progression are related to the size of the CAG expansion. DM1 is caused by a CTG repeat expansion to at least 90 repeats in the 3′-untranslated region of the DMPK gene, which produces a toxic-gain-of-function CUG RNA. DM1 expansions are frequently expanded to thousands of units (1). For DM1, as for HD, the severity of disease symptoms, age of onset and progression are related to the size of the CTG expansion. Data from patients and model systems support a contribution of somatic instability at these disease loci to age of onset, disease severity and progression (1–3).

Although HD and DM1 are caused by dynamic mutations of complementary repeats CAG/CTG and thus share common characteristics, as noted above, there are also striking
differences. The degree of instability of the repeat depends on its size, on its genomic context and on the tissue studied (2–4).

Intergenerational instability occurs during gametogenesis when the repeat is over a certain threshold size and can change (often increasing in size), leading to the phenomenon of genetic anticipation (i.e. the aggravation of the symptoms and earlier age of onset from one generation to the next) (3,5–8).

In somatic tissues, the HD CAG repeat is stable, except for the brain, where the largest expansions are observed in the striatum (9,10). In contrast, the DM1 repeat presents high levels of somatic instability, with length differences as great as 5000 repeats between different tissues (11). In Huntington’s fetuses, no repeat instability can be detected between tissues (12), whereas in DM1 fetuses considerable levels of instability have been detected (13–15). While inter-tissue repeat length differences are limited in early fetuses, some instability has been detected. However, the precise timing of the onset of somatic instability is unclear (13–15). Repeat instability has been shown in one undifferentiated DM1 human embryonic stem cell (hESC) line (16) and one differentiated HD hESC line (17).

Various processes have been implicated in non-human model systems to contribute to CTG/CAG instability. DNA replication, repair as well as transcription are suggested to drive repeat instability (1,4,18,19). Proteins in the DNA mismatch repair (MMR) pathway are involved in TNR instability (1,20,21). The MMR machinery is composed of various interacting proteins, including MSH2, MSH3, MSH6, MLH1, PMS1, PMS2 and MLH3. The MSH2, MSH3 and PMS2 proteins have been shown to be involved in TNR expansion in transgenic DM1 and HD mice. MSH2 forms a heterodimer with MSH3, to direct the repair of insertion–deletion loops. MSH2 also forms a heterodimer with MSH6, to repair base substitutions and small insertion/deletion loops up to 12 nucleotides. MLH1 can form a heterodimer with either PMS1, PMS2 or MLH3, which interacts with MSH2-containing complexes bound to mismatches. The involvement of these proteins in TNR instability has been proven in numerous experiments. For instance, Msh2−/− and Msh3−/− HD or DM1 mouse models show stabilization or contractions of the expanded CTG/CAG repeat (22–26); PMS2 is known to be an enhancer of somatic mosaicism of trinucleotide CAG•CTG repeat (27). Importantly, the concentration of Msh3 in DM1 mice determines the level of CTG expansions (25). Curiously, in the absence of either Msh2 or Msh3, large expanded CTG repeats show 90% contractions, as opposed to the 90% expansion in the presence of these proteins (24,25,28). A mechanistic role of the human hMSH2–hMSH3 (the hMutSβ complex) in CTG/CAG instability has been revealed: short DNA slip-outs of 1–3 CTG units are repaired in a manner depending upon hMutSβ. At higher hMutSβ protein concentrations, the proper repair of slipped-CTG repeats is hampered and likely error-prone leading to expansions (21), similar to the MSH3 concentration sensitivity in DM1 mice (25).

Human cell models where MMR proteins were knocked down by siRNAs revealed a requirement of these proteins for the instability of integrated CTG tracts (29). However, there has been no demonstration of a natural down-regulation of MMR proteins with instability of an endogenous disease locus.

hESCs are pluripotent cells derived from surplus or discarded embryos after in vitro fertilization treatments. The cells possess the ability to differentiate into the different cell types present in the adult individual and are thus considered pluripotent (30). Next to their obvious potential in regenerative medicine, hESCs have also been proposed as a valuable tool to study early development, lineage commitment, tissue growth and maturation during embryonic development. They can also be used for the identification of biomarkers of disease and could be models to test the toxicity and efficacy of new drugs and chemicals (31,32). This is of particular interest in disorders such as DM1 and HD, for which the existing mouse and cell models do not fully represent the human pathogenesis or the culture of the relevant cell types is difficult.

The aims of this study were to characterize the behavior of the TNR in the DM1- and HD-derived hESC lines in undifferentiated and differentiated cells [osteogenic progenitor-like cells (OPL), neural progenitor cells and teratoma cells]. We also studied the expression of various MMR proteins and correlated this with the repeat instability.

RESULTS

**HD and DM1 repeats in the hESC lines**

The analysis of the CAG repeat in *HTT*, in passages 3, 12, 20, 30, 31, 41, 51, 58, 65, 75, 86, 97, 103 and 106 of VUB05_HD, showed a stable genotype of 23 and 44 repeats (Supplementary Material, Figs S1A and B).

On the other hand, the study of the size of the CTG expansion in *DMPK* in VUB19_DM1 and VUB24_DM1 showed an unstable genotype, presenting both expansions and contractions (Fig. 1). VUB19_DM1 carried an expansion of approximately 250 repeats at passage 1, which enlarged during cell culture, to give rise to a population of hESC carrying mostly small expanded CTG repeats at passage 54. VUB24_DM1 started with a much larger expansion of 1800 repeats, first measured at passage 5. Over time in culture, predominantly contractions of this repeat were detected. Wild-type alleles remained unchanged through the whole time period studied.

The results for VUB03_DM1 were previously published and are here summarized for the clarity of the other results in this work (16). At the initial passages, VUB03_DM1 carried a (CTG)470. Small-pool PCR of later passages (up to passage 120) showed an increasing number of alleles, with predominantly expansions going up to 2100 repeats (upper detection limit of the method).

Quantification of the relationship between repeat size and passage number using multiple regression analysis indicated that this relationship was statistically significant (*P* < 0.001), with a standardized regression coefficient of 0.91 for VUB19_DM1 (Fig. 1A) and of −0.499 for VUB24_DM1 (Fig. 1B). The sign of the standardized regression coefficient describes the direction of the relationship between repeat size (Y, dependent variable) and passage number (X, independent variable), i.e. repeat numbers in the higher passages are
significantly higher in VUB19_DM1 and lower in VUB24_DM1.

HD and DM1 repeats in differentiated cells

VUB03_DM1, VUB05_HD and VUB19_DM1 were differentiated into OPL (see Materials and Methods). This particular differentiation protocol was chosen because it yields a very homogeneous population of differentiated cells and is easily reproducible. DNA and RNA samples were collected at most passages, until the senescence of the OPL cells at passage 11.

VUB05_HD did not show any changes in repeat size during the OPL differentiation (Fig. 2A–C). On the other hand, VUB03_DM1 and VUB19_DM1 showed stabilization of the repeat. Figure 3 shows the results for the OPL-derived cells of VUB03_DM1 and VUB19_DM1. The undifferentiated hESCs were considered as passage 0, in which a large variety of alleles could be seen. Within two passages, most of the alleles were lost due to the clonal and focal growth of the culture during these first two passages. Morphologically, the culture becomes homogeneous at passage 3 (33), which coincides with the genetic homogenization. Through the subsequent passages, no significant repeat instability was detected.

Since we hypothesized that the HD repeat could be unstable in neural progenitors-derived from hESC, we tested by small-pool PCR the DNA obtained from a teratoma formed by VUB05_HD (which should contain a mix of cell types, including neural progenitors) in a SCID mouse and DNA from different passages of neural progenitor cells, obtained by directed in vitro differentiation of VUB05_HD. No change in repeat size was detected in any of the samples analyzed, at least for the time course assessed (Fig. 2D–I).

Gene expression of MMR

The semi-quantitative study of gene expression of the MMR genes hMSH2, hMSH3, hMSH6, hMLH1, hMLH3, hPMS1 and hPMS2 showed a statistically significant down-regulation of each of their transcripts upon differentiation into OPL cells (results for VUB03_DM1 in Fig. 4A and B, results for VUB05_HD were analogous and can be found in the Supplementary Material, Fig. S2). For VUB03_DM1, MMR expression was significantly different between undifferentiated and differentiated cells \( P < 0.001, \) one-way analysis of variance (ANOVA)]. Subsequent pair-wise comparisons indicated that expression in undifferentiated cells was significantly higher than the differentiated stages p1–p11 \( (P < 0.001, \) Bonferroni test). No significant differences were observed among the various stages post-differentiation. Similar findings were observed for VUB05_HD \( (P < 0.001) \).

These transcript levels suggest that MMR protein levels are also reduced. However, the protein levels of several MMR
Figure 2. Representative results of the small-pool PCR analysis of the HD repeat in VUB05_HD. The horizontal scale indicates fragment size in base pairs and the vertical scale shows fluorescent signal intensity. The arrows indicate the CAG alleles. (A–C) Results for the OPL cells obtained from VUB05_HD. (D–F) VUB05_HD derived neural cells. (G–I) DNA from a VUB05_HD derived teratoma.
factors, particularly the relative levels of hMSH2, hMSH3 and hMSH6, do not always reflect the level of the transcript, but are dependent upon protein–protein interactions (21,34). To this end, we assessed the levels of MMR proteins.

Mismatch repair protein expression

The undifferentiated control (VUB01) and DM1 (VUB03_DM1) hESC lines show similar levels of hMSH2, hMSH3 and hMSH6; comparable to the well-characterized MMR-proficient HeLa cell line (Fig. 4C), these expression changes are highly reproducible (Supplementary Material, Fig. S3). Protein levels did not vary with passage number of the undifferentiated hESCs (data not shown). Upon differentiation of the hESCs, the protein expression remained relatively unchanged at the first passage following differentiation (VUB03_DM1 P1). However, in later passages of the differentiated VUB03_DM1 (P3, P7 and P10), hMSH2, hMSH3 and hMSH6 proteins are not detectable (Fig. 4C; Supplementary Material, Fig. S3). This result is coincident with gene expression profiles obtained. The delay in protein loss, relative to transcript down-regulation, is likely due to the time for protein turnover. This analysis reveals that the levels of these MMR proteins do not vary with passage number of the undifferentiated hESCs, but dramatically drops upon differentiation.

The levels of hPMS1, hPMS2, hMLH1 and hMLH3 were also assessed by western blotting (Fig. 4B). The levels of hMLH3 were low in the hESCs, as previously noted for most cell lines and tissues (35,36), with higher levels in HeLa extracts (35). The levels of hPMS1, hMLH1 and hMLH3 did not vary in the control and DM1 undifferentiated hESCs and the earliest differentiated passage, P1. A subsequent reduction in hPMS1 and hMLH1 levels was observed in later differentiated passages (P3, P7 and P10). Curiously,
hMLH1 showed a triplet banding pattern in the post-differentiation passages, possibly due to degradation or post-translational modification. The level of the hPMS2 protein was somewhat higher in the control hESC lines compared with the DM1 hESC, and this level was mildly increased in the earliest and diminished in later post-differentiation passages. Together, these results suggest that the levels of many of the MMR genes are down-regulated at the transcriptional level and proteins are degraded to varying degrees.

**DISCUSSION**

In DM1 hESCs, the expanded DM1 CTG repeat is highly unstable from very early passages onward and is apparent both between different passages and between cells within the same colony. When comparing the instability results of VUB03_DM1 (16), with those presented herein for VUB19_DM1 and VUB24_DM1, it is apparent that, although all lines show some levels of instability from the beginning, at later passages VUB03_DM1 and VUB24_DM1 present a much broader range of alleles than VUB19_DM1, with very large size changes. On the other hand, VUB19_DM1 shows a progressive increase in size for the most common allele in the cell culture, without such large and sudden changes. Furthermore, while VUB03_DM1 and VUB19_DM1 show a trend towards an increase in repeat size, VUB24_DM1 seems to present rather a tendency towards contractions. Possible factors influencing these differences include the differences in the size of the original expansion, the different ‘parental origins’ of the lines (the affected donor of the embryos from which these lines were derived were female in the case of VUB03_DM1 and VUB24_DM1, and male in the case of VUB19_DM1), or the genetic backgrounds of each line. Regarding the tendency for DM1 CTG contractions, rare large transmitted contractions have been observed in some DM1 families particularly when large alleles are paternally transmitted (37), which contrasts with the very large expansions that often occur by maternal DM1 transmission.

Since hESCs are derived from preimplantation embryos, which have been shown to have a stable CTG repeat (7,8), one would expect the TNR to be stable in hESC. The results presented here suggest that hESCs do not always behave...
one explanation for these counter-intuitive findings is that the in vitro culture could be influencing the stability of an already expanded repeat. Repeat instability has been observed in other in vitro cultures of diverse DM1 cell types such as fetal tissues (14,38) and lymphoblastoid cell lines (39,40). In our DM1 hESC lines, we observed the same two types of mutations as previously described (38,39), namely frequent small changes of the repeat size and more rare large changes, although the frequency of these changes differed from line to line.

Another explanation could be that this unstable behavior is not culture related, but typical for stem cells. For instance, it has been recently shown that the CGG repeat causing fragile X syndrome has a very particular behavior in a male hESC line carrying a large CGG expansion (41). The repeat is unstable and unmethylated in the undifferentiated state, and stabilizes and becomes methylated upon differentiation; consistent with experimental systems revealing a role of CpG methylation upon CCG instability (42). Moreover, in our work, the stem cells display a similar pattern of stabilization of the expansion upon differentiation. The analysis of the CTG repeat in the OPL cells derived from VUB03_DM1 and VUB19_DM1 showed that the repeat undergoes stabilization, when compared with the undifferentiated source hESCs. After a few passages, only a few allele sizes remain observable in the culture, and no new allele lengths tend to appear. The stabilization of repeat instability with the arrest of proliferation coincident with differentiation may indicate a link to cell growth (4) or variations of trans-factors.

Since several publications point towards the involvement of the MMR proteins Msh2, Msh3 and Pms2 in the instability of large CTG/CAG tracts in transgenic mice (22–28,43,44), we tested the evolution of the relative gene expression of these genes in the different hESC passages. We found that the cultured hESCs expressed many of the MMR proteins, as previously reported by large-scale expression microarray gene expression in hESC (45). We show that upon differentiation of control-, DM1- and HD-derived hESCs, the cells quickly lose expression of MMR genes. Interestingly, this reduced expression is concomitant with the stabilization of the DM1 repeat. At the protein level, the reduction was most dramatic for the hMS2, hMsh3 and hMSH6 proteins, the former two which are known to dramatically contribute to CTG/CAG instability in transgenic mice. The down-regulation of MMR protein expression and the coincident loss of high levels of CTG instability present in the DM1 undifferentiated hESCs is consistent with the current model that indicates a requirement of MMR proteins for the instability of large CTG/CAG tracts (24–27,43,44,46). Direct evidence supports that altered TNR instability can be induced by variations in the levels of DNA repair proteins (18,19). Our observation is particularly relevant as it is the first time a correlation between altered repeat instability is seen in cells undergoing a pseudo-natural biologically induced reduction in MMR expression.

In contrast to the results for the DM1-derived hESC lines, the HD repeat of VUB05_HD proved very stable in all studied conditions. This finding is similar to the in vivo behavior of the HD repeat, where only germ cells and brain tissue present limited levels of instability (10,47). Higher levels of somatic instability arise in various tissues of DM1 patients, but not in HD patients. In cultured DM1 cells (14,38–40) high levels of instability was observed as opposed to limited changes in HD cells (46,49). Two explanations for this difference have been put forward: the HD CAG/CTG expansion is considerably shorter than the DM1 CTG/CAG expansion making it less prone to mutation. Secondly, the DM1 locus may harbor a stronger cis-element that drives its instability compared with the HD locus (4). Data favoring this is the much higher levels of instability observed in HD transgenic mice that are likely due to the larger CAG expansions in the transgenes relative to those present in HD patients (180–225 repeats compared with <80 repeats) (48). Various attempts have failed to observe high levels of CAG instability in patient-derived HD cells (49) and only limited instability was observed in cells derived from transgenic HD mice with >155 repeats (46). The absence of instability of the (CAG)44 HD tract in our HD hESCs may be due to either the short expansion length, insufficient culture time and/or the absence of a strong cis-element. Recently, Niclis et al. (17) reported the same CAG stability in undifferentiated hESC cells carrying HD expansions of (CAG)37 or (CAG)51. On the other hand, they detected low-level instability in neurospheres derived from these hESC lines. In our case, it is probable that none of the cells we used as differentiated models was close enough to the in vivo cell type to mimic the somatic instability of this particular repeat. Another possibility is that the cells were not kept in culture long enough to mimic the age-related gains in length in HD neural tissue.

In summary, in this study we have shown that the expanded CTG/CAG tract is highly unstable in DM1- but not HD-derived hESCs. Differences exist between different hESC lines in the general behavior of the CTG expansion, some lines showing a preference for expansions whereas others show predominantly contractions. This trait may be variable and it cannot be excluded that other hESC lines carrying these mutations behave differently. In our hands, upon differentiation, the DM1 repeat looses instability, and this process occurs at the same time as a loss of expression of the MMR machinery. This is the first demonstration of the correlation between a biologically natural down-regulation of the MMR proteins and changes in the stability of an endogenous disease locus.

**MATERIALS AND METHODS**

**Human embryonic stem cell culture and differentiation**

For this study, we used the hESC lines VUB03_DM1, VUB19_DM1 and VUB24_DM1 carrying DM1, and VUB05_HD carrying HD, all of them derived in our laboratory. The hESC lines are registered at the European Human Embryonic Stem Cell Registry (www.hescreg.eu) and are available upon request. The hESCs were derived from human preimplantation embryos that were donated for research after in vitro fertilization and/or preimplantation genetic diagnosis treatments. All donor couples gave informed consent and the local ethical committee as well as the Belgian Federal Commission for Embryo Research approved the different studies involving the derivation and study of the
hESC. Derivation, culture, cryopreservation and characterization of their stemness properties were performed as described by Mateizel et al. (50). Details on the culture conditions can be found in the Supplementary experimental procedures.

Teratomas were obtained by injection of undifferentiated hESC into the rear leg muscle of SCID-beige mice (50). During teratoma formation, the hESCs differentiate into tissues of the three germ cell layers: endoderm, ectoderm and mesoderm. The presence of these three layers was investigated in the teratomas to prove the pluripotency of the hESC lines used in this work. DNA was extracted from the teratoma formed by VUB05_HD to study the behavior of the HD repeat.

The hESC lines VUB03_DM1, VUB05_HD and VUB19_DM1 were differentiated into OPL (33). Briefly, the hESCs were collected using collagenase type IV (Invitrogen, Carlsbad, CA, USA), and the cell clumps re-suspended in differentiation medium and plated on 0.1% gelatin-coated plastic dishes (passage 1). The differentiation medium contained Knockout Dulbecco’s modified Eagle’s medium, 2 mmol/l L-glutamine, 1% non-essential amino acids, 0.1 mmol/l β-mercaptoethanol and 20% heat-inactivated fetal calf serum (Gibco, Invitrogen). The differentiation medium was changed every 2 days, and after 24 days in culture, the cells were passaged and maintained in the same conditions. When possible, during passaging, a sample was collected for DNA and for RNA extraction.

DNA and RNA samples from neural progenitors derived from VUB05_HD were kindly provided to us by the group of M. Peschanski, I-stem, Evry, France (51).

Analysis of TNR instability

Details on all PCR and detection procedures can be found in the Supplementary experimental procedures. The amplification of the CTG repeat in DMPK was performed as previously described (16). Passages 1 and 3 of the hESC line VUB19_DM1 were studied using fragments of colonies. From passage 4 of VUB19_DM1 onwards, and for all samples of VUB24_DM1, as well as for all samples from differentiated cells, analysis was done by small-pool PCR on 200 pg of DNA (between 4 and 27 replicates). The number of CAG repeats in the HTT gene was studied in all DNA samples by small-pool PCR on 200 pg of DNA (between 4 and 27 replicates) using the primer set and PCR conditions described by Sermon et al. (52).

Fragment size of the wild-type allele of the CTG in DMPK and of both alleles of the CAG repeat in HTT was established using an ABI PRISM 3100—Avant Genetic analyser (Applied Biosystems, Life Technologies, Carlsbad, CA, USA). The expanded CAG repeats in DMPK were detected by Southern blot using a (CAG)5 probe.

Gene transcript expression analysis by real-time PCR

Real-time PCR (RT-PCR) was used to quantify the mRNA levels of hMSH2, hMSH3, hMSH6, hPMS1, hPMS2, hMLH3 and hMLH1 in undifferentiated and differentiated cultures from VUB03_DM1 and VUB05_HD. The RT-PCR reactions were performed on RNA obtained from undifferentiated hESCs and OPLs. Cells were collected using collagenase type IV or trypsin-EDTA (Gibco, Invitrogen), and RNA was extracted using the RNeasy micro or RNeasy mini kits from Qiagen, including a DNAse treatment and according to the instructions of the manufacturer (Qiagen, Venlo, The Netherlands). cDNA was synthesized using the first-strand cDNA synthesis kit, following the protocol provided by the manufacturer (GE Healthcare, Uppsala, Sweden).

RT-PCR reactions were performed in a final volume of 25 μl containing 1× TaqMan Universal PCR Master Mix (Life Technologies), 900 nM of the forward and reverse primers (Eurogentec, Seraing, Belgium) and 250 nM of the probe (Applied Biosystems, Life Technologies), or a TaqMan Assay (Applied Biosystems, Life Technologies). Sequences and assay numbers can be found in the Supplementary information. RT-PCR was performed on the Sequence Detector System ABI-Prism 7500 (Applied Biosystems, Life Technologies) under the following conditions: 2 min at 50°C and 10 min at 95°C, followed by 40 cycles of 15 s of denaturation and 1 min of annealing/extension at 60°C. For each test, two endogenous controls were used (GAPDH and UBC) and mouse cDNA samples and no-template control were taken along.

Data analysis was done using the 7500 System SDS Software (Applied Biosystems, Life Technologies). Relative quantification of gene expression was achieved by normalization against the endogenous control GAPDH or UBC using the △△Ct method. Fold changes were calculated as 2−△△Ct.

Protein expression analysis by western blotting

Western blot for hMSH2, hMSH3 and hMSH6 was performed as follows. Thirty micrograms of cell lysate and multiple dilutions of hMutSc and hMutSβ were separated at 170 V on an 8% SDS–PAGE. Proteins were transferred to a PVDF membrane at 300 mAmp. The membranes were blocked and then simultaneously probed overnight at 4°C in primary antibodies anti-MSH3 (BD Transduction Laboratories, San Jose, CA, USA, cat. no. 611390), anti-MSH2 (Calbiochem, Merck KGaA, Darmstadt, Germany, cat. no. CA8000-852), anti-MSH6 (BD Transduction Laboratories, cat. no. 610919) and anti-actin (BD Transduction Laboratories, cat. no. 612656). Immunoblots were washed briefly and incubated in HRP-conjugated sheep anti-mouse secondary antibody. Chemiluminescent signals were generated using GE Healthcare ECLTM Western Blotting Detection Reagent (GE Healthcare). Images were captured on Denville HyBlot film (Denville Scientific Inc., NJ, USA) with multiple exposures.

For hPMS1, hPMS2, hMLH1 and hMLH3, 30 μg of each cell lysate was separated and transferred as above, membranes were probed independently with anti-hPMS1 (Santa Cruz, Santa Cruz Biotechnology, Inc., Santa Cruz, CA, USA, cat. no. SC-615), anti-hPMS2 (BD Transduction Laboratories, cat. no. 556415), anti-hMLH1 (BD Transduction Laboratories, cat. no. 554073) and anti-hMLH3 (Santa Cruz, cat. no. SC-25313). Membranes were washed briefly and incubated in HRP-conjugated secondary antibodies. Chemiluminescent signals were generated using GE Healthcare ECLTM Western Blotting Detection Reagent. Images were captured on Denville HyBlot film with multiple exposures.
Statistical analysis
A multiple regression analysis was conducted to explore the potential relationship between passage number (X, independent variable) and repeat size in the DMPK gene (Y, dependent variable). The magnitude of the strength of this relationship was quantified by the standardized regression coefficient (labeled Beta). Tests were two-tailed, and a P-value of less than 0.05 was considered to indicate statistical significance.

In the case of the gene expression study, one-way ANOVA was used to test the null hypothesis that the mean relative quantification values are the same for all categories of samples, i.e. the differentiated samples labeled p1 to p11 and the undifferentiated sample. The Bonferroni test procedure was used for subsequent multiple comparisons. One-way ANOVA and Bonferroni testing were conducted, in turn, for VUB05_HD and VUB03_DM1.

WEB RESOURCES

AUTHORS’ CONTRIBUTIONS
A.S. and C.S. contributed to the design of the study. A.S. cultured and differentiated the cells and C.S. performed and interpreted molecular analysis. Both A.S. and C.S. interpreted the data and co-wrote the manuscript.

J.P.S. performed all western-blot analysis.
P.Hi. carried out all Southern-blot analysis.
P.Ha. was responsible for the statistical analysis of the data.
C.E.P. contributed to the design of the study and to the writing of the manuscript.
K.S. contributed to the design of the study and to the writing of the manuscript.

SUPPLEMENTARY MATERIAL
Supplementary Material is available at HMG online.

ACKNOWLEDGEMENTS
The authors acknowledge their colleagues from the human embryonic stem cell laboratory for the derivation and culture of the cell lines, and their colleagues from the Centers for Medical Genetics and Reproductive Medicine for the fruitful collaboration. We thank the group of M. Peschanski, I-stem (Evry, France) for kindly providing the VUB05_HD derived neural progenitor samples.

FUNDING
This work was supported by the Fund for Scientific Research Flanders [Fonds voor Wetenschappelijk Onderzoek (FWO) Vlaanderen], the STEM-HD (STREP EU FP6 program) and the Canadian Institutes of Health Research (to C.E.P.), the Muscular Dystrophy Canada (to C.E.P.). C.S. is a postdoctoral fellow at the FWO Vlaanderen and A.S. was funded by STEM-HD and the Aeren grant at the Vrije Universiteit Brussel.

REFERENCES


