Hsa-miR-34b is a plasma-stable microRNA that is elevated in pre-manifest Huntington’s disease

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Huntington’s disease (HD) is a devastating, neurodegenerative condition, which lacks effective treatment. Normal Huntingtin (HTT) and mutant Huntingtin (mHTT) are expressed in multiple tissues and can alter transcription of microRNAs (miRs). Importantly, miRs are present in a bio-stable form in human peripheral blood plasma and have recently been shown to be useful biomarkers in other diseases. We therefore sought to identify potential miR biomarkers of HD that are present in, and have functional consequences for, neuronal and non-neuronal tissues. In a cell line over-expressing mHTT-Exon-1, miR microarray analysis was used to identify candidate miRs. We then examined their presence and bio-stability in control and HD plasma. We found that miR-34b is significantly elevated in response to mHTT-Exon-1, and its blockade alters the toxicity of mHTT-Exon-1 in vitro. We also show that miR-34b is detectable in plasma from small input volumes and is insensitive to freeze-thaw-induced RNA degradation. Interestingly, miR-34b is significantly elevated in plasma from HD gene carriers prior to symptom onset. This is the first study suggesting that plasma miRs might be used as biomarkers for HD.

INTRODUCTION

Huntington’s disease (HD) is an autosomal dominant neurodegenerative disorder characterized by choreiform movements, personality changes, dementia and weight loss. It is caused by a CAG repeat expansion in the gene encoding Huntingtin (HTT) (1) and currently lacks effective treatment (2,3). Neuronal death is extensive, particularly in the striatum and cerebral cortex. Degenerative changes and cell death also occur in other brain regions and outside the central nervous system (4,5). The age at onset of motor symptoms is typically in mid-life, and non-motor symptoms may appear earlier (6). Although the age of onset is inversely related to the length of the CAG repeat, the correlation is not absolute, indicating that other factors also affect the age of symptomatic onset. Furthermore, progression of symptoms varies significantly between patients (7). Therefore, it would be valuable to have biomarkers that reflect disease mechanisms and predict symptom onset and progression (2). Plasma markers that track with disease progression would be particularly valuable in clinical treatment trials.

The normal function of HTT protein is poorly understood. It is expressed in multiple tissues (4,8) and interacts with many other proteins (9–11). Mutant HTT (mHTT Protein) promotes death of cortical and striatal neurons in several cell culture paradigms (12,13), by altering gene transcription and translation (8). Intriguingly, recent studies demonstrate the toxicity of mHTT is cell type-dependent. mHTT does not compromise the viability of pluripotent, undifferentiated human embryonic stem cell (hESC) lines (14) and induced pluripotent stem cell (iPSC) lines (15).

MicroRNAs (miRs) are transcribed from a diverse and expanding gene population encoding small, single-stranded RNA molecules that mediate the post-transcriptional regulation of gene expression (16). Recent studies demonstrate that miRs are present in normal human plasma and are...
our knowledge, no study has examined whether plasma-stable miR levels correlate with symptoms or signs of HD. Clearly, the measurement of bio-stable miR levels in plasma derived from HD patients is pertinent to the development of much-needed biomarkers.

Here, we present evidence that hsa-miR-34b (miR-34b), a P53-regulated miR (21), is up-regulated in response to mHTT in both pluripotent and neuronally differentiated human cells and in human plasma. We show that miR-34b is significantly elevated in plasma from HD gene carriers already prior to symptom onset. Its detection by Taqman quantitative polymerase chain reaction (qPCR) assays is linear and robust, and is comparatively insensitive to RNA degradation. We also report that miR-34b levels may influence mHTT cytoplasmic distribution and toxicity in vitro. Our study is the first to show that plasma miRs are potential biomarkers for HD.

RESULTS

NT2 cells are a viable model to study mHTT-Exon-1 activity

We first developed a human, cell-based model to identify mHTT-induced miRs that may represent potential biomarker candidates. Previous studies have used mHTT-Exon-1 expression to model the transcriptional dysregulation observed in response to mHTT (22). Based on previous studies (23,24), we selected NT2 cells, an embryonal carcinoma-derived, pluripotent cell line capable of directed, neuronal differentiation (25,26) to identify mHTT-induced miRs. We performed transient transfection of mHTT-Exon-1 constructs [23 polyglutamine repeat-bearing mHTT-Exon-1 (23Q-HTT), 73Q-HTT and 145Q-HTT] (Supplementary Material, Fig. S1A), followed by G418 selection of undifferentiated NT2 cells (Supplementary Material, Fig. S1B). We observed strong and comparable expression of ‘mHTT-Exon-1’ constructs and green fluorescent protein (GFP), at the level of transcription (Fig. 1A, Supplementary Material, Fig. S2A–C).

To control for the possibility that mHTT-Exon-1 over-expression altered nominal gene expression in undifferentiated NT2 cells, we evaluated messenger ribonucleic acid (mRNA) levels of OCT4 and NANOG. mRNA levels of OCT4 and NANOG did not alter in response to 145Q-HTT construct transfection (Fig. 1B) consistent with observations in hESCs and hiPSCs (14,15). In contrast, 145Q-HTT significantly reduced the low mRNA levels of dopamine receptor D2 (DRD2) (Fig. 1C, P < 0.05 relative to 23Q-HTT, n = 4 replicates: threshold cycles in 23Q-HTT cells, mean ± standard deviation: ACTB, 16.7 ± 0.4, brain-derived neurotrophic factor (BDNF) 26.7 ± 0.2, DRD2 34.5 ± 0.5). At the level of translation, we detected robust immunoreactivity for IC2 (mHTT-associated polyglutamine expansion antibody) in 23Q-HTT (Fig. 1D and E), 73Q-HTT (data not shown) and 145Q-HTT (Fig. 1G and H) transfected cells in addition to GFP expression (Fig. 1F and I). IC2 immuno reactivity was not observed in non-transfected NT2 cells (data not shown). The pattern of polyglutamine immuno reactivity observed using the IC2 antibody was qualitatively similar to that observed using the monoclonal antibody MAB5942/2B4, which targets mHTT-Exon-1.

mHTT-Exon-1-transfected NT2 cells readily differentiate into neurons

The transcriptional targets of mHTT in pluripotent cells are poorly understood. However, mHTT-Exon-1 has been shown to modulate neuron-enriched transcripts (8). Therefore, we created stably transfected NT2 cells and differentiated them into mature neurons with the addition of retinoic acid (RA) (26) (Supplementary Material, Fig. S1D). Importantly, the mRNA levels for GFP (Fig. 2A) and mHTT-Exon-1 (Fig. 2B) remained stable during RA-induced differentiation. Strikingly, 145Q-HTT transfection significantly attenuated the increase in neurogenic differentiation 1 (NEUROD) (Fig. 2E), microtubule-associated protein tau (MAPT) (Fig. 2F), BDNF (Fig. 2G) and DRD2 (Fig. 2H) mRNA transcript levels during neuronal differentiation (P < 0.05, n = 6 replicates). Furthermore, 145Q-HTT transfection delayed the down-regulation of pluripotency markers OCT4 (Fig. 2C) and NANOG (Fig. 2D). Thus, the application of mHTT-Exon-1-transfected NT2 cells as a model of mHTT-induced transcription is supported by IC2-positive, nuclear immuno staining and the reduction in pluripotent and neuron-specific transcript levels, including known mHTT targets (BDNF and DRD2).

miR-34b and miR-1285 are up-regulated by mHTT in vitro

We next applied this cell-based model to identify novel, mHTT-induced miRs that could be potentially detectable in plasma. We performed a miR microarray analysis of mHTT-Exon-1 transfected, undifferentiated NT2 cells. Surprisingly, few miRs were significantly elevated in response to 145Q-HTT and 73Q-HTT transfection, relative to 23Q-HTT transfection (Fig. 3A). Indeed, only two annotated miRs (miR-34b, miR-1285) and one predicted miR (hsa-miRPLUS-F1024) exhibited significant up-regulation with a fold change of ≥1.2 relative to 23Q-HTT (Table 1, Fig. 3B and Supplementary Material, Fig. S3). We next examined the expression and bio-stability of miR-34b and miR-1285 mature transcript levels in HD patient plasma, using hsa-miR-16 as an internal normalization control. Levels of mature miR-34b and miR-1285 were detectable over a plasma input range of 10–250 μl for both miRs with comparable efficiency to miR-16 (Fig. 3C). Thereafter, we examined the bio-stability of miR-34b and miR-1285 in response to sequential rounds of plasma freeze–thaw. Importantly, we observed no significant change in miR-34b or miR-1285 transcript levels relative to miR-16 (Fig. 3D). miR-34b and miR-1285, therefore, represent ideal potential biomarkers for plasma detection, as they are bio-stable relative to protein-based markers and their detection is linear for even low amounts of input plasma.
miR-34b is elevated in pre-manifest HD plasma and in mHTT-expressing, NT2-derived neurons

We next measured miR-34b or miR-1285 in age-matched controls and in HD patient plasma samples (Table 2). Most interestingly, levels of miR-34b were significantly elevated in pre-manifest HD patient plasma ($P < 0.05$, $n = 11$ samples) relative to age-matched controls ($n = 12$ samples) (Fig. 4A). In contrast, miR-1285 plasma levels were not statistically significant in pre-manifest samples compared with age-matched controls ($P = 0.48$). To explore the relevance of miR-34b changes in neuronal populations, we next examined the expression of miR-34b in differentiating NT2 cells (Fig. 4B). Consistent with the plasma and microarray observations, miR-34b levels were significantly higher in differentiating, 145Q-HTT-transfected, NT2-derived neurons relative to 23Q-HTT-transfected cells ($P < 0.05$, $n = 4$ replicates). These results suggest that miR-34b constitutes a novel potential biomarker of HD that is induced by mHTT and is detectable before the onset of clinical symptoms. Clearly, this observation is based on a small patient cohort, and additional observations...
in larger patient numbers are required to establish the biomarker potential of miR-34b.

mHTT over-expression has pro-survival effects in undifferentiated NT2 cells

Previous studies have not explored the functional consequences of mHTT-Exon-1 over-expression in pluripotent cells. Therefore, we examined whether 145Q-HTT significantly altered survival or proliferation of undifferentiated NT2 cells. We used quantitative assays of cell survival (caspase-3/7 activity), cell proliferation [5'-bromo-2'-deoxyuridine (BrDU) incorporation] and pluripotency [alkaline phosphatase (AP) activity]. Surprisingly, we found that 145Q-HTT expression increased all these parameters. Thus, 145Q-HTT significantly reduced caspase-3/7 activity relative to 23Q-HTT-transfected cells (Fig. 5A, *P < 0.05, n = 6 replicates). 145Q-HTT-transfected, GFP+ cells also had significantly reduced immuno staining for caspase-3 (Fig. 5B, C and Supplementary Material, Fig. S4A, *P < 0.05, n = 6 replicates). 145Q-HTT transfection also significantly increased BrDU incorporation (Fig. 5F, *P < 0.05, n = 6 replicates) and AP levels (Fig. 5G, *P < 0.05, n = 6 replicates) relative to 23Q-HTT-transfected cultures. We observed a non-significant (Supplementary Material, Fig. S4B, P = 0.18) trend for a
similar difference with Ki67 immuno staining of 145Q-HTT-transfected cells (Supplementary Material, Fig. S4C–F). 73Q-HTT-transfected cell measurements for cell death, cell proliferation and AP levels were not significantly different from 23Q-HTT. This is an intriguing and novel finding that suggests mHTT expression can have a pro-survival effect in a pluripotent human cell line.

mHTT over-expression is toxic for NT2-derived neurons

In contrast to the results obtained in pluripotent cells (14,15), mHTT-Exon-1 expression is selectively cytotoxic to neurons (27). Therefore, we measured neuronal differentiation (MAPT, SMI312), caspase-3 immuno reactivity and phosphorylated neurofilament length (SMI312) as indicators of neuronal survival in NT2-derived neurons (Supplementary Material, Fig. S1D). 145Q-HTT-transfected, SMI312+ cells had greater caspase-3 immuno reactivity (Fig. 6A and H; $P < 0.05$, $n = 4$ replicates) and reduced axon length (Fig. 6B and I; $P < 0.05$, $n = 4$ replicates), relative to 23Q-HTT-transfected cells (Fig. 6F and G). Importantly, the relative numbers of 23Q-HTT- and 145Q-HTT-transfected, NT2-derived neurons (examined after 21 days of RA-induced differentiation) were not significantly different when examined by immuno staining.
Table 1. Candidate miRs elevated in response to mHTT over-expression in undifferentiated NT2 cells

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<th>145Q/73Q</th>
<th>23Q/145Q</th>
<th>F</th>
<th>P (raw)</th>
<th>P (B–H)</th>
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Pair-wise comparisons of 145Q-HTT up-regulated (>0.2-fold) miRs relative to 23Q-HTT and 73Q-HTT (Fig. 3A, n = 4 replicates per transfection). 23Q/145Q, fold change relative to 145Q-HTT. AveExpr, average signal intensity; F, F-statistic; P, P-value significance of difference between 145Q and 23Q as determined by one-way ANOVA. P-value adjusted for Benjamini–Hochberg correction for multiple testing (B–H). *) indicates miRs with a fold change (LogFC) >1.2 or a non-adjusted P-value P < 0.001. miRs in bold were selected for further analysis.

for axonal MAPT (Fig. 6C–E) and the phosphorylated neurofilament marker SMI312 (Fig. 6G and I). These findings indicate that, subsequent to directed differentiation, 145Q-HTT exerts toxic effects on survival and function in neurons derived from NT2 cells, in marked contrast to their effects on undifferentiated, pluripotent NT2 cells.
amplification is linear over a large range of plasma input volumes. To our knowledge, this is the first identification of a miR associated with the pre-clinical manifestation of HD that is detectable outside of the nervous system. It is anticipated that these observations will serve as a platform for further studies evaluating the role of miRNAs as potential biomarkers of neurodegenerative disease.

Recent studies have demonstrated the value of biomechanical tests of motor function as reliable biomarkers of HD progression (2,30). Molecular correlates of disease progression provide insight into potential therapeutic targets and dysregulation of gene expression in response to mHTT (31,32). This is especially true in HD, which is caused by mHTT, a protein capable of modulating gene transcription and translation in a systemic manner in different somatic cell populations (8). In HD patient-derived whole blood, Borovecki et al. (33) identified multiple miRNAs that were significantly elevated relative to control subjects. However, this finding has not been replicated (34). We concede that our findings are based on a small cohort of patients and it will be critical to evaluate the significance of this enrichment in larger patient samples and in longitudinal samples.

Neurons exhibit exquisite sensitivity to mHTT. We (4) and others (8) have also observed changes consistent with mHTT-induced cytotoxicity in several somatic cell types that may precede symptomatic neuronal dysfunction. Few studies have examined mHTT toxicity in pluripotent mouse and human cells. NT2 cells share key features of pluripotent hESCs and hiPSCs and their somatic derivatives that may precede symptomatic neuronal dysfunction. Few studies have examined mHTT toxicity in pluripotent mouse and human cells. NT2 cells share key features of pluripotent hESCs and hiPSCs and their somatic derivatives.

**DISCUSSION**

There is a strong need for bio-stable measures that change consistently with disease pathology in HD. Although the mutant HD gene constitutes the ultimate trait biomarker, attempts to find mRNA- and protein-based state biomarkers have not yet yielded desired results. Biomarkers that are observable in the pre-manifest period are especially important, as they coincide with a window of opportunity for disease-modifying interventions. Here, we have developed a novel human cell line-based assay to identify miR biomarkers that may be of functional significance in HD progression in both neuronal and non-neuronal cell populations. We show that miR-34b constitutes a plasma bio-stable marker that affords robust detection from volumes as low as 10 μL. Multiple RNAs can be isolated from a single plasma sample, and
in pluripotent cells. miR-34b is known to represses translation of MYC (42) and CAMP-responsive element binding (CREB) (29), among other putative targets (43,44). Interestingly, MYC promotes apoptosis and differentiation of hESCs, in marked contrast to mESCs (45). The present study has not addressed whether mHTT-induced, miR-34b up-regulation has different effects in neurons or other cell types. Clearly, NT2 cells and emerging mHTT-dominant-negative hESC or hiPSC cell lines and their differentiated progeny represent powerful technology platforms to identify novel miR-34b targets and address the context-specific effects of miR-34b in future studies.

We did not observe significant changes in any of the miRs previously associated with REST (46–48). REST interacts with distinct genes in pluripotent and somatic populations (49). It appears to be dispensable for ESC pluripotency or self-

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**Figure 4.** miR-34b is elevated in pre-manifest HD plasma and differentiating NT2 cells. Levels of miR-1285 (black bars) and miR-34b (white bars) were quantified from 100 µl of blinded HD clinical samples (pre-manifest HD, n = 11 samples; early HD, n = 8 samples; moderate HD, n = 8 samples) and age-matched controls (n = 12 samples). A significant increase in miR-34b was observed in pre-manifest HD samples (A). A similar up-regulation of miR-34b relative to day 0 miRNA levels was observed in (B) differentiating NT2 cells in vitro (Supplementary Material, Fig. S1D). 145Q-HTT-transfected cells (white bars) exhibited higher levels of miR-34b than 23Q-HTT transfected cells (black bars). *P < 0.05 relative to (A) control plasma samples and (B) undifferentiated, mHTT-Exon-1 transfected cells.

**Figure 5.** mHTT exerts a pro-survival effect on undifferentiated NT2 cells. 145Q-HTT transfection significantly reduced (A) caspase-3/7 activity and (D–E) caspase-3 immuno reactivity relative to (B and C) 23Q-HTT-transfected cells. Also, 145Q-HTT also associated with increased (F) BrDU incorporation and (G) AP protein levels relative to 23Q-HTT transfected cells. *P < 0.05 relative to 23Q-HTT-transfected cells. Scale bar, 25 µm.
renewal (50,51), although this is disputed (52). However, mHTT may alter expression of neuron-associated, REST-repressed miRs and transcripts that are de-repressed or activated by REST on neuronal differentiation (53). It is likely that the homogenous cellular assay used here has underestimated the diversity of miRs that might be elevated or decreased in human plasma. To identify such genes, next-generation sequencing of HD patient plasma and age-matched control samples might identify populations of plasma-enriched miRs in a quantitative manner, as has been previously described (17). These strategies would also allow for comparative analysis of mHTT-induced changes in pluripotent and differentiated NT2 cells at the level of transcription. We hope our study will provide impetus for these experiments and that the functional significance of miR changes can be further elucidated in both in vitro and in vivo models of HD.

MATERIALS AND METHODS

Human plasma samples

Blood samples were obtained from control subjects and genetically diagnosed HD subjects at National Hospital for Neurology and Neurosurgery, Queen Square, London, UK, and processed as previously described (54). The study population consisted of 12 control subjects, 11 pre-manifest HD subjects, 8 early and 8 moderate HD subjects (Table 2). The study was conducted in accordance with the Declaration of Helsinki and approved by the ION/NHNN ethical review board and the NHNN Research and Development Committee. Institutional Review Board (IRB) and consent permitted the use of samples for biomarker studies. The operator was unaware of the disease state of each sample during processing and statistical analysis. To examine miR linearity of amplification, we used samples from a single, known HD patient and isolated RNA from serial dilutions of plasma (10, 25, 50, 100 and 250 μl v/v RNAse-free H2O to a final volume of 250 μl). To examine miR bio-stability, a separate series of known HD patient plasma samples were pooled, aliquoted (100 μl/sample, n = 6 samples per freeze-thaw round) and subjected to multiple rounds (1–3) of freeze–thaw in dry ice (10 min) followed by incubation at room temperature (15 min). To examine blinded samples, 100 μl of patient and control plasma was used for miRNA extraction and reverse transcription/polymerase chain reaction.

mHTT-Exon-1 constructs

pCDNA3.1 constructs were obtained (Supplementary Material, Fig. S1A) that encode the first 90 amino acids (Exon1) of human HTT with short (23Q, CH0017), medium (73Q, CH0018) and long (145Q, CH0019) variant polyglutamine repeats (translated from a mixed codon where mix = [CAG, CAA, CAG, CAA, CAA]n) and Neomycin resistance (Fig. 1A) (Coriell, Camden, NJ, USA; www.coriell.org), both under the cytomegalovirus promoter. A separate

Figure 6. mHTT exerts a toxic effect on NT2-derived neurons. Stably transfected NT2 cells were differentiated in RA for 21 days and cellular survival and differentiation assayed by immuno staining and analysis of nuclear morphology. Relative to 23Q-HTT-transfected cells, 145Q-HTT increased the number of (A) SMI312 immuno-reactive cells co-stained for caspase-3 (F–I) relative to 23Q-HTT. 145Q-HTT transfection also significantly reduced the length of SMI312-positive neuronal processes (B) but did not alter the total number of neurons (C). This was also observed using MAPT immuno staining (C, D–E). *P < 0.05. Scale bar, 50 μm.
expression cassette encoding GFP downstream of the phosphoglycerate kinase promoter was added (courtesy of Dr T. Hjalt, Lund University). Plasmids were amplified in Escherichia coli strand in lysogeny broth with ampicillin overnight at 37°C with shaking. For isolation of plasmid, we used MaxiPrep Kit (Qiagen GmbH, Hilden, Germany).

Cell culture
All cell culture reagents were supplied by Invitrogen Ltd (Carlsbad, CA, USA) unless otherwise specified. The NTera2 (NT2, #CRL-1973) cell line was obtained from ATCC (c/o LGC Standards Office, Boras, Sweden; www.atcc.org). Cells were cultured in 75 cm² flasks (BD Biosciences, San Jose, CA, USA) in standard culture media (Dulbecco’s modified Eagle’s medium supplemented with 10% fetal bovine serum, 100 U penicillin, 100 ìg streptomycin and 1× minimal essential medium non-essential amino acids supplement) in 5% CO₂/O₂ at 37°C.

Cell transfection and nucleofection
Low-passage NT2 cells, with a minimum of three biological replicates derived from two temporally separate passages, were used for all studies unless otherwise stated. For transient transfection of undifferentiated NT2 cells, Lipofectamine2000™ transfection reagent was used to deliver plasmids (1.0 ìg/1 × 10⁶ cells) or oligonucleotide antagonists of miRs (Anti-miRs, Applied Biosystems, Foster City, CA, USA; 10 nm/1 × 10⁶ cells) in plasma-free media with 100% media change 30 min post-transfection. Twenty-four hours post-selection, G418 (1 ìg/ml) was applied and media were changed every other day for 6 days. Nucleofection of NT2 cells for differentiation was performed using the Cell Line Nucleofector Kit (Lonza AG, Basel, Switzerland) essentially according to the manufacturers protocol but using programme A01 instead of X01. One day after transfection, G418 (1.0 ìg/ml) was applied for 6 days. Afterwards, cells were harvested and re-plated on poly-l-lysine-coated (1 ìg/ml) 6-well or 96-well black-walled optical plates (Nunc™, ThermoFisher Scientific, Waltham, MA, USA) in standard culture media with 10 ìM RA (Sigma-Aldrich, St Louis, MO, USA) and 0.5 ìg/ml G418. Cells were cultivated for 3 weeks (7, 14 and 21 day time points) with media changed every other day.

Analysis of cell death, cell proliferation and AP activity
All assays were performed according to the manufacturer’s protocol using transfected/nucleofected cells post-G418 selection in 96-well black-walled optical plates in the standard culture medium. To normalize cell density prior to the assay, cell number was counted by haemocytometer and aliquoted at 2 × 10⁶ cells/well. Cell death was measured with the Apo-ONE Homogenous Caspase-3/7 Assay at 1, 6 and 18 h post-lysis (Promega Inc., Madison, WI, USA). Cell proliferation was measured using the BrDU incorporation assay (Millipore Inc., Billerica, MA, USA) after an 18 h BrDU (20 ìM) incubation period. Alkaline phosphatase (AP) activity
was measured using the Alkaline Phosphatase ES Characterization Kit (Millipore) after 1 day of culture. AP measurements were normalized to a standard curve based on supplied recombinant protein (data not shown). Fluorescence and absorbance measurements were made using a FluoroOPTIMA microplate reader (BMC Labtech, Germany).

RNA isolation

All molecular biology reagents were supplied by Invitrogen unless otherwise stated. To isolate RNA from cell lines, transfected/nucleofected NT2 cells were washed twice with phosphate-buffered saline (PBS) and harvested using 0.25% trypsin, inactivated with the standard culture medium and pelleted by centrifuging for 5 min at 600g. Cells were then lysed using TRIzol reagent. To isolate RNA from plasma, samples (10–250 µl, v/v H2O) were aliquoted on ice and lysed in 5 volumes (1.0 ml) of TRIzol. RNA isolation from TRIzol was performed essentially according to the manufacturer’s instructions but with 100% ethanol precipitation followed by column purification (miRNeasy RNA Purification Kit, Qiagen). On-column RNase-free DNaseI treatment (Qiagen) was performed according to the manufacturer’s protocols. Total RNA/miRNA was eluted in 25 µl ddH2O and stored at −80°C. For qPCR assays, RNA concentration was determined by the absorbance at 260 nm and the 260/280 nm and 260/230 nm ratios using Nandrop3300 (ThermoFisher Scientific). Typical RNA yields for plasma isolation were variable, but were in the range of 0.5–5 ng/µl.

mRNA reverse transcription and PCR

All PCRs were performed using a Bio-Rad Mini-Opticon 48-well real-time PCR machine in low-profile optical tubes (20 µl per reaction). SuperScript III reverse transcriptase was used with Oligo-dT primers and 1.0 µg of total RNA according to the manufacturer’s protocols in a volume of 25 µl. PCR (n = 3–4 replicates per sample) was performed using a 100× dilution of cDNA, 20 pmol primers and 2x Maxima® SYBR Green Master Mix (Fermentas GmbH, St Leon-Rot, Germany) in a reaction volume of 20 µl. mRNA amplification conditions were as follows: hold, 95°C; 5 min; 25–40 cycles (95°C for 20 s, 62°C 20 s, 72°C 30 s). Primer sequences (obtained from the Realtime PCR Primer Database, http://medgen.ugent.be/rprimerdb/; 55) were as follows (all written in 5’−3’ orientation with the length of the product in base pairs, bp): ACTB-F, AACGGACTTCTCTGAAACATGA, ACTB-R, CTGGAA CGTGAAAGGTGACA, 321 bp; BDNF-F, AGTGC CCAAC TACCACTGCTGA, BDNF-R, CCTATGAATGC CCACGCA AAAT, 75 bp; DRD2-F, AGCACCAC ACGCTGACTCT, DRD2-R, GGCGA TGCTGTGCTATCACA, 140 bp; GFP-F, AAATCCAAAGCGACACGAC, GFP-R, GTTGGTGTGTTGTA GTTGGC, 127 bp; HITE exon1-F, ACAGGC CGCTGCTGGCTCT, HITE exon1 R, CGCGT CTCAGGCCACA, 78 bp; IL6-F, GCC ACTGGCAGA ACAAACC, IL6-R, GCAATGCT CTATTGAC, 84 bp; NANO-G-F, AATACCTACAC CAGCTGAG, NANO-G-R, TGGGCTC CCTATTCTTCTT, 150 bp; NEUROD1-F, TCAATCATGAGCGGAT CATGA, NEUROD1-R, TGAAACCTGGCGTGC TTCTAA, 69 bp; OCT4-F, GAGGAAACCGAGTGAGGCACC, OCT4-R, CATAGTCGTCTGCTTGTGCTTG, 156 bp; MAPT-F, TTTGGTGGGTTGTAAGATATGC, MAPT-R, CCGAGGT GCCTGAAAGATG, 72 bp. The success of each reaction was deduced based on the observation of a single reaction product on an agarose gel and/or a single peak on the DNA melting temperature curve determined at the end of the reaction (Supplementary Material, Fig. S2). For some reactions, semi-qPCR was performed based on measurement of intensity of bands with ImageJ software after 2% agarose gel electrophoresis, with comparable results to fluorescent mRNA detection. All results are presented as percent of cytoskeletal Actin (ACTB primer set) expression before normalization to 23Q-HTT levels.

miR reverse transcription and PCR

For miR bio-stability and linearity assays, input total RNA was normalized to 5 ng for pooled HD patient plasma. For blinded samples, 5 µl of the RNA eluate was used without prior RNA normalization. For NT2 samples, input was normalized to 10 ng. Multiscribe Reverse Transcriptionase (Applied Biosystems) was used with inventoried miR-specific primers in a total volume of 7.5 µl according to the manufacturer’s protocol. PCR (n = 3 replicates per sample) was performed in a reaction volume of 20 µl using 2.0 µl cDNA, 1.0 µl inventoried Taqman primers for hsa-miR-16, hsa-miR-34b and hsa-miR-1285 (Applied Biosystems), 10 µl× NoAmpErase UNG Master Mix (Applied Biosystems) and 7.0 µl of RNase-free H2O. miR amplification conditions were as follows: hold, 95°C, 5 min; 50 cycles (95°C for 15 s, 60°C 60 s). For SYBR green and Taqman miRNA primers, PCR amplicon cut-off levels were established at 35 cycles. For Taqman miRNA amplicons from NT2 cells and human plasma samples, PCR amplicon cut-off levels were established at 45 cycles (excluding dilution assays, Fig. 3C). Negative RT samples did not exhibit amplicons at 35 and 45 cycles, respectively (data not shown).

Locked nucleic acid (LNA) microarray

Total RNA including the miRNA fraction from four replicate experiments was directly labelled (Cy3) and hybridized to miRCURY LNA miR v 11.0 arrays (Exiqon Microarray Services, Vedbaek, Denmark). Combined input RNA from all samples (Cy5) was measured using the Alkaline Phosphatase ES Characterization Kit (Millipore) after 1 day of culture. AP measurements were normalized to 10 ng. Multiscribe Reverse Transcriptionase (Applied Biosystems) was used with inventoried miR-specific primers in a total volume of 7.5 µl according to the manufacturer’s protocol. PCR (n = 3 replicates per sample) was performed in a reaction volume of 20 µl using 2.0 µl cDNA, 1.0 µl inventoried Taqman primers for hsa-miR-16, hsa-miR-34b and hsa-miR-1285 (Applied Biosystems), 10 µl× NoAmpErase UNG Master Mix (Applied Biosystems) and 7.0 µl of RNase-free H2O. miR amplification conditions were as follows: hold, 95°C, 5 min; 50 cycles (95°C for 15 s, 60°C 60 s). For SYBR green and Taqman miRNA primers, PCR amplicon cut-off levels were established at 35 cycles. For Taqman miRNA amplicons from NT2 cells and human plasma samples, PCR amplicon cut-off levels were established at 45 cycles (excluding dilution assays, Fig. 3C). Negative RT samples did not exhibit amplicons at 35 and 45 cycles, respectively (data not shown).

Immunocytochemistry

Cells were cultured in uncoated (undifferentiated NT2 cells) or poly-l-lysine-coated (differentiated NT2 cells) eight-well chamber slides (BD Biosciences), fixed overnight in 4% paraformaldehyde (Sigma) at 4°C and treated with 95% ethanol v/v PBS for 10 min at −20°C. After three washes with PBS, cells were blocked with 5% normal goat plasma (Sigma) and 0.05% Triton X-100 (Sigma) in PBS for 30 min and then incubated with primary antibody (see below) overnight at 4°C. After extensive washing, the cells were incubated with the
secondary antibody (Alexa Fluor 488 goat anti-rabbit IgG or anti-chicken IgY, Alexa Fluor 594 goat anti-mouse IgG or anti-rabbit IgG; Invitrogen) for 2 h at room temperature. Cells were washed and the nuclei counterstained with Hoechst33342 (Sigma). The following primary antibodies were used: anti-caspase-3 (mouse IgG, Cell Signalling Technology, Beverly, MA, USA, 9662, 1:1000), anti-cleaved-caspase-3 (Rabbit IgG, Cell Signalling Technology, 9661; 1:1000), anti-GFP (Chicken IgY, Invitrogen, A10262; 1:500), anti-HTT-Exon-1 [mouse IgG, Millipore, MAB5492/2B4 (recognizing N-terminus amino acids 1–82); 1: 500], anti-Ki67 (Rabbit IgG, Abcam, Cambridge, UK; 1: 1000), anti-polylglutamine (Mouse IgG, Clone 5TF1-1C2, MAB1574; Millipore, 1: 1000), anti-phosphorylated neurofilament (Mouse IgG2a, Covance, Emeryville, CA, USA, SM1312; 1:500) and anti-Tau (anti-MAPT) (Rabbit IgG, Sigma, T6402; 1:2000). Slides were analysed using laser scanning confocal (Carl Zeiss) or Nikon Eclipse 80i (Nikon, Tokyo, Japan) fluorescent microscopes with analysis on NIS-Elements software (Nikon) and ImageJ-64.

Statistical analysis
Analyses of microarray normalization and significance of relative changes in gene expression were performed with Bioconductor and the use of the Limma, marray, pvclust and statmod packages. Significance was determined by one-way analysis of variance (ANOVA) and Benjamini–Hochberg (B–H) correction. For cell culture experiments, all data are expressed at the mean ± standard error of the mean (SEM). Significance was assayed by unpaired Student’s t-test (α = 0.05) or one-way ANOVA (α = 0.05).

SUPPLEMENTARY MATERIAL
Supplementary Material is available at HMG online.

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Conflict of Interest statement. None declared.

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