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# siRNA-mediated inhibition of endogenous Huntington disease gene expression induces an aberrant configuration of the ER network in vitro

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**Abstract**

Huntingtin is a ubiquitously expressed cytoplasmic protein encoded by the Huntington disease (HD) gene, in which a CAG expansion induces an autosomal dominant progressive neurodegenerative disorder; however, its biological function has not been completely elucidated. Here, we report for the first time that short interfering RNA (siRNA)-mediated inhibition of endogenous Hdh (a mouse homologue of huntingtin) gene expression induced an aberrant configuration of the endoplasmic reticulum (ER) network in vitro. Studies using immunofluorescence microscopy with several ER markers revealed that the ER network appeared to be congregated in various types of cell lines transfected with siRNA directed against Hdh, but not with other siRNAs so far tested. Other subcellular organelles and structures, including the nucleus, Golgi apparatus, mitochondria, lysosomes, microtubules, actin cytoskeletons, cytoplasm, lipid rafts, and plasma membrane, exhibited normal configurations. Western blot analysis of cellular prion protein ($PrP^C$) revealed normal glycosylation, which is a simple marker of post-translational modification in the ER and Golgi compartments, and immunofluorescence microscopy detected no altered subcellular distribution of $PrP^C$ in the post-ER compartments. Further investigation is required to determine whether the distorted ER network, i.e., loss of the huntingtin function, participates in the development of HD.


*Keywords:* Huntingtin; Huntington disease; Mouse homologue of huntingtin; Short interfering RNA; Endoplasmic reticulum; Microtubules

Huntingtin [1] is a ubiquitously expressed 348-kDa cytoplasmic protein encoded by the Huntington disease (HD) gene. A CAG expansion in this gene induces an autosomal dominant progressive neurodegenerative disorder characterized by the appearance of progressive chorea and dementia, usually in adult life [2]. Since the cloning of the HD gene in 1993, most efforts directed toward understanding the pathogenic mechanism of this disease have focused on the analyses of the mutant huntingtin; the basis of these analyses is the gain-of-function hypothesis. However, the biological function of huntingtin has not been fully elucidated [3].

Recent studies have indicated that wild-type huntingtin is essential for embryonic development and normal function in adulthood [4]. Several independent studies using knockout mice that do not express a mouse homologue of the HD (Hdh) gene demonstrated that wild-type huntingtin plays a crucial role in embryogenesis because, in all cases, nullizygous mouse embryos died at approximately embryonic day E7.5 [5–7]. Alternatively, conditional inactivation of wild-type huntingtin resulted in neurological deficits and neurodegeneration, indicating the importance of the wild-type huntingtin function in adulthood [8].

Although a number of techniques, including an antisense method [9,10] and a catalytic DNA strategy [11], have been used to inhibit huntingtin expression, their efficiency is quite low. On the other hand, short interfering RNA (siRNA) has proven to be a much more powerful tool for

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suppressing the expression of genes of interest in various mammalian cells [12,13]. Indeed, several groups have reported the suppression of human HD transgene using siRNA [14–16]; however, these studies did not examine loss of endogenous Hdh function.

In order to collect information on its physiological function, we inhibited endogenous Hdh expression using siRNA and examined the morphology of subcellular organelles including the nucleus, endoplasmic reticulum (ER), Golgi apparatus, mitochondria, lysosomes, microtubules, actin cytoskeletons, cytoplasm, lipid rafts, and plasma membrane in various cultured cells. Consequently, the siRNA-mediated inhibition of endogenous Hdh expression induced an aberrant configuration of the ER network with no remarkable secretion deficit in vitro.

## Materials and methods

*Synthetic siRNA duplexes.* siRNAs against the Hdh gene were designed using the algorithm proposed by Naito et al. [17]. The sense strand sequences of newly synthesized siRNA were as follows: siHdh1, 5′-CCGUGUGAA UCAUUGUCUAAC-3′; siHdh2, 5′-GGAGCAUCGUGGAGCUUUU AG-3′; and control (non-silencing) siRNA, 5′-UUCUCCGAACCUGUCA CGUUU-3′. The *Photinus* luciferase (La2 [18]), green fluorescent protein (GFP, Qiagen, Hilden, Germany), amyloid precursor protein (APP, Ambion, Austin, TX, USA), and glyceraldehyde-3-phosphate dehydrogenase (GAPDH, Ambion, Austin, TX, USA) siRNAs were purchased and used. For preparation of siRNA duplexes, sense- and antisense-stranded oligonucleotides were mixed in an annealing buffer (30 μM Hepes, pH 7.4; 100 μM potassium acetate; and 2 μM magnesium acetate), heat denatured at 95 °C for 5 min, and annealed at 37 °C overnight.

*Cell culture and transfection.* Mouse neuroblastoma neuro2a (N2a) and human glioblastoma T98G cells were obtained from the American Tissue Culture Collection. N2a cells were grown and maintained at 37 °C in minimum essential medium (MEM) supplemented with 10% fetal bovine serum (FBS). T98G cells were grown and maintained at 37 °C in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% FBS. Synthetic siRNA duplexes (40 nM) were transfected into N2a cells by using jetSI (Qbiogene, Irvine, CA, USA) and into T98G cells by using Lipofectamine 2000 (Invitrogen, Carlsbad, CA, USA) reagents.

*Real-time PCR.* After the transfection of siRNA duplexes, total RNA was extracted with Trizol (Invitrogen, Carlsbad, CA, USA) and subjected to cDNA synthesis by using oligo(dT) primers and a SuperScript II reverse transcriptase (Invitrogen, Carlsbad, CA, USA). The resultant cDNAs were examined by real-time PCR using the ABI 7000 sequence detection system (Applied Biosystems, Foster City, CA, USA) with a SYBR Green PCR Master Mix (Applied Biosystems, Foster City, CA, USA) and a TaqMan Assay-on-Demand Gene Expression Product (Applied Biosystems, Foster City, CA, USA) for the mouse GAPDH gene that functioned as a control, according to the manufacturer's instructions. The PCR primers for detection of the Hdh transcript were as follows: Mm.Hdh-F, 5′-TTCTGCTTGAATTGTACAGC-3′ and Mm.Hdh-R, 5′-AATGAGG ATCTCATCTTCTG-3′. The expression level of the Hdh gene was normalized to that of the GAPDH gene.

*Antibodies and organelle markers.* Anti-huntingtin antibody MAB2166 was purchased from Chemicon (Temecula, CA, USA). As ER markers, anti-Calnexin (Stressgen, San Diego, CA, USA), anti-PDI antibody (Stressgen, San Diego, California, USA), ER-Tracker Blue-White DPX (Molecular Probes, Carlsbad, CA, USA), and pECFP-ER (Clontech, Mountain View, CA, USA) were purchased and used. Other organelle markers, including a nuclear marker, DAPI (Molecular Probes, Carlsbad, CA, USA); a Golgi marker, anti-GM130 antibody (BD Biosciences, San Jose, CA, USA); a mitochondrial marker, MitoTracker Red CMXROS (Molecular Probes, Carlsbad, CA, USA) and anti-porin antibody (Calbiochem, San Jose, CA, USA) ; a lysosomal marker, LysoTracker Red (Molecular Probes, Carlsbad, CA, USA); a marker for microtubules, anti-tubulin antibody DM1A (Sigma, Tokyo, Japan); a marker for actin cytoskeleton, rhodamine phalloidin (Molecular Probes, Carlsbad, CA, USA); a cytoplasmic marker, anti-Hsp70 antibody (BD Biosciences, San Jose, CA, USA); a marker for lipid rafts, anti-GM1 antibody (Chemicon, Temecula, CA, USA); a plasma membrane marker, FM 1-43FX (Molecular Probes, Carlsbad, CA, USA); and a microsome marker, anti-Erp57 antibody (Stressgen, San Diego, CA, USA) were also used for the experiments. Anti-prion protein (PrP) antibody SAF-32 was purchased from Cayman Chemical (Michigan, USA). Western blot analyses were performed as described [19].

*Immunofluorescence and fluorescence microscopy.* For indirect immunofluorescence analysis, cells with siRNAs were rinsed with phosphate-buffered saline (PBS) $Ca^{2+}$ and $Mg^{2+}$ (PBS(+)) and then fixed with 10% formalin in 70% PBS(+). After four washes with PBS(−), the fixed cells were incubated with 10 % FBS in PBS(−) at room temperature for 30 min. They were then incubated at room temperature for 1 h with primary antibodies at the desired concentration. After four washes with PBS(−), the cells were incubated with Alexa 488 Fluor-conjugated anti-mouse or anti-rabbit IgG (Molecular Probes, Carlsbad, CA, USA), diluted 1:100 in PBS, at room temperature for 2.5 h. The stained cells were washed four times with PBS(−) and mounted with SLOW FADE (Molecular Probes, Carlsbad, CA, USA). Immunofluorescent or autofluorescent samples were imaged using a DeltaVision microscopy system (Applied Precision, Washington, USA); out of focus light of the visualized images was eliminated by interactive deconvolution.

*Preparation of mitochondrial, microsome, and cytosolic fractions [20]* Cells were homogenized with 4 volumes of homogenize buffer (10 mM Hepes–KOH, pH 7.4, 250 mM sucrose, 1 mM EDTA, and 1 mM DTT) and centrifuged at 800*g* for 5 min at 4 °C, and the supernatant was further centrifuged at 8000*g* for 5 min at 4 °C. The supernatant was used as a post-mitochondrial supernatant. The resulted pellet was washed three times with homogenize buffer, resuspended in 4 volumes of the same buffer, and then centrifuged at 5000*g* for 10 min at 4 °C. The pellet was recovered and used as mitochondrial fraction. The post-mitochondrial supernatant was further centrifuged at 100,000*g* for 1 h at 4 °C, and the supernatant was used as cytosolic fraction, and the pellet was resuspended in homogenize buffer (microsome fraction). Western blots were performed at 3 μg of total protein/lane.

## Results

### *siHdhl and siHdh2 inhibit endogenous Hdh expression in vitro*

In vitro screening was used to identify an effective siRNA directed against endogenous Hdh mRNA. Two siRNAs (siHdh1 and siHdh2) reduced endogenous Hdh mRNA levels by 75% and 60%, respectively, in comparison with those in the control siRNA-treated mouse N2a cells (Fig. 1A). After the transfection with siHdh1 or siHdh2 in N2a cells, Western blot analysis with an anti-huntingtin antibody (MAB2166) revealed a significant reduction in endogenous Hdh expression (Fig. 1B), of which the subcellular localization was predominantly in a microsome fraction using a subcellular fractionation method (Fig. 1C).

### *siHdh1-mediated inhibition of endogenous Hdh expression induces an aberrant configuration of the ER network in vitro*

After siHdh1 was introduced into mouse N2a cells, immunofluorescence and fluorescence studies were performed.