Rescue of gene expression by modified REST decoy oligonucleotides in a cellular model of Huntington's disease

Article
Published Version

Open Access (Wiley Online Open)


It is advisable to refer to the publisher's version if you intend to cite from the work. See Guidance on citing.

To link to this article DOI: http://dx.doi.org/10.1111/j.1471-4159.2010.07122.x

Publisher: Wiley-Blackwell

All outputs in CentAUR are protected by Intellectual Property Rights law, including copyright law. Copyright and IPR is retained by the creators or other copyright holders. Terms and conditions for use of this material are defined in the End User Agreement.

www.reading.ac.uk/centaur
CentAUR
Central Archive at the University of Reading
Reading's research outputs online
Rescue of gene expression by modified REST decoy oligonucleotides in a cellular model of Huntington's disease

Chiara Soldati,* Angela Bithell,* Paola Conforti,† Elena Cattaneo† and Noel J. Buckley*

*Department of Neuroscience and Centre for the Cellular Basis of Behaviour, Institute of Psychiatry, King’s College London, The James Black Centre, London, UK
†Department of Pharmacological Sciences and Centre for Stem Cell Research, University of Milano, Milan, Italy

Abstract

Transcriptional dysfunction is a prominent hallmark of Huntington’s disease (HD). Several transcription factors have been implicated in the aetiology of HD progression and one of the most prominent is repressor element 1 (RE1) silencing transcription factor (REST). REST is a global repressor of neuronal gene expression and in the presence of mutant Huntington increased nuclear REST levels lead to elevated RE1 occupancy and a concomitant increase in target gene repression, including brain-derived neurotrophic factor. It is of great interest to devise strategies to reverse transcriptional dysregulation caused by increased nuclear REST and determine the consequences in HD. Thus far, such strategies have involved RNAi or mutant REST constructs. Decoys are double-stranded oligodeoxynucleotides corresponding to the DNA-binding element of a transcription factor and act to sequester it, thereby abrogating its transcriptional activity. Here, we report the use of a novel decoy strategy to rescue REST target gene expression in a cellular model of HD. We show that delivery of the decoy in cells expressing mutant Huntington leads to its specific interaction with REST, a reduction in REST occupancy of RE1s and rescue of target gene expression, including Bdnf. These data point to an alternative strategy for rebalancing the transcriptional dysregulation in HD.

Keywords: brain-derived neurotrophic factor, decoy oligonucleotides, histone modification, Huntington’s disease, repressor element 1 silencing transcription factor, transcriptional dysregulation.

(Huang et al. 1998), Sp1 (Dunah et al. 2002), p53 (Steffan et al. 2000; Bae et al. 2005), transcription initiation factor TFID 130 kDa subunit (Dunah et al. 2002) and TATA-binding protein (Huang et al. 1998) and these interactions are frequently disrupted in the presence of muHtt (Rubinsztein and Carmichael 2003). Recent work has highlighted repressor element 1 (RE1) silencing transcription factor (REST) (also known as neuron-restrictive silencing factor) as one transcription factor, whose function is disrupted in HD (Zuccato et al. 2003, 2007; Bithell et al. 2009; Johnson and Buckley 2009). REST was first identified as a regulator of neuronal genes in non-neuronal cells (Kraner et al. 1992; Mori et al. 1992; Schoenherr and Anderson 1995). Genome-wide chromatin immunoprecipitation (ChIP) and bioinformatic studies have subsequently revealed several thousand genomic REST binding loci (RE1 sites), which are linked to genes that encode non-neuronal as well as neuronal proteins (Bruce et al. 2004; Johnson et al. 2006, 2007, 2008a). Further, REST regulates target genes in both neural progenitors (Johnson et al. 2008a) and in mature neurons and glia (Palm et al. 1998; Abrajano et al. 2009a,b). Indeed REST dysfunction in neurons has been implicated in several neurological and neurodegenerative pathologies, including epileptic seizure (Palm et al. 1998; Garriga-Canut et al. 2006; Spencer et al. 2006), ischaemia (Calderone et al. 2003) and HD (Zuccato et al. 2003, 2007; Buckley et al. 2010).

Previous work has shown that wild-type Htt can sequester REST in the cytoplasm whereas in the presence of muHtt, this association is disrupted and leads to increased levels of nuclear REST resulting in increased REST occupancy of RE1 sites and repression of target genes (Zuccato et al. 2007). One key REST target gene that is repressed in the presence of muHtt is Bdnf. Brain-derived neurotrophic factor (BDNF) is supplied by cortical projections to the striatum where it is necessary for the maintenance and survival of medium spiny projection neurons, the most prominent neuronal population that dies during HD (Altar et al. 1997; Canals et al. 2004; Zala et al. 2005; Zuccato and Cattaneo 2009). Several studies have shown reduced BDNF levels in both animal and cellular models of HD. These studies indicate that a decrease in cortical BDNF occurs early in the disease process and is followed by a progressive decline, strongly implicating the role of this neurotrophin in HD pathology (Zuccato et al. 2001, 2007, 2008). REST occupancy of RE1 sites, including at the Bdnf promoter, is increased in HD models including Hdh109/109 knock-in and R6/2 mice (Zuccato et al. 2007). Importantly, in the case of Hdh109/109 mice, this increased occupancy precedes the development of a neuropathological phenotype (Zuccato et al. 2007). This suggests that dysregulation of REST target gene expression might be an early molecular event in HD and thus attenuating REST binding during early disease stages could be of therapeutic benefit.

Subsequently, we have shown that use of a dominant-negative REST construct, comprising only the eight zinc finger DNA-binding domain and lacking any co-repressor interaction domains, can rescue BDNF levels (mRNA and protein) in cellular models of HD (Zuccato et al. 2007). Although such mutant constructs are useful tools, they are large and require viral delivery for optimal results. An alternative strategy is deployment of decoy oligodeoxy nucleotides (ODNs) that act as surrogate binding sites for transcription factors and sequester the native transcription factor from its genomic binding sites. Decoy ODNs have been used successfully to inhibit the binding and transcriptional action of several transcription factors including nuclear factor-kappaB protein, Sp1, activator protein 1, signal transducer and activator of transcription 1/3 and nuclear factor of activated T-cells 1c (Griesenbach et al. 2002; Chae et al. 2006; Stadlbauer et al. 2008). Furthermore, this approach has been used to successfully deliver and attenuate transcription factor function in neural cells in culture (Steiger et al. 2004; Meller et al. 2005; Yao et al. 2005; Aharoni-Simon et al. 2006), in brain slice preparations and in vivo (Ye and Johnson 2001; Kubo et al. 2003; Dash et al. 2005). The main limitation of unmodified ODNs is their susceptibility to degradation by nucleases prevalent in sera and cells. To rectify this problem, ODNs with modified linkages, such as phosphorothioate and methylphosphonate, have been developed (Lee et al. 2003; Osako et al. 2007). Here, we describe the development of modified decoy ODNs to inhibit REST-mediated gene repression in a cellular model of HD and show efficient rescue of aberrant gene expression.

Methods

Hdh109/109 and Hdh7/7 cell culture and transfection
Hdh7/7 and Hdh109/109 cell lines were previously generated from the striata of wild-type and homozygous knock-in mice with each Htt allele bearing 7 or 109 CAG repeats, respectively, as described by Trettel et al. (2000). Both cell lines were propagated in Dulbecco's modified Eagle's medium supplemented with 10% foetal calf serum at 33°C in a humidified 5% CO2 atmosphere. Transfection with ICAFection (Eurogentec, Southampton, UK) was performed according to manufacturer’s instructions and optimal transfection efficiency (50–60%) was achieved using 0.5 μg of DNA/μL of ICAFection. Transfections were carried out in the presence of serum and medium was replaced after 12–24 h.

Construction of phosphorothioilated RE1 decoy ODN
Single stranded oligonucleotides were synthesised by Sigma Genosys (St. Louis, MO, USA). Annealing was performed in 10× annealing buffer (100 mM Tris-HCl, pH 8.0, 10 mM EDTA, 1 mM NaCl) by heating to at least 5–10°C above their Tm and cooling slowly to 4°C over 3–4 h using a heatblock. A RE1 decoy (++D) was designed corresponding to a canonical REST-binding site (Johnson et al. 2006). A negative decoy control (−−D) was generated using a sequence corresponding to a non-canonical RE1
that has been shown not to bind REST (Bruce et al. 2004). The decoy ODN sequences, including a phosphorothiolate modification on the first three nucleotides were: Positive decoy (+D): 5'-GPPC'PTPTTCAGCACCCAGGCAGC5'-; Negative decoy (−D): 5'-GPPC'PTPTTCCAGCACGTTGAGCAC-3'.

DNA binding and electromobility shift assay (EMSA) For gel shift analysis, 12.5 nM of infrared (IR700) fluorescence-labelled decoy (IR-Deoxy) was incubated with 0.25 μg HeLa cell nuclear extract in binding buffer (40 mM Tris-HCl, pH8, 0.4 mg/mL bovine serum albumin, 200 mM ZnCl2, 400 mM KCl, 2 mM MgCl2, 40% glycerol, 0.4% NP-40, Sigma, St. Louis, MO, USA) and 2 μM β-mercaptoethanol for 1 h at 4°C in the dark. For supershift experiments, 2 μL of anti-REST rabbit polyclonal (P-18X, 2 mg/mL; Santa Cruz Biotechnology, Santa Cruz, CA, USA) was added to the mixture. For excess negative decoy assays, 50 nM decoy binding site was added to the mixture. To test for competition, decoy ODN sequences, including a phosphorothiolate modification on the first three nucleotides were: Positive decoy (++D): 5'-GGATTGCCATGTCTGA-3'; Negative decoy (−D): 5'-GPPC'PTPTCCAGCACGTTGAGCAC-3'.

Real-time PCR and quantification of gene expression Polymerase chain reactions were run with the following condition: 95°C for 3 min, then 45 cycles × (30 s 95°C, 30 s 60°C, 30 s 72°C). Real-time PCR was performed using iQ SYBR Green Supermix (Bio-Rad Laboratories, Hercules, CA, USA) with 0.5 μM primers. Expression levels were analysed according to the Pfaffl method (Pfaffl 2002) and then normalised to levels of Gapdh.

Immunocytochemistry Cells were fixed for 10 min in 4% paraformaldehyde, permeabilised in 0.1% Triton X-100 (Sigma) for 5 min then incubated with anti-REST (1 : 200; Upstate Biotechnology, Lake Placid, NY, USA) for 1 h at 20–25°C in phosphate-buffered saline containing 10% normal goat serum. Secondary antibody incubation was performed for 30 min at 20–25°C with Cy5-conjugated rabbit anti-goat IgG (1 : 500; Jackson Labs, CA, USA). Nuclei were counter-stained with 4',6-diamidino-2-phenylindole (DAPI) and coverslips were mounted in Fluorount-G (Southern Biotech, AL, USA).

BDNF ELISA assays Cell lysates were prepared in lysis buffer consisting of 10% glycerol, 25 mM Tris HCl, pH 7.5, 150 mM NaCl, 1% Triton X-100, 5 mM EDTA and 1 mM EGTA supplemented with 1 : 100 Protease Inhibitor Mixture (Sigma-Aldrich, St Louis, MO, USA). Samples were homogenised, sonicated and centrifuged at maximum speed for 15 min at 4°C. The supernatants were collected and stored at −80°C. Samples were assayed for BDNF using the ImmunoAssay System (Promega) according to manufacturer’s instructions.

Chromatin immunoprecipitation Cells were fixed in 1% formaldehyde at 20–25°C for 5 min before washing in cold phosphate-buffered saline and centrifugation at 2200 g for 5 min. Cells were lysed in lysis buffer for 30 min on ice, cell nuclei pelleted and resuspended in shearing buffer before sonication in a Bioruptor (Diagenode, Belgium) to obtain an average chromatin shear size of 200–500 bp. All steps included protease inhibitor cocktail (Roche Molecular Biochemicals, Indianapolis, IN, USA). Pre-cleared chromatin (20 μg) was immunoprecipitated with 1–2 μg of antibody at 4°C for 3 h and 25 μL magnetic protein-G beads (Active Motif; Carlsbad, CA, USA) was added for 1 h at 4°C before magnetic capture. Following a series of washes, eluted chromatin was de-crosslinked, proteinase K-treated and ChIP DNA was purified using QIAquick PCR purification kit (Qiagen, Valencia, CA, USA). Eluted ChIP DNA was analysed using quantitative PCR (qPCR). An anti-REST rabbit polyclonal antibody (Millipore Corporation, Bedford, MA, USA) was used to precipitate chromatin. Non-specific binding was assessed by immunoprecipitation with non-immune rabbit IgG and enrichments were normalised to those at the Chrm4 coding region, a locus greater than 10 kb distal to any known RE1 site. RE1 flanking primer sequences were as follows: Bdnf RE1 [Fwd: 5'-GGATTTCGAGGGTGATGAG-3'; Rev: 5'-CGGAAAGAAGCCGTTTTAAGG-3'] (these primers are designed to amplify exon 2, present in all Bdnf transcripts); Syn1 RE1 (Fwd: 5'-GGACACTCAGAGGGAACAT-3'; Rev: 5'-CTCTTTCTTTGGCAGAC-3'); Chrm4 RE1 (Fwd: 5'-AGAGACTGTCGCGCATCTTC-3'; Rev: 5'-CGAGCACTCACCCTCTGC-3').
Trans-Blot system (Bio-Rad) before blocking in 1× Tris-buffered saline with 0.1% Tween-20 with 5% skimmed-milk for 1 h at 20–25°C. Membranes were then incubated with rabbit anti-REST (Millipore, 1 : 2000), mouse anti-actin (1 : 2000; Abcam, UK) or rabbit anti-RNA Polymerase II (PolIISS, 1 : 2000; Abcam) followed by goat anti-rabbit horseradish peroxidase (1 : 7500; Santa Cruz) or goat anti-mouse horseradish peroxidase (1 : 2000; Dako, Carpinteria, CA, USA) secondary antibodies. Chemiluminescence was performed using Lumi-Light Western Blot Substrate and Lumi-Film (Roche) developed using a CompactX4 X-ray developer. Membranes were stripped between detections using a Re-Blot Plus Kit (Chemicon) according to manufacturer’s instructions.

**Results**

Changes in gene and protein expression following decoy ODN delivery in Hdh109/109 cells

Hdh109/109 cells provide an ideal model system in which to test the effect of a decoy ODN on REST-mediated gene dysregulation since these cells have reduced Bdnf mRNA and protein levels compared with Hdh7/7 cells and show de-repression of Bdnf and other REST target genes following infection with a dominant-negative REST (DN : REST) adenovirus (Zuccato et al. 2007). Compared with wild-type Hdh7/7 cells, mutant Hdh109/109 cells displayed increased nuclear localisation of REST by both immunofluorescence (Fig. 1a) and nuclear and cytoplasmic fraction western blotting (Fig. 1b) as previously reported (Zuccato et al. 2003). To target REST function in this system, we designed a decoy ODN (++)D that contained a canonical RE1 sequence, which can be bound by the REST DNA-binding domain. We additionally designed a negative control decoy ODN (---D) carrying a non-canonical RE1 site that cannot be bound by REST (Bruce et al. 2004). Both sense and antisense oligonucleotides were synthesised with phosphorothioate bases at their 5’-ends to protect against exonuclease action. To demonstrate the efficacy and specificity of the REST decoy ODN, we investigated the expression of three REST target genes: Bdnf, Chrm4 and Syn1 together with β-actin. Bdnf, Chrm4 and Syn1 have all been shown to be aberrantly repressed by REST in Hdh109/109 cells (Zuccato et al. 2007), whereas β-actin is neither bound nor regulated by REST.

Hdh109/109 cells were transfected with the REST decoy ODN (++)D or non-canonical decoy (---D) and expression of Bdnf, Syn1, Chrm4 and Rest was analysed by qPCR in untransfected Hdh7/7 and Hdh109/109 cells and in Hdh109/109 cells transfected with decoy ODNs 48 h following transfection (Fig. 1c). Bdnf, Syn1 and Chrm4 all showed significantly reduced expression in Hdh109/109 compared with control Hdh7/7 cells. Hdh109/109 cells transfected with REST decoy ODN showed significant up-regulation of all three REST target genes compared with untransfected and comparing with cells transfected with ---D (Fig. 1c). ---D transfected cells showed no significant change in expression of Bdnf, Syn1 or Chrm4 compared with untransfected cells (p-value = 0.11, 0.12 and 0.05, respectively). REST and actin expression were unaltered in all conditions (Fig 1c). The observed level of decoy-mediated de-repression of target genes was comparable with that obtained using DN : REST and Rest shRNA strategies (Figure S1). In addition, rescue of Bdnf gene expression was accompanied by increased Bdnf protein levels as measured by ELISA assays (Fig. 1d). Untransfected Hdh109/109 cells expressed approximately 26% of Hdh7/7 Bdnf levels but following delivery of REST decoy ODN, Bdnf protein levels were significantly increased to 65% of wild-type levels (Fig. 1d, ++D) compared with levels following negative decoy (---D) transfection. Our results, therefore, indicate that by attenuating REST binding with modified decoy ODNs, it is possible to de-repress multiple REST target genes and to partially restore Bdnf protein levels in HD cells.

REST occupancy of RE1s following decoy ODN delivery

To directly test whether treatment with decoy ODNs decreased occupancy of REST at its RE1 targets in the genome, we carried out ChIP-qPCR analysis using anti-REST IgG and primers that flank RE1 sites of the Bdnf, Syn1 and Chrm4 genes (Fig. 2). Chromatin from decoy-transfected cells (++D or ---D) was collected 48 h post-transfection, similar to gene and protein expression analyses described above earlier, since this time-point showed the highest level of efficacy in rescue of target gene expression (data not shown). REST occupancy was assessed at three REST target gene RE1s: Bdnf, Syn1 and Chrm4 (Fig. 2). Higher levels of REST occupancy were seen at all three loci in untransfected Hdh109/109 cells compared with Hdh7/7 cells, consistent with the higher levels of REST seen in Hdh109/109 cells. No change in REST occupancy was detected in cells transfected with the negative decoy (Fig. 2, ---D). However, following delivery of the REST RE1 decoy ODN, REST occupancy was significantly decreased at all three loci in Hdh109/109 cells to essentially wild-type (Hdh7/7) levels (Fig. 2, ++D). We again performed parallel experiments using DN : REST and Rest shRNA for comparison and showed that RE1 decoy ODN was equally as effective at decreasing REST recruitment to these RE1s (Figure S2). Therefore, modified decoy ODNs that contain an RE1 consensus binding site are able to sequester REST protein from target RE1 sites in the genome, resulting in decreased Rest occupancy at several target genes, including Bdnf.

Changes to histone acetylation of RE1s following decoy delivery

Transcriptionally active or transcriptionally silent chromatin states are accompanied by specific histone amino-terminal modifications conferring distinct epigenetic signatures (for review, see Jenuwein and Allis 2001). Numerous studies have demonstrated that REST is able to maintain low levels of
histone H3 lysine 9 acetylation (H3K9ac), an epigenetic marker associated with active chromatin (Dhalluin et al. 1999), at the promoter region of target genes via recruitment of histone deacetylase (HDAC) activity (Grimes et al. 2000; Roopra et al. 2000). To better understand the mechanisms of REST silencing and repression in Hdh7/7 and Hdh109/109 cells, we looked at H3K9ac levels around the RE1s in response to changing REST occupancy using our decoy strategy. Figure 3 shows ChIP-qPCR analysis of H3K9ac levels of REST target genes in untransfected Hdh7/7 and Hdh109/109 cells and Hdh109/109 cells 48 h following delivery of decoy ODNs (++D or −−D). Bdnf and Chrm4 showed more than twofold and Syn1 almost 1.8-fold lower enrichments of H3K9ac in Hdh109/109 cells compared with Hdh7/7 cells (Fig. 3, all expressions).
We observed a significant increase in H3K9ac enrichments at all three loci, to essentially wild-type levels or greater, following REST decoy ODN delivery (+D) compared with untransfected or with negative decoy ODN (−−D) transfected cells (Fig. 3, all \( p \leq 0.05 \)). Our results indicate that REST is able to maintain low levels of H3K9ac around REST binding sites. We also show that regulation of the local chromatin environment is associated with levels of REST occupancy and reduced H3K9ac observed in \( Hdh^{109/109} \) cells can be restored by the use of modified decoy ODN technology.

**RE1-containing decoy ODN interact directly with REST**

To demonstrate a direct interaction between REST and the specific REST RE1 decoy ODN we performed an EMSA with a fluorescent (5'-IRD700) labelled decoy ODN (Fig. 4, 0.05 using Student’s \( t \)-test). We observed a significant increase in H3K9ac enrichments at all three loci, to essentially wild-type levels or greater, following REST decoy ODN delivery (+D) compared with untransfected or with negative decoy ODN (−−D) transfected cells (Fig. 3, all \( p \leq 0.05 \)). Our results indicate that REST is able to maintain low levels of H3K9ac around REST binding sites. We also show that regulation of the local chromatin environment is associated with levels of REST occupancy and reduced H3K9ac observed in \( Hdh^{109/109} \) cells can be restored by the use of modified decoy ODN technology.

**Fig. 2** Repressor element 1 (RE1) silencing transcription factor (REST) occupancy in response to decoy delivery. Chromatin immunoprecipitation (ChIP)-quantitative PCR (qPCR) analysis at three known RE1 sites in untransfected wild-type (\( Hdh^{7/7} \)) and mutant (\( Hdh^{109/109} \)) cells and in mutant cells following delivery of canonical RE1-containing decoy (++D) or a non-REST binding RE1-containing decoy (−−D). REST occupancy of the \( Bdnf \), \( Syn1 \) and \( Chrm4 \) RE1 loci was decreased in \( Hdh^{109/109} \) cells transfected with +D compared with untransfected cells and those transfected with −−D. Data show REST enrichment relative to both enrichment at a non-specific region that does not contain an RE1 site and to input levels and represent the average of three independent experiments, error bars show SDs, **\( p \leq 0.01 \), ***\( p \leq 0.001 \) (using Student’s \( t \)-test).

**Fig. 4** Decoy oligonucleotides (ODNs) interact directly with repressor element 1 (RE1) silencing transcription factor (REST) protein. The figure shows a fluorescence western blot image with visualisation of an infra-red fluorescence-labelled decoy ODNs containing an RE1-binding site known to bind REST protein (IR-Decoy ++D). The following samples were loaded in duplicate: IR-Decoy ++D alone (lanes 1 and 2); IR-Decoy ++D incubated with HeLa nuclear extract (lanes 3 and 4); IR-Decoy ++D incubated with HeLa nuclear extract and anti-Rest IgG (lanes 5 and 6); HeLa nuclear extract incubated with anti-Rest IgG and excess, non-specific (un-labelled) Decoy ODNs (−−D). Free (unbound) IR-Decoy ++D is shown in the bottom panel and alone serves as a negative control (lanes 1 and 2). Incubation of IR-Decoy ++D with HeLa nuclear extract shows an additional band shifted to a higher molecular weight corresponding to a complex of REST protein and IR-Decoy ++D (REST : IR-Decoy). The presence of REST in this complex is confirmed by the super-shifted bands of complexes co-incubated with anti-Rest IgG (lanes 5 and 6). HeLa nuclear extract incubated with anti-Rest IgG and excess, non-specific (un-labelled) Decoy ODNs (−−D). Free (unbound) IR-Decoy ++D is shown in the bottom panel and alone serves as a negative control (lanes 1 and 2). Incubation of IR-Decoy ++D with HeLa nuclear extract shows an additional band shifted to a higher molecular weight corresponding to a complex of REST protein and IR-Decoy ++D (REST : IR-Decoy). The presence of REST in this complex is confirmed by the super-shifted bands of complexes co-incubated with anti-Rest IgG (lanes 5 and 6). HeLa nuclear extract incubated with anti-Rest IgG and excess, non-specific (un-labelled) Decoy ODNs (−−D). Free (unbound) IR-Decoy ++D is shown in the bottom panel and alone serves as a negative control (lanes 1 and 2). Incubation of IR-Decoy ++D with HeLa nuclear extract shows an additional band shifted to a higher molecular weight corresponding to a complex of REST protein and IR-Decoy ++D (REST : IR-Decoy). The presence of REST in this complex is confirmed by the super-shifted bands of complexes co-incubated with anti-Rest IgG (lanes 5 and 6). HeLa nuclear extract incubated with anti-Rest IgG and excess, non-specific (un-labelled) Decoy ODNs (−−D). Free (unbound) IR-Decoy ++D is shown in the bottom panel and alone serves as a negative control (lanes 1 and 2). Incubation of IR-Decoy ++D with HeLa nuclear extract shows an additional band shifted to a higher molecular weight corresponding to a complex of REST protein and IR-Decoy ++D (REST : IR-Decoy). The presence of REST in this complex is confirmed by the super-shifted bands of complexes co-incubated with anti-Rest IgG (lanes 5 and 6). HeLa nuclear extract incubated with anti-Rest IgG and excess, non-specific (un-labelled) Decoy ODNs (−−D). Free (unbound) IR-Decoy ++D is shown in the bottom panel and alone serves as a negative control (lanes 1 and 2). Incubation of IR-Decoy ++D with HeLa nuclear extract shows an additional band shifted to a higher molecular weight corresponding to a complex of REST protein and IR-Decoy ++D (REST : IR-Decoy). The presence of REST in this complex is confirmed by the super-shifted bands of complexes co-incubated with anti-Rest IgG (lanes 5 and 6). HeLa nuclear extract incubated with anti-Rest IgG and excess, non-specific (un-labelled) Decoy ODNs (−−D). Free (unbound) IR-Decoy ++D is shown in the bottom panel and alone serves as a negative control (lanes 1 and 2). Incubation of IR-Decoy ++D with HeLa nuclear extract shows an additional band shifted to a higher molecular weight corresponding to a complex of REST protein and IR-Decoy ++D (REST : IR-Decoy). The presence of REST in this complex is confirmed by the super-shifted bands of complexes co-incubated with anti-Rest IgG (lanes 5 and 6). HeLa nuclear extract incubated with anti-Rest IgG and excess, non-specific (un-labelled) Decoy ODNs (−−D). Free (unbound) IR-Decoy ++D is shown in the bottom panel and alone serves as a negative control (lanes 1 and 2). Incubation of IR-Decoy ++D with HeLa nuclear extract shows an additional band shifted to a higher molecular weight corresponding to a complex of REST protein and IR-Decoy ++D (REST : IR-Decoy). The presence of REST in this complex is confirmed by the super-shifted bands of complexes co-incubated with anti-Rest IgG (lanes 5 and 6). HeLa nuclear extract incubated with anti-Rest IgG and excess, non-specific (un-labelled) Decoy ODNs (−−D). Free (unbound) IR-Decoy ++D is shown in the bottom panel and alone serves as a negative control (lanes 1 and 2). Incubation of IR-Decoy ++D with HeLa nuclear extract shows an additional band shifted to a higher molecular weight corresponding to a complex of REST protein and IR-Decoy ++D (REST : IR-Decoy). The presence of REST in this complex is confirmed by the super-shifted bands of complexes co-incubated with anti-Rest IgG (lanes 5 and 6). HeLa nuclear extract incubated with anti-Rest IgG and excess, non-specific (un-labelled) Decoy ODNs (−−D). Free (unbound) IR-Decoy ++D is shown in the bottom panel and alone serves as a negative control (lanes 1 and 2). Incubation of IR-Decoy ++D with HeLa nuclear extract shows an additional band shifted to a higher molecular weight corresponding to a complex of REST protein and IR-Decoy ++D (REST : IR-Decoy). The presence of REST in this complex is confirmed by the super-shifted bands of complexes co-incubated with anti-Rest IgG (lanes 5 and 6). HeLa nuclear extract incubated with anti-Rest IgG and excess, non-specific (un-labelled) Decoy ODNs (−−D). Free (unbound) IR-Decoy ++D is shown in the bottom panel and alone serves as a negative control (lanes 1 and 2). Incubation of IR-Decoy ++D with HeLa nuclear extract shows an additional band shifted to a higher molecular weight corresponding to a complex of REST protein and IR-Decoy ++D (REST : IR-Decoy). The presence of REST in this complex is confirmed by the super-shifted bands of complexes co-incubated with anti-Rest IgG (lanes 5 and 6). HeLa nuclear extract incubated with anti-Rest IgG and excess, non-specific (un-labelled) Decoy ODNs (−−D). Free (unbound) IR-Decoy ++D is shown in the bottom panel and alone serves as a negative control (lanes 1 and 2). Incubation of IR-Decoy ++D with HeLa nuclear extract shows an additional band shifted to a higher molecular weight corresponding to a complex of REST protein and IR-Decoy ++D (REST : IR-Decoy). The presence of REST in this complex is confirmed by the super-shifted bands of complexes co-incubated with anti-Rest IgG (lanes 5 and 6). HeLa nuclear extract incubated with anti-Rest IgG and excess, non-specific (un-labelled) Decoy ODNs (−−D). Free (unbound) IR-Decoy ++D is shown in the bottom panel and alone serves as a negative control (lanes 1 and 2). Incubation of IR-Decoy ++D with HeLa nuclear extract shows an additional band shifted to a higher molecular weight corresponding to a complex of REST protein and IR-Decoy ++D (REST : IR-Decoy). The presence of REST in this complex is confirmed by the super-shifted bands of complexes co-incubated with anti-Rest IgG (lanes 5 and 6).
IR-Decoy ++D). The IR-Decoy was incubated with or without HeLa cell nuclear extract and the binding reactions were run on a native polyacrylamide gel. Incubation of the decoy with HeLa cell nuclear extract resulted in a specific band shift corresponding to a protein : decoy (REST : IR-Decoy) interaction (Fig. 4, lanes 3–4). Inclusion of specific anti-REST IgG resulted in a supershift of the protein : decoy complex (Fig. 4, lanes 5–6) that confirmed that complexes shown by the band shifts in lanes 3 and 4 contained REST protein. Moreover, REST : IR-Decoy complexes were not disrupted when binding reactions were co-incubated with excess negative decoy (Fig. 4, lanes 7–8, --D). The EMSA assays confirm the ability and specificity of the modified REST RE1 decoy ODNs to directly interact with REST protein. Therefore, the observed changes to REST target gene expression, protein expression, REST occupancy and the local chromatin environment in Hdh109/109 cells following introduction of ++D can be attributed to ++D specific sequestration of REST protein.

**Discussion**

Use of modified decoy ODNs to abrogate the action of transcription factors is a promising approach to rescuing phenotypic defects because of aberrant transcription factor function. In this work, we have developed a decoy strategy to block the activity of REST based on its binding of the conserved 21bp RE1 (Chong et al. 1995; Schoenherr et al. 1996; Bruce et al. 2004; Johnson et al. 2006). The Bdnf promoter contains an RE1-binding site that has previously been shown to bind to and be regulated by REST both in vitro and in vivo (Zuccato et al. 2001, 2003; Bruce et al. 2004) and recent work has highlighted a critical role for REST in HD through its regulation of BDNF (Zuccato et al. 2007). Here, EMSA show we have the ability of a modified double-stranded ODNs that contains a canonical RE1 site to block binding of REST to endogenous binding sites by sequestration of REST protein and to subsequently rescue levels of BDNF mRNA and protein in Hdh109/109 cells. Moreover, using ChIP, we show that there is a concomitant decrease in REST occupancy of the RE1 sites in the Bdnf promoter and other target genes. In addition, a concomitant local increase in H3K9ac is suggestive of a more open chromatin environment characteristic of activated gene loci. Collectively, these data show that modified decoy ODNs can rescue REST-mediated transcriptional and epigenetic repression in an in vitro model of HD.

**Decoy ODN can rescue REST target gene expression including BDNF**

Widespread transcriptional dysregulation is a central pathogenic mechanism in HD and several transcription factors have been implicated in this disease state (Zuccato et al. 2010). Perhaps, the most robust mechanistic link between transcriptional dysfunction and the pathology of HD is provided by REST (Zuccato and Cattaneo 2007). In vitro studies have demonstrated that in normal cells, wild-type Htt sequesters REST in the cytoplasm thereby preventing its binding and repression of target genes (Zuccato et al. 2003). However, in the presence of muHtt, this cytoplasmic complex is disrupted and REST migrates to the nucleus where it can bind to and repress its target genes. Although the initial report focussed on the subsequent repression of the BDNF gene (Zuccato et al. 2003), we have since demonstrated that REST can binds to and repress numerous genes whose expression is altered in the brain and periphery of HD patients, many of which may also be involved in mediating underlying pathological mechanisms (Zuccato et al. 2007; Johnson et al. 2008b; Marullo et al. 2008). Although many studies, including this study have used cell lines to study the transcriptional effects of mutant Htt, it is important to realise that levels of REST tend to be higher in progenitor lines than differentiated neurons. Nevertheless, several studies have indicated the presence of REST protein in the cytoplasm and nucleus of differentiated neurons (Calderone et al. 2003; Kuwabara et al. 2004; Sun et al. 2005; Spencer et al. 2006). Furthermore, one recent study showed that endogenous REST levels were sufficient to repress a RE1-containing BDNF promoter construct in cortical neurons (Hara et al. 2009). Accordingly, we propose that abrogation of REST activity offers a potential strategy to rescue aberrantly repressed REST target genes in differentiated neurons and in HD might ultimately restore globally dysregulated neuronal gene transcription.

Using adenoviral delivery of a dominant-negative form of REST, we have previously shown that in addition to Bdnf, other REST target genes dysregulated in HD, including Syn1 and Chrm4 (encoding synapsin-1 and the M4 muscarinic receptor, respectively), are also de-repressed in parallel with decreased REST occupancy at their RE1 sites (Greenway et al. 2007; Zuccato et al. 2007; Johnson et al. 2008b). Here, we show that transcription of these genes can also be effectively de-repressed using a decoy ODN strategy, thus offering an exciting alternative to the use of viral or plasmid vectors. Further, the efficacy of decoys relies only on having access to the nuclear transcription factor, whereas dominant-negative constructs based on DNA binding domains need to access the chromatin of target genes.

**REST acts as an epigenetic regulator in HD**

One mechanism by which transcription can be impaired is through altered nucleosome dynamics; in this context, histone methylation and acetylation status is closely linked with transcriptional activity, regulating transcription factor access to promoter regions in DNA (Hake et al. 2004; Shogren-Knaak et al. 2006). Recent studies have linked HD transcriptional dysregulation to changes in histone modifications (Sadri-Vakili et al. 2007; Kim et al. 2008), including
hypo-acetylation of histone H3 at promoters of downregulated genes in R6/2 mice (Sadri-Vakili et al. 2007). Moreover, treatment with HDAC inhibitors corrects dysregulated gene expression through modification of histone proteins (Ferrante et al. 2003; Sadri-Vakili et al. 2007; Thomas et al. 2008). This may prove to be of therapeutic value in HD, particularly since several HDAC inhibitors are already approved or being validated in clinical trials (for recent review, see Copeland et al. 2010).

REST has been shown to interact with a number of proteins by recruitment of co-repressor platforms via its N- and C-terminal repressor domains (for review, see Ooi and Wood 2007). Many of these proteins are required for REST function via their enzymatic activities that serve to repress or silence gene expression. For example, HDAC1 and HDAC2 are able to repress gene expression by removal of acetyl groups on core histones, including H3K9 (Roopra et al. 2001; Ballas et al. 2000). Similarly REST recruits H3K4 demethylase activity in the form of lysyergic acid diethylamide 1, H3K9 methylase activity via G9a and chromatin re-modelling activity, including brahma-related gene 1 and BRAF35 (Battaglioli et al. 2002; Hakimi et al. 2002; Roopra et al. 2004; Lee et al. 2005; Ooi et al. 2006). Therefore, REST is able to both silence and repress neuronal genes by creating a chromatin environment bearing a repressive local epigenetic signature (Greenway et al. 2007).

Since REST repression in other in vitro systems has been shown to be characterised by low levels of H3K9ac (Greenway et al. 2007), we used ChIP to assess changes in H3K9ac following delivery of our decoy ODNs into Hdh109/109 cells. The low levels of H3K9ac observed around the RE1 sites of REST target genes in Hdh109/109 cells compared with wild-type Hdh123 cells is in keeping with the parallel decreases observed in gene expression and the reciprocally increased occupancy of REST at these sites. ++D decoy-transfected cells, however, showed significantly increased enrichment of H3K9ac at these loci to essentially wild-type levels. These data suggest that REST may have a dual function in target gene repression, including Bdnf, acting both as a transcriptional repressor and epigenetic modulator. This likely occurs through direct binding to its RE1 site and subsequent modification of the surrounding chromatin environment. In this regard, we propose that REST may be considered as much an epigenetic regulator as a transcriptional repressor during the pathological state of HD.

Potential of modified decoy ODN technology in HD

Over 3000 REST binding sites have been identified in the human and murine genomes, and many target genes encode for proteins that are fundamental for neuronal development and differentiation (Bruce et al. 2004; Johnson et al. 2006). REST is the only known transcription factor able to bind the highly conserved RE1 sequence. Owing to the specificity of this binding and the long 21 bp length of the canonical RE1 sequence, modulation of aberrant REST function associated with HD represents an ideal model for the application of decoy ODN strategies. One of the main limitations of decoy technology has been the rapid degradation of unmodified ODNs by nuclease digestion (Gao et al. 1992; Brown et al. 1994). Different strategies have evolved to render the decoys nuclease-resistant, such as the use of locked nucleic acids (Crinelli et al. 2002) or addition of phosphorothioated nucleotides (Yang et al. 2002). We chose the latter approach and used a phosphorothioated decoy ODN based on a canonical RE1 site (REST decoy) as defined by previous bioinformatic and ChIP studies (Bruce et al. 2004; Johnson et al. 2006). Results presented here demonstrate that a phosphorothioated decoy is sufficiently stable and specific to enable efficient blockade of REST activity. In addition, our results suggest that decoy ODNs are as effective at attenuating REST activity as other previously employed strategies, such as adenoviral delivery of DN: REST or transfection of Rest shRNAs (Greenway et al. 2007; Zuccato et al. 2007 and data not shown). They also avoid the need for large DNA constructs and viral delivery systems and they side-step the issue of potential off-target effects encountered using RNAi technology. Moreover, their ability to directly interfere with transcription factor binding can be directly inferred by using labelled ODNs to serve as probes in EMSAs (Hara et al. 2003; Kubo et al. 2003). With a fluorescence-labelled decoy ODN carrying the canonical RE1 sequence, we were able to directly show the interaction between REST and the decoy and we confirmed the presence of REST in this complex through the use of a specific anti-REST antibody to supershift the protein/DNA complex.

Widespread gene dysregulation is a hallmark of HD and in some cases precedes many of the well-known physical symptoms of the disease (for recent review, see Buckley et al. 2010). The functional products of many of these dysregulated genes have provided new insights into possible mechanisms underlying HD pathology, including the well-known role of BDNF and concomitant loss of medium spiny neurons (reviewed in Zuccato and Cattaneo 2007). As well as transcriptional dysregulation, it is becoming increasingly clear that epigenetic disruption occurs in HD and, indeed, targeting HDAC activity has become a major platform for HD drug development (Ryu et al. 2006; Kazantsev and Hersch 2007; Kazantsev and Thompson 2008). Many REST functions are attributed to its recruitment of HDAC activity and it is likely that the causative role of REST in HD occurs through an inextricable linkage of transcriptional and epigenetic dysregulation (Buckley et al. 2010). The use of a modified decoy strategy presented here that specifically targets REST function provides a novel and effective means by which it is possible to show effective rescue of both transcriptional and epigenetic effects of REST in an in vitro HD model; this included restoration of Bdnf gene and protein expression and H3K9ac levels at its promoter. Modified
decoys thus offer an attractive alternative to plasmid- or viral-based vectors for interfering with target protein function and provide an ideal strategy to investigate the contribution of REST to HD pathology in well-established mouse models. Perhaps, more intriguingly, since modified ODNs targeting transcription factor function have been shown to be of potential therapeutic value in other disorders (Kubo et al. 2003; De Stefano et al. 2010), they may be useful in future clinical intervention for early-stage HD. The work presented here presents a proof-of-concept that our decoy strategy is capable of rescuing REST-mediated repression of neuronal gene expression a cell line model of HD. Nevertheless, these findings need to be taken forward and the effectiveness of decoys needs to be tested in neuronal cells, where endogenous levels of REST are lower than those found in Hdh cells. Future studies will address this by using in vivo models of HD.

Acknowledgements

This work is funded by a fellowship from Fondazione Cenci Bolognetti (to CS), the Wellcome Trust (to NJB), the Huntington’s Disease Society of America (H.D.S.A., New York) to EC and the Cure HD Initiative (CHDI) Foundation (New York) to EC and NJB. The authors have no competing interests to declare.

Supporting information

Additional Supporting information may be found in the online version of this article:

Appendix S1. Supplementary Materials and methods.

Figure S1. Reduced REST target gene repression in Hdh109/109 cells using dominant-negative REST or Rest shRNA.

Figure S2. Reduced REST occupancy of RE1 sites in Hdh109/109 cells using dominant-negative REST or Rest shRNA.

As a service to our authors and readers, this journal provides supporting information supplied by the authors. Such materials are peer-reviewed and may be re-organised for online delivery, but are not copy-edited or typeset. Technical support issues arising from peer-reviewed and may be re-organised for online delivery, but are supporting information supplied by the authors. Such materials are

capable of rescuing REST-mediated repression of neuronal gene expression a cell line model of HD. Nevertheless, these findings need to be taken forward and the effectiveness of decoys needs to be tested in neuronal cells, where endogenous levels of REST are lower than those found in Hdh cells. Future studies will address this by using in vivo models of HD.

References


