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Hsp40 Gene Therapy Exerts Therapeutic Effects on Polyglutamine Disease Mice via a Non-Cell Autonomous Mechanism

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Abstract

The polyglutamine (polyQ) diseases such as Huntington's disease (HD), are neurodegenerative diseases caused by proteins with an expanded polyQ stretch, which misfold and aggregate, and eventually accumulate as inclusion bodies within neurons. Molecules that inhibit polyQ protein misfolding/aggregation, such as Polyglutamine Binding Peptide 1 (QBP1) and molecular chaperones, have been shown to exert therapeutic effects *in vivo* by crossing of transgenic animals. Towards developing a therapy using these aggregation inhibitors, we here investigated the effect of viral vector-mediated gene therapy using QBP1 and molecular chaperones on polyQ disease model mice. We found that injection of adeno-associated virus type 5 (AAV5) expressing QBP1 or Hsp40 into the striatum both dramatically suppresses inclusion body formation in the HD mouse R6/2. AAV5-Hsp40 injection also ameliorated the motor impairment and extended the lifespan of R6/2 mice. Unexpectedly, we found even in virus non-infected cells that AAV5-Hsp40 appreciably suppresses inclusion body formation, suggesting a non-cell autonomous therapeutic effect. We further show that Hsp40 inhibits secretion of the polyQ protein from cultured cells, implying that it inhibits the recently suggested cell-cell transmission of the polyQ protein. Our results demonstrate for the first time the therapeutic effect of Hsp40 gene therapy on the neurological phenotypes of polyQ disease mice.

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Introduction

The polyglutamine (polyQ) diseases are a group of inherited neurodegenerative disorders that are all caused by a common genetic mutation, namely an expansion (>40) of a polyQ-encoding CAG repeat in each unrelated disease-causing gene [1,2]. Nine polyQ diseases have been identified to date, including Huntington's disease (HD), spinocerebellar ataxia (SCA) types 1, 2, 3, 6, 7, 17, dentatorubral pallidoluysian atrophy (DRPLA), and spinobulbar muscular atrophy (SBMA). In these disorders, progressive degeneration of neurons in brain areas specific for each disorder occurs, causing various neurological and psychiatric symptoms corresponding to each affected brain area [1,2].

In the common molecular pathogenesis of the polyQ diseases, proteins with an expanded polyQ stretch become misfolded and form aggregates, and subsequently accumulate as inclusion bodies within neurons, eventually resulting in neurodegeneration [3–7]. Moreover, recent studies suggest that prion-like transmission of aggregation-prone proteins between cells is involved in neuropathological spreading during disease progression in not only the polyQ diseases but also various other neurodegenerative diseases [8–11]. Although various therapeutic strategies against downstream targets of the pathogenic cascade have been investigated, misfolding and aggregation of the polyQ protein are ideal therapeutic targets since they are the most initial pathogenic events, and therefore their inhibition is expected to result in the suppression of a broad range of downstream pathogenic events [3,5,12,13].

In our attempt to establish a therapy for the polyQ diseases, we hypothesized that molecules that specifically bind to the expanded polyQ stretch would suppress misfolding and aggregation of the expanded polyQ protein. Accordingly, by phage display screening of combinatorial peptide libraries, we identified PolyQ Binding Peptide 1 (QBP1), and proved that QBP1 indeed inhibits polyQ protein misfolding/aggregation *in vitro* [14–16]. Furthermore, we

demonstrated that expression of QBP1 suppresses neurodegeneration *in vivo* in polyQ disease model animals [16,17]. Another approach for targeting misfolding and aggregation of the expanded polyQ protein is to utilize molecular chaperones, which are a group of biomolecules that assist the proper folding of proteins and prevent protein misfolding/aggregation [18–20]. Indeed, overexpression of molecular chaperones such as Hsp40 and Hsp70 has been shown to suppress polyQ protein aggregation and polyQ-induced neurodegeneration in various animal models of the polyQ diseases, such as flies [21–24], worms [25] and mice [26–30]. However, most studies showing the therapeutic efficacy of these aggregation inhibitors have been performed by crossing transgenic animals so far. To develop a therapy using aggregation inhibitors such as QBP1 and molecular chaperones, transgenes need to be delivered in affected individuals by administration, rather than be expressed in the next generation by crossing transgenic animals.

In this study, we employed a viral vector to deliver these transgenes into the brain and investigated their therapeutic effects on polyQ disease model mice. Among various viral vectors, we chose to use adeno-associated virus vector (AAV) because of its widespread infection throughout the brain, its long-term expression of transgenes, and its safety [31,32]. We successfully demonstrate the therapeutic effects of AAV5-QBP1 and AAV5-Hsp40 injections on a mouse model of HD. Most interestingly, we found that AAV5-Hsp40 exerts a non-cell autonomous therapeutic effect, possibly via inhibition of the recently-suggested cell-cell transmission of the polyQ protein, indicating a novel therapeutic mode of action of Hsp40.

Results

AAV5-QBP1 and AAV5-Hsp40 Inhibit Inclusion Body Formation in polyQ Disease Mouse Neurons

We employed the R6/2 HD mouse model [33] to investigate the therapeutic effect of AAV-mediated expression of QBP1 and molecular chaperones. We first tested the effect on accumulation of the pathogenic polyQ protein into inclusion bodies in the neurons of R6/2 mice by AAV5 injections. Injections were performed on mice at postnatal day 7 (P7) using an infusion pump (see Materials and Methods), which has been shown to lead to widespread delivery of the injected molecules in the brain [34], and indeed resulted in widespread expression of the transgene throughout the injected brain hemisphere with ~30% infection efficiencies (Fig. S1). R6/2 mice were injected with AAV5-GFP on one side of the striatum and AAV5-QBP1 on the other side, and htt inclusion body formation was compared between the two sides of both the striatum and the cortex. Inclusion bodies were already formed in 36.0% of AAV5-GFP infected neurons in the striatum at 4 weeks of age, which increased to 68.5% at 8 weeks and 73.8% at 14 weeks, and an age dependent increase in inclusion bodies was also observed in the cortex (Fig. S2). In contrast, AAV5-QBP1 infected neurons had significantly less inclusion bodies at most time points (Fig. S2), and the rates of neurons with inclusion bodies at 8 weeks, for example, were 68.5% for GFP vs 33.7% for QBP1 ($p<0.001$) in the striatum, and 49.3% for GFP vs 27.4% for QBP1 ($p<0.001$) in the cortex (Figs. 1A,B). These results demonstrate a significant inhibitory effect of AAV5-QBP1 on inclusion body formation.

We also tested the effect of AAV5-mediated expression of a molecular chaperone on inclusion body formation. Among the various molecular chaperones, we chose to use a member of the Hsp40 family, namely DNAJB1 (referred to as Hsp40 in this study), since members of the DNAJB subfamily have been

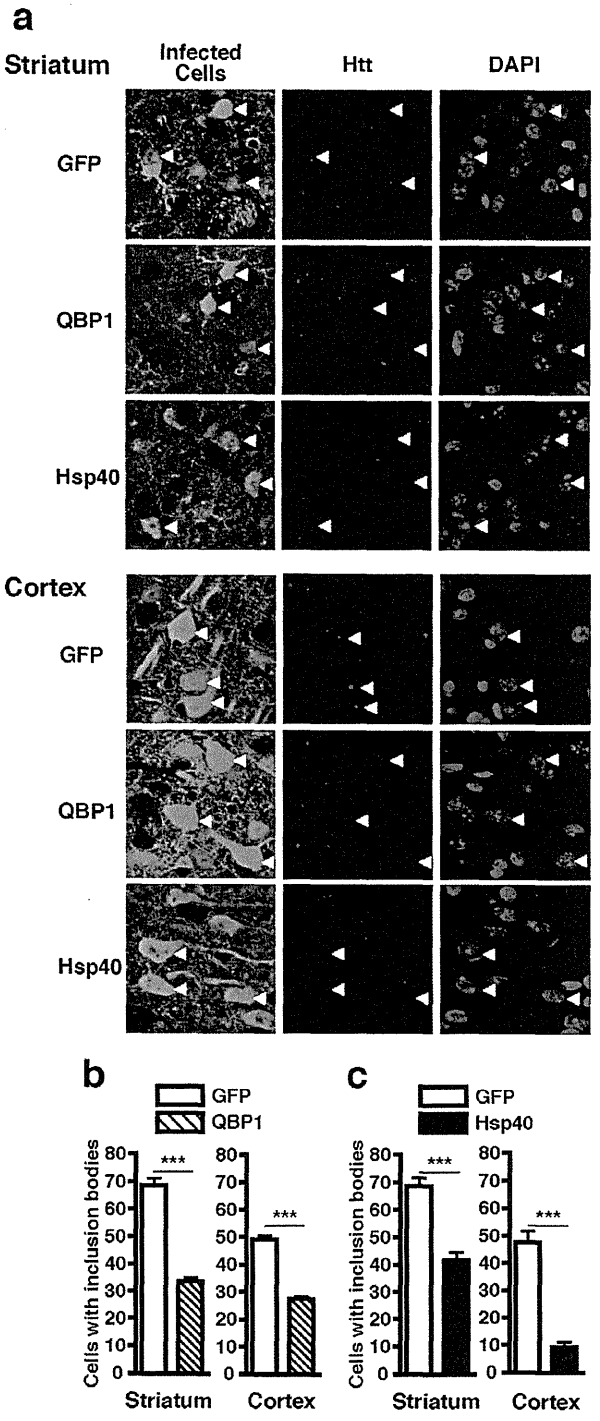

Figure 1. AAV5-QBP1 and AAV5-Hsp40 inhibit polyQ inclusion body formation in virus infected neurons of polyQ disease mice. R6/2 mice at P7 were injected with AAV5-GFP on one side of the striatum and either AAV5-QBP1 or AAV5-Hsp40 on the other side, and htt inclusion body formation in virus infected neurons was assessed at 8 weeks of age by immunohistochemistry of brain sections. (A) Representative photographs of striatal (top panels) and cortical (bottom panels) sections of R6/2 mice, injected with the indicated viruses. Green; virus infected cells, red; htt, and blue; nuclei visualized by DAPI staining. In each panel, representative virus infected cells are indicated by white

arrowheads. (B) Inclusion body formation in AAV5-QBP1 infected neurons in the striatum (left) and cortex (right). (C) Inclusion body formation in AAV5-Hsp40 infected neurons in the striatum (left) and cortex (right). In (B) and (C), data are shown as means ± SEM of ≥6 fields of view, in which over 180 cells were counted (*$p<0.05$, ***$p<0.001$). Representative results of two mice analyzed are shown.
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reported to be the most potent suppressors of expanded polyQ protein aggregation and toxicity [35]. The effect of AAV5-Hsp40 on polyQ inclusion body formation in R6/2 mice was investigated at 8 weeks, an age at which AAV5-QBP1 showed a clear inhibitory effect. AAV5-Hsp40 also exerted a robust effect on inclusion body formation, and the rates of virus infected neurons with inclusion bodies were 68.6% for GFP vs 41.7% for Hsp40 ($p<0.001$) in the striatum, and 47.5% for GFP vs 9.2% for Hsp40 ($p<0.001$) in the cortex (Figs. 1A,C). This difference in the effectiveness of Hsp40 between the two brain areas may be due to differences in the expression levels of its partner Hsp70. Although whether polyQ inclusion bodies themselves are cytotoxic or cytoprotective has been controversial [36], we assume that suppression of inclusion body formation by QBP1 and Hsp40 can be regarded as a therapeutic effect, since they act by preventing the initial toxic misfolding of the polyQ protein and promoting its refolding, respectively [15,16,19,20]. Therefore, these results collectively demonstrate that QBP1 and Hsp40 exert therapeutic effects in polyQ disease mouse neurons, via their widespread and long-term expression using AAV5.

AAV5-Hsp40 Improves Neurological Phenotypes of polyQ Disease Mice

Since our above results demonstrated that AAV5-QBP1 and AAV5-Hsp40 inhibit accumulation of the pathogenic polyQ protein into inclusions, we next tested whether this could also lead to amelioration of the neurological phenotypes of R6/2 mice. For this purpose we injected AAV5-GFP, AAV5-QBP1, or AAV5-Hsp40 into both sides of the striatum of P7 R6/2 mice, and analyzed their effects on various neurological phenotypes of these mice.

To evaluate motor impairments of R6/2 mice, we first tested their forced locomotor activity using the rotarod. Although the reduction in rotarod performance of R6/2 mice compared with wild-type (WT) mice becomes evident by 7 weeks of age, the performance of AAV5-QBP1 injected and AAV5-Hsp40 injected R6/2 mice did not significantly differ from AAV5-GFP injected mice (Fig. 2A). We further tested the effect of these viruses on spontaneous locomotor activity. R6/2 mice demonstrated significantly less open-field activity and rearing compared with WT mice as early as 5 weeks of age, which further decreased by 8 weeks. Although we could not detect a significant improvement in AAV5-QBP1 injected mice, AAV5-Hsp40 injected R6/2 mice exhibited significantly higher open-field activity and rearing compared with AAV5-GFP injected mice at 8 weeks of age (Activity: GFP 134.8±10.2 vs Hsp40 196.4±8.4 counts/min, $p<0.001$; Rearing: GFP 2.0±0.44 vs<Hsp40 6.3±0.67 counts/min, $p<0.001$) (Figs. 2B,C). These results demonstrate the therapeutic effect of AAV5-Hsp40 on the decreased spontaneous locomotor activity of R6/2 mice.

We also tested the effect of each virus on the grip strength abnormality of R6/2 mice. Grip strength of R6/2 mice was significantly weaker than that of WT mice at 9 weeks of age, and further weakened by 12 weeks of age. AAV5-QBP1 injection did not have a significant effect on grip strength at either age. In contrast, AAV5-Hsp40 injected R6/2 mice exhibited significantly

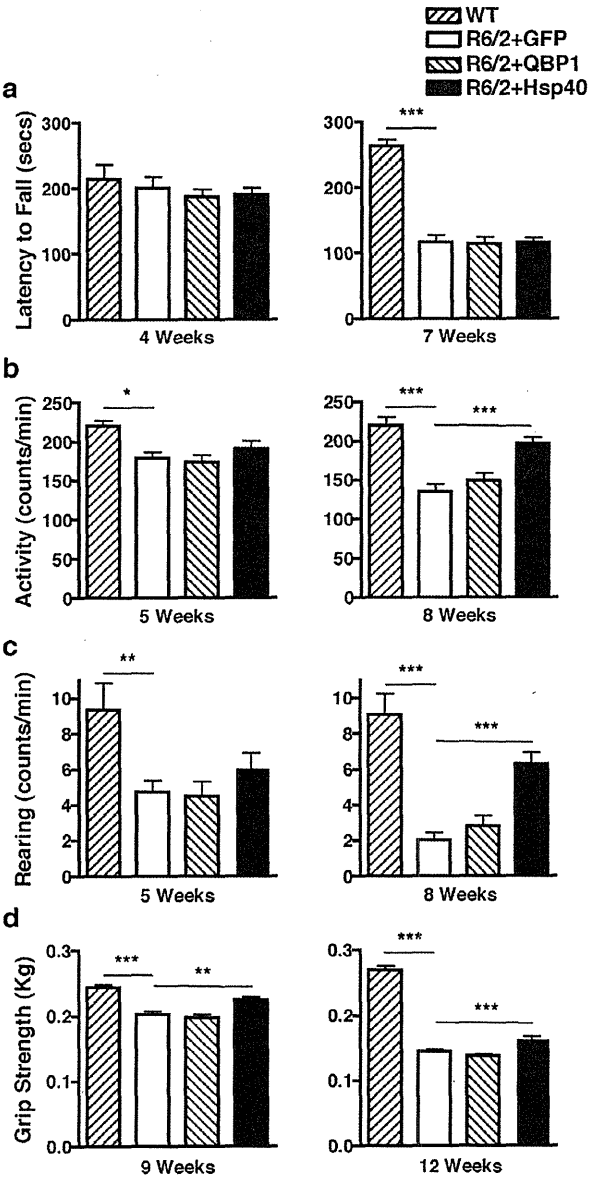

Figure 2. AAV5-Hsp40 improves spontaneous locomotor activity and grip strength abnormalities of polyQ disease mice. R6/2 mice at P7 were injected in both sides of the striatum with AAV5-GFP, AAV5-QBP1, or AAV5-Hsp40, and the following phenotypes were analyzed. (A) Rotarod performance measured at 4 (left) and 7 weeks (right) of age. (B, C) Open-field activity (B) and rearing (C) measured at 5 (left) and 8 weeks (right) of age. (D) Grip strength measured at 9 (left) and 12 weeks (right) of age. Values represent mean ± SEM, n≥9 mice (*$p<0.05$, **$p<0.01$, ***$p<0.001$).
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greater grip strengths than AAV5-GFP injected mice both at 9 weeks (GFP 0.202±0.005 vs Hsp40 0.225±0.005 Kg, $p<0.01$) and 12 weeks of age (GFP 0.145±0.003 vs Hsp40 0.165±0.003 Kg, $p<0.001$), demonstrating the therapeutic effect of AAV5-Hsp40 (Fig. 2D).

We also evaluated the body weight loss of AAV5-injected R6/2 mice. AAV5-GFP injected R6/2 mice demonstrated significantly lower body weights than WT mice after 9 weeks of age. The

weights of AAV5-QBP1 injected R6/2 mice were almost indistinguishable from AAV5-GFP injected mice at each time point. In contrast, the weights of AAV5-Hsp40 injected R6/2 mice showed a significant improvement compared to AAV5-GFP injected mice between 9 and 13 weeks of age (Fig. 3A). These results demonstrate the therapeutic effect of AAV5-Hsp40 also against the weight loss of R6/2 mice.

We then assessed the effect of AAV5 injections on the lifespan of R6/2 mice. The survival of AAV5-QBP1 injected R6/2 mice (median lifespan 100 days) was not significantly different from AAV5-GFP injected mice (median lifespan 95 days). In contrast, AAV5-Hsp40 injection resulted in a rightward shift of the survival curve to a median lifespan of 112 days, demonstrating the therapeutic effect of AAV5-Hsp40 on the decreased survival of R6/2 mice (Fig. 3B). Taken together, these results clearly demonstrate that AAV5-Hsp40 significantly improves many neurological phenotypes of R6/2 mice.

AAV5-Hsp40 Inhibits Inclusion Body Formation also in Virus Non-infected Neurons of polyQ Disease Mice

When analyzing the effect of the viruses on inclusion body formation in R6/2 mice (Fig. 1), we suspected that on the AAV5-Hsp40 injected side, not only virus infected neurons, but even virus non-infected neurons appeared to have fewer inclusion bodies than those on the AAV5-GFP injected side. To clarify our suspicion, we focused on the virus non-infected neurons that are not stained with Hsp40 or GFP antibodies (see Materials and Methods and Fig. S3), and reanalyzed inclusion body formation in the virus non-infected neurons. On the AAV5-QBP1 injected side, virus non-infected neurons showed a similar rate of inclusion body formation as non-infected neurons on the AAV5-GFP injected side, in both the striatum and cortex, as expected (Fig. 4). Surprisingly, we found that on the AAV5-Hsp40 injected side, virus non-infected neurons had strikingly fewer inclusion bodies compared with non-infected neurons on the AAV5-GFP injected side in both the striatum (GFP side 69.0% vs Hsp40 side 49.8%, $p<0.01$) and cortex (GFP side 59.1% vs Hsp40 side 39.6%,

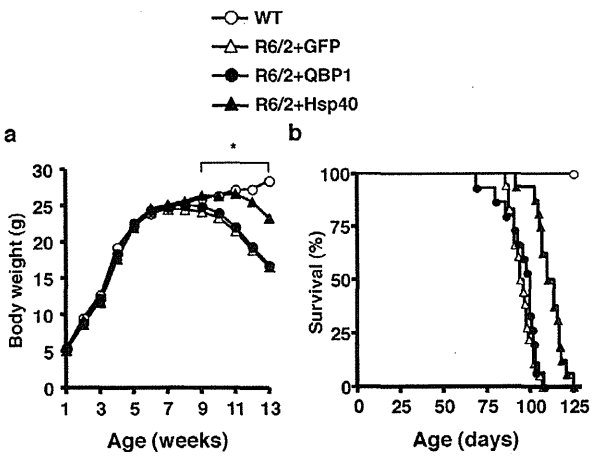

Figure 3. AAV5-Hsp40 improves body weight loss and extends the lifespan of polyQ disease mice. R6/2 mice at P7 were injected in both sides of the striatum with AAV5-GFP, AAV5-QBP1, or AAV5-Hsp40, and the following phenotypes were analyzed. (A) Body weight measured weekly. Values represent the mean (*$p<0.05$, R6/2+GFP vs R6/2+Hsp40). (B) Survival ($p<0.0001$, R6/2+GFP vs R6/2+Hsp40, Log-rank test). In both (A) and (B), n≥9 mice.
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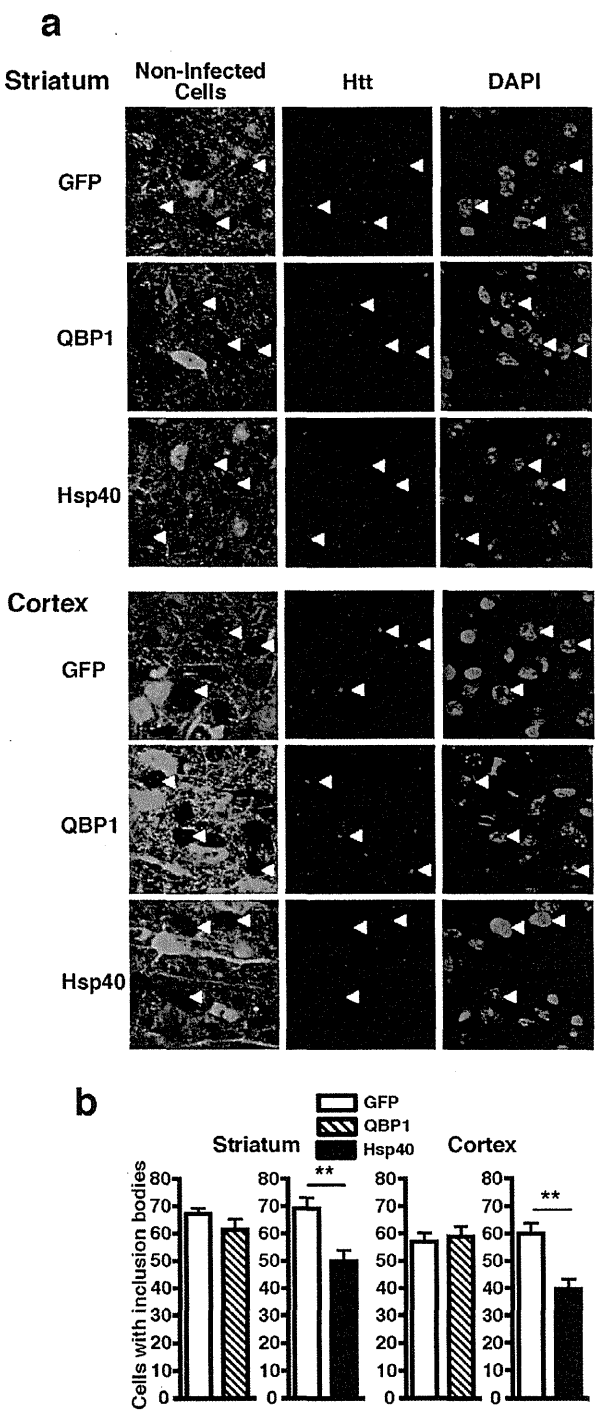

Figure 4. AAV5-Hsp40 also inhibits polyQ inclusion body formation in virus non-infected neurons of polyQ disease mouse brains. Htt inclusion body formation in 8 week old R6/2 mouse brains injected with AAV5-GFP on one side of the striatum and AAV5-QBP1 or AAV5-Hsp40 on the other side, was analyzed as in Fig. 1, but in the virus non-infected cells on each side of the brain. (A) Representative photographs of striatal (top panels) and cortical (bottom panels) sections of R6/2 mice injected with the indicated viruses. Green; virus infected cells, red; htt, and blue; nuclei visualized by DAPI staining. Representative virus non-infected cells in each panel are indicated by

white arrowheads. (B) Inclusion body formation in virus non-infected cells on the AAV5-QBP1 injected side and AAV5-Hsp40 injected side in the striatum (left) and cortex (right). Data are shown as means ± SEM of ≥6 fields of view, in which over 180 cells were counted (**$p<0.01$). Representative results of two mice analyzed are shown.
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$p<0.01$). The degree of inhibition was not as robust as in AAV5-Hsp40 infected neurons, but was still significant. These results raise a possibility that Hsp40 can exert a non-cell autonomous therapeutic effect on virus non-infected neurons in the brains of R6/2 mice, which is not observed with QBP1.

Hsp40 Inhibits Secretion of Pathogenic polyQ Proteins from Cultured Cells

We next aimed to elucidate the mechanism by which Hsp40 exerts its non-cell autonomous therapeutic effect in the brains of R6/2 mice. Recent studies suggest that prion-like cell-cell transmission of aggregation-prone proteins via their release from cells and subsequent uptake into neighboring cells, is involved in the spreading of neuropathology in the polyQ diseases as well as other neurodegenerative diseases [8–11]. We therefore hypothesized that Hsp40 may inhibit secretion of the pathogenic polyQ protein from cells to exert its non-cell autonomous therapeutic effect.

We used a cell culture model to test whether Hsp40 could inhibit secretion of a pathogenic polyQ protein from cells. An expanded polyQ stretch of 81 repeats fused with CFP and a V5 tag (Q81-CFP-V5) was co-expressed together with the GFP control, QBP1, or Hsp40 in Neuro2A cells. Twenty-four h later, the culture media were replaced with fresh media to remove all of the dead cells, and after a further 6 h of incubation, culture media were collected and concentrated using centrifugal filters, and subjected to Western blot analysis. Q81-CFP-V5 was detected in the culture medium of cells (Fig. 5A), suggesting that pathogenic polyQ proteins are secreted from cells. In cells co-expressing QBP1, the amount of Q81-CFP-V5 detected in the culture medium was similar to that in cells co-expressing GFP. In contrast, Neuro2A cells co-expressing Hsp40 showed ~40% less Q81-CFP-V5 in the culture medium compared with cells co-expressing GFP, suggesting that Hsp40 inhibits secretion of the pathogenic polyQ protein from cells (Figs. 5A,B). Furthermore, siRNA-mediated knockdown of endogenous Hsp40 increased the secretion of Q81-CFP-V5 by ~40% compared with cells treated with a control siRNA (Figs. 5C,D), indeed confirming that Hsp40 inhibits polyQ protein secretion. These results imply that inhibition of secretion of the polyQ protein from cells by Hsp40 results in a non cell-autonomous therapeutic effect in R6/2 mouse brains, possibly via inhibition of the cell-cell transmission of the pathogenic polyQ protein.

Discussion

In this study we show for the first time that viral vector-mediated expression of a molecular chaperone, namely Hsp40 significantly improves the neurological phenotypes of a mouse model of the polyQ diseases. Although a recent study reported the effectiveness of another Hsp40 family member, HSJ1a (DNAJB2a) in R6/2 mice, this study cannot be directly translated to a therapy since it was performed by the crossing of transgenic mice [30]. In addition, although lentiviral vector-mediated delivery of DNAJB2a to a polyQ disease rat model has been investigated [37], it did not demonstrate the therapeutic effect on the neurological phenotypes, perhaps because of the limited spread of lentiviruses. We successfully overcame these above problems by using AAV, which infects a widespread area of the brain, and is already used in human patients [38].

We did not detect significant therapeutic effects of AAV5-Hsp70 on R6/2 mice unlike AAV5-Hsp40, possibly due to the very low infection rate of our AAV5-Hsp70 (data not shown), or differences in the effectiveness of Hsp40 and Hsp70 against mutant htt. Indeed, previous studies examining the effect of Hsp70 in R6/2 mice have shown no or very modest therapeutic effects [28,29]. Furthermore, a cell culture study demonstrated that Hsp40 family members are effective against the toxicity of mutant htt, while Hsp70 family members are ineffective [35]. Taken together, these studies indicate that Hsp40 family members may be more effective than Hsp70 family members against the toxic effects of mutant htt.

We surprisingly found that AAV5-Hsp40 inhibits inclusion body formation also in virus non-infected cells (Fig. 4), suggesting a non cell-autonomous therapeutic effect. Aggregation prone proteins that cause neurodegenerative diseases including pathogenic polyQ proteins, as well as α-synuclein which causes Parkinson's disease, and tau which causes the tauopathies have recently been suggested to be transmitted between cells, and this may be the mechanism leading to the progressive spread of neuropathology in these diseases [8–11]. We detected a significant amount of the pathogenic polyQ protein in the culture medium of Neuro2A cells (Fig. 5A), suggesting its cell-cell transmission, which is compatible with previous studies [39,40]. We further found that Hsp40 suppresses secretion of the pathogenic polyQ protein from cells (Fig. 5), suggesting that it may inhibit the cell-cell transmission of the pathogenic polyQ protein. The non-cell autonomous therapeutic effect of Hsp40 may also involve other mechanisms, such as (1) AAV5-Hsp40 infected neurons may create a better environment for contacting non-infected neurons [41] or (2) Hsp40 itself may be secreted to exert therapeutic effects on neighboring non-infected neurons, as is suggested for Hsp70 [42].

We were unable to detect the therapeutic effect of AAV5-QBP1 on the neurological phenotypes of R6/2 mice, although we successfully detected its inhibition of inclusion body formation. However, we and others have previously shown that expression of QBP1 exerts therapeutic effects on the neurological phenotypes of *Drosophila* and mouse models of the polyQ diseases [16,17,43]. Since the infection efficiency of all of the viruses used in this study was quite low (~30%), the extent of neurons expressing QBP1 in the R6/2 mouse brains was probably insufficient to exert a detectable effect on the phenotypes. On the other hand, in the case of AAV5-Hsp40, inhibition of polyQ protein secretion which should lead to an increase in the number of rescued neurons, likely contributed to its improvement of the neurological phenotypes. Other possibilities may also contribute to their varying effects, for example (1) Hsp40 is more effective than QBP1 in inhibiting polyQ protein misfolding/aggregation, and (2) Hsp40 can also support the degradation of misfolded proteins [19], while QBP1 cannot.

In this study we demonstrate a therapeutic strategy against the polyQ diseases using AAV5-Hsp40, which has great potential for clinical application, since AAVs are safe and are widely utilized in clinical trials [38]. We further suggest a novel therapeutic mode of action of Hsp40, namely suppression of pathogenic polyQ protein secretion from cells, which may consequently suppress its cell-cell transmission. Since the transmission of aggregation-prone proteins is thought to be involved also in other neurodegenerative diseases, Hsp40 may exert a non-cell autonomous therapeutic effect on these other diseases. Elucidation of how Hsp40 inhibits polyQ protein secretion should reveal new therapeutic targets and

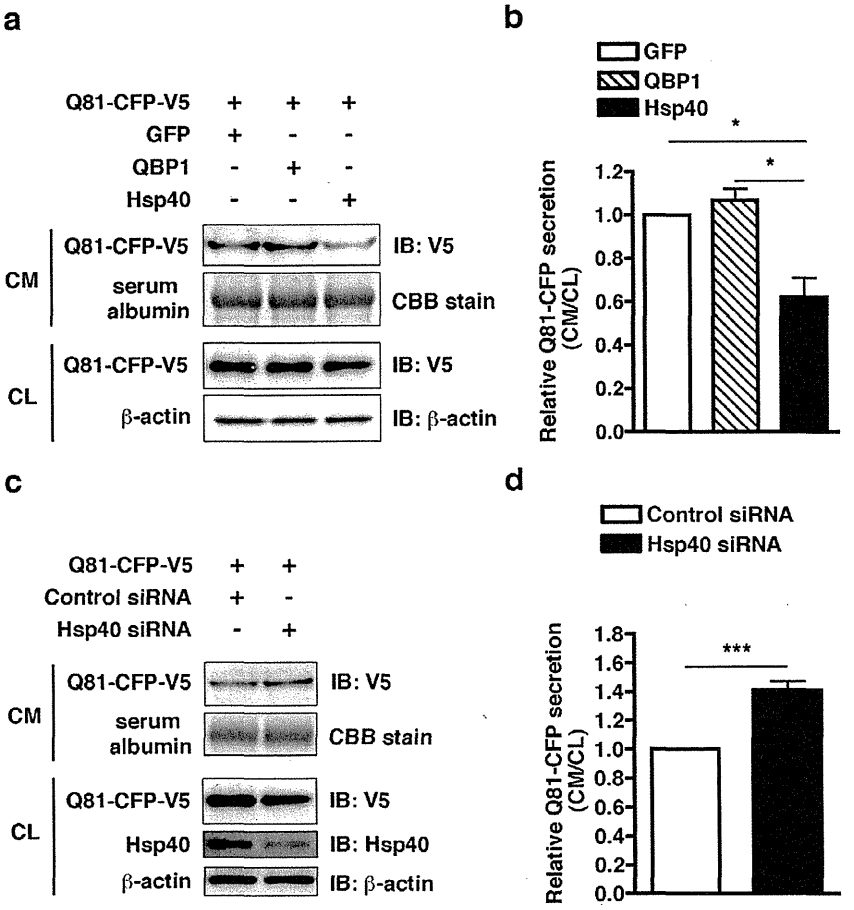

Figure 5. Hsp40 inhibits polyQ protein secretion in cultured cells. (A) Neuro2A cells were co-transfected with plasmids expressing Q81-CFP-V5 and either GFP, QBP1, or Hsp40, and the culture media (CM) and cell lysates (CL) were subjected to Western blot analysis with a V5 antibody to detect the Q81-CFP-V5 protein. (B) Relative amounts of Q81-CFP-V5 secreted into the culture media, calculated from the band intensities in (A), with the amount of Q81-CFP-V5 secreted from cells co-expressing GFP set to 1. (C) Neuro2A cells were transfected with a plasmid expressing Q81-CFP-V5 and siRNA against Hsp40 or a control siRNA, and the culture media (CM) and cell lysates (CL) were subjected to Western blot analysis with a V5 antibody to detect the Q81-CFP-V5 protein, and with an Hsp40 antibody. The loading controls are as in (A). (D) Relative amounts of Q81-CFP-V5 secreted into the cuture media, calculated from the band intensities in (C). In (A) and (C), serum albumin is shown as a loading control for the culture media, and β-actin as a loading control for the cell lysates. In (B) and (D), data are shown as means ± SEM of ≥ four independent experiments (*$p<0.05$, ***$p<0.001$).
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strategies for neurodegenerative diseases caused by aggregation-prone proteins.

Materials and Methods

Viral Vectors

Adeno-associated virus type 5 (AAV5) vector plasmids contained an expression cassette with a human cytomegalovirus enhancer/chicken β actin (CAG) promoter followed by the first intron of human growth hormone, target cDNA (either a tandem repeat of QBP1 fused to GFP [14], human Hsp40 (DNAJB1) [44], or GFP), and a simian virus 40 polyadenylation signal sequence, all positioned between the inverted terminal repeats of the AAV5 genome. AAV5 vectors were produced using the AAV5 plasmid, the AAV5 helper plasmid containing the rep and cap sequences from AAV5, as well as the pHelper plasmid from the AAV Helper-Free System containing the E2A, E4, and VA RNA genes of the adenovirus genome (Stratagene, La Jolla, CA). HEK293 cells were co-transfected with the AAV5 plasmid and two helper plasmids by the calcium phosphate method. Seventy-two h later, the cells were harvested and subjected to three rounds of freeze-thaw lysis. AAV5 vectors were then purified by two rounds of cesium chloride density gradient centrifugation. Vector titers were estimated by quantitative DNA dot-blot hybridization to be ~$0.2–1.6\times10^{13}$ genome copies/ml.

Animals

All animal experiments were performed in accordance with the guidelines of the Animal Ethics Committee of the National Institute of Neuroscience, National Center of Neurology and Psychiatry, Japan, and performed in accordance with the guidelines. Mice transgenic for human *huntingtin* exon 1 with approximately 150 CAG repeats (strain R6/2) [33] were obtained from the Jackson Laboratory (Bar Harbor, ME), and maintained on a B6CBAF1 background. Genotypes were analyzed and CAG repeat numbers of the transgenic mice were confirmed to be

similar by PCR as previously described [33]. Mice were housed on a 12-hour light/dark cycle, with food and water provided *ad libitum*. At least nine male R6/2 mice per group and wild-type littermate (WT) controls were used for the phenotype analyses, and two R6/2 mice were used for the inclusion body analyses.

AAV Injections

P7 old R6/2 mice were stereotaxically injected with 1 µl of virus solution (AAV5-GFP, AAV5-QBP1, or AAV5-Hsp40) into the striatum (coordinates 1 mm anterior to bregma, 2.25 mm lateral to the midline, and 3 mm ventral to the skull surface) at a rate of 0.1 µl/min using a 10 µl Hamilton syringe (Hamilton Company, Reno, NV) and an infusion pump (KD Scientific, Holliston, MA). For inclusion body analyses, AAV5-GFP was injected into one side of the striatum and AAV5-QBP1 or AAV5-Hsp40 into the other side. For phenotype analyses, the same virus was injected into both sides of the striatum.

Mouse Phenotype Analyses

For rotarod analysis, mice were tested at 4 and 7 weeks of age on an accelerating rotarod apparatus (Ugo Basile, Comerio, Italy) set to accelerate from 4 to 40 rpm over a period of 300 seconds. The time it took for each mouse to either fall off the rod or cling onto the rod for one full rotation was recorded. Mice were tested on the rod for three consecutive days, with three trials per day. The first day was regarded as training, and only the data from the second and third day were used. The highest and lowest values were excluded, and the middle four values were averaged. Grip strength analysis was performed at 9 and 12 weeks of age using a grip strength meter (Muromachi Kikai, Tokyo, Japan). Mice were placed gently by their tail on the metal grid of the meter so that they grip the grid with both forelimbs and hindlimbs, at which point they were pulled back gently with their tails, exerting a tension that is measured by the meter. Five trials were performed for each mouse, and the highest and lowest values were excluded and the middle three values were averaged. To measure open-field activity and rearing, mice at 5 and 8 weeks of age were tested using a spontaneous locomotor activity monitor (Supermex, Muromachi Kikai, Tokyo, Japan), consisting of an acrylic box of dimensions 40 cm×28 cm×31 cm with an activity sensor placed at the top and rearing sensors placed at the sides of the box. Mice were left in the box for a total of 15 min, and their activity count and rearing frequency were measured. For body weight analysis mice were weighed weekly. Mice were followed until their deaths in order to calculate their lifespans, a widely accepted and valuable parameter to assess therapeutic effects in these mice, which was approved by the institution's Animal Ethics Committee.

Tissue Preparation and Immunohistochemical Analyses

Mice were deeply anesthetized with sodium pentobarbital (100 mg/kg), and then perfused intercardially with saline followed by 4% paraformaldehyde (PFA) in phosphate-buffered saline (PBS). Brains were removed, post-fixed in 4% PFA in PBS at 4°C overnight, and then cryoprotected in 30% sucrose in PBS at 4°C for 24 h. Frozen 10 µm sections were cut using a cryostat. For immunohistochemical analysis, sections were blocked in PBS containing 5% goat serum and 0.1% Triton X-100 for 1 h at room temperature. The sections were then incubated with a mouse anti-huntingtin antibody (1:250; MAB5374, Millipore, Billerica, MA), and either a rabbit anti-Hsp40 antibody (1:500; SPA-400, Enzo Life Sciences, Farmingdale, NY) or a rabbit anti-GFP antibody (1:250; A11122, Invitrogen, Carlsbad, CA) at 4°C overnight, followed by an Alexa Fluor 596-conjugated goat anti-mouse IgG antibody and an Alexa Fluor 488 goat anti-rabbit IgG antibody (1:1000, Invitrogen) for 1 h at room temperature. Sections were mounted with Slowfade Gold antifade reagent with DAPI (Invitrogen) and examined using a confocal laser scanning microscope (FV1000; Olympus, Tokyo, Japan). The average fluorescence intensity of representative cells that were regarded as either "infected" or "non-infected" in each sample were measured using the Image J software, confirming that the two population of cells can readily be distinguished in the images (Fig. S3).

Cell Culture, Transfection and Western Blot Analysis

Neuro2A cells (obtained from ATCC) were grown and maintained in DMEM supplemented with 10% (v/v) FBS. Cells were plated on a 35-mm dish at a density of 4×10^5 cells per dish on the day before transfection. For the overexpression experiment, a plasmid vector encoding a tandem repeat of QBP1 fused to GFP, human Hsp40 [44], or GFP was transiently co-transfected with the Q81-CFP-V5 vector encoding an expanded polyQ stretch of 81 repeats fused with CFP and a V5 tag, using Lipofectamine LTX with PLUS reagent (Invitrogen). For the knockdown experiment, siRNA targeted against mouse Hsp40 (Santa Cruz Biotechnology, Santa Cruz, CA) or a control siRNA (RNAi Inc, Tokyo, Japan) was cotransfected with the Q81-CFP-V5 vector using Lipofectamine 2000 reagent (Invitrogen). At 24 h after transfection, culture media were replaced with fresh media, and after a further 6 h of incubation, culture media were collected and whole cell lysates were prepared with 1% Triton X in tris-buffered saline. For Western blot analysis, the culture media were concentrated using 30 kDa cutoff Amicon Ultra filters (Millipore). Q81-CFP-V5 in the concentrated culture media and whole cell lysates was separated using 10% SDS-PAGE gels and transferred onto PVDF membranes (Bio-Rad, Hercules, CA). The membranes were incubated overnight with an HRP-conjugated anti-V5 antibody (1:2500, Invitrogen) at 4°C. The HRP signal was visualized with SuperSignal West Femto Chemiluminescent Substrate (Thermo Fisher Scientific Inc, Rockford, IL), captured with a LAS-3000 mini CCD imaging system (Fujifilm, Tokyo, Japan), and band intensities were quantified using the Image J software. For the siRNA experiments, the membranes were then stripped and incubated with a rabbit anti-Hsp40 antibody (1:5000, SPA-400, Enzo Life Sciences Inc, Farmingdale, NY) at 4°C followed by an HRP-conjugated anti-rabbit IgG secondary antibody (1:10000, Thermo Fisher Scientific), and visualized as above.

Statistical Analyses

For the rotarod, open-field activity, rearing, grip strength and weight data, statistical analyses were performed by using one-way analysis of variance followed by Tukey's multiple comparison test to assess for significant differences between individual groups. The survival data was analyzed using the Log-rank test. For the inclusion body formation and Western blot analyses, Student's *t*-test was used. For all analyses, $p<0.05$ was considered as significant.

Supporting Information

Figure S1 AAV5 injection into the mouse striatum at P7 results in widespread expression of the transgene. R6/2 mice at P7 were injected in the right striatum with 1 µl of AAV5-QBP1, and 2 weeks later the expression of QBP1 was analyzed by immunohistochemistry. This widespread expression of QBP1 throughout the brain lasts for at least 13 weeks (data not shown). (PDF)

Figure S2 AAV5-QBP1 inhibits polyQ inclusion body formation in virus infected neurons of polyQ disease

mice. R6/2 mice at P7 were injected with AAV5-GFP on one side of the striatum and AAV5-QBP1 on the other side, and at 4, 8, and 14 weeks of age htt inclusion body formation in virus infected neurons of the striatum (left) and cortex (right) was assessed by immunohistochemistry. Data are shown as means ± SEM of ≥ 6 fields of view, in which over 180 cells were counted (*$p<0.05$, ***$p<0.001$). Representative results of two mice analyzed are shown.
(PDF)

Figure S3 AAV5 "infected" and "non-infected" cells can be clearly distinguished from their fluorescence intensity. The fluorescence intensity of representative cells that were regarded as either "infected" or "non-infected" in photographs of immunostained brain sections of R6/2 mice injected with either AAV5-GFP (left), AAV5-QBP1 (middle) or AAV5-Hsp40 (right). For each sample, a total of over 100 representative cells were analyzed from 5–6 fields of view.
(PDF)

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Author Contributions

Conceived and designed the experiments: HAP T. Takeuchi SM T. Toda KW YN. Performed the experiments: HAP T. Takeuchi HF KY CI HY. Analyzed the data: HAP T. Takeuchi T. Toda KW YN. Contributed reagents/materials/analysis tools: SM. Wrote the paper: HAP T. Takeuchi YN.

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Two Cohort and Three Independent Anonymous Twin Projects at the Keio Twin Research Center (KoTReC)

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The Keio Twin Research Center has conducted two longitudinal twin cohort projects and has collected three independent and anonymous twin data sets for studies of phenotypes related to psychological, socio-economic, and mental health factors. The Keio Twin Study has examined adolescent and adult cohorts, with a total of over 2,400 pairs of twins and their parents. DNA samples are available for approximately 600 of these twin pairs. The Tokyo Twin Cohort Project has followed a total of 1,600 twin pairs from infancy to early childhood. The large-scale cross-sectional twin study (CROSS) has collected data from over 4,000 twin pairs, from 3 to 26 years of age, and from two high school twin cohorts containing a total of 1,000 pairs of twins. These data sets of anonymous twin studies have mainly targeted academic performance, attitude, and social environment. The present article introduces the research designs and major findings of our center, such as genetic structures of cognitive abilities, personality traits, and academic performances, developmental effects of genes and environment on attitude, socio-cognitive ability and parenting, genes x environment interaction on attitude and conduct problem, and statistical methodological challenges and so on. We discuss the challenges in conducting twin research in Japan.

Keywords: twin cohort, longitudinal, infancy, childhood, adolescence, adulthood

The Keio Twin Research Center (KoTReC) was established in 2009 as an integrated organization of two twin cohort projects at Keio University; the Keio Twin Study (KTS) for adolescence and adulthood, and the Tokyo Twin Cohort Project (ToTCoP) for infancy and childhood. These two

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twin projects have been independently conducting various psychological, behavioral, neurophysiological, and molecular genetic studies for several years, and have involved a range of funding sources and research teams. The early work of the KTS and ToTCoP was reported by Shikishima et al. (2006) and Ando et al. (2006), respectively. The KoTReC has also collected three anonymous one-shot twin data sets, one of which uses a cross-sectional design.

The current article provides a brief outline of the current status and main findings at the KoTReC.

Purpose of Twin Studies

Twin studies typically have three main aims: to obtain relevant information *of* twins, *by* twins, and *for* twins. Studies *of* twins are designed to collect information about factors differentiating twins from singletons, such as the development of linguistic abilities and sibling relationships. Studies *by* twins are typically behavioral genetic studies in which genetically and environmentally systematic information of twins are utilized as a biometrical method. This second type of research is the focus of the KoTReC. Studies *for* twins are focused on providing evidence-based support for nursing and educating twins, mainly in infancy and childhood, by producing relevant information about the causes of parenting stress and environmental effects on infant growth and development.

The age ranges of the two cohorts in the KTS and ToTCoP are different, and the aims of the projects also differ. In the KTS, which includes participants between 15 and 40 years of age, almost all the research is conducted with a behavioral genetic focus (i.e., a *by*-twins study), including psychological, psychiatric, sociological, socio-economic, neurological, and molecular genetic characteristics. On the other hand, the ToTCoP, which examines twin participants from birth to 6 years of age, includes all three of the main aims of twin studies. For studies *of* twins, a set of singleton data that is comparable to twin data were obtained for several important variables.

Recruitment of Twin Participants

The strategy to recruit twins and their families in both the KTS and ToTCoP is to send letters to twin families identified by the Basic Resident Register (BRR; nation-wide census). The BRR is a quasi-complete (i.e., complete at a specific time in a specific area) residential record of each municipal area, which contains each resident's name, gender, residential address, and date of birth. This information gathering is authorized by each municipal area's regulations, and data are available at city halls. Twins or higher multiples can be identified as individuals who share the same date of birth and address. With this method, it is difficult to recruit newborn and adult twins because it takes several months for newborns to be registered on the BRR and because most adult twins live apart. Because these data are not obtained electronically, but rather by printed documents with a substantial cost, well-trained staff are required to identify multiple births and transfer the information manually. This strategy has a number of methodological shortcomings (see Ando et al., 2006), but it is the only way to obtain 'population-based', rather than hospital-based or twin support group-based, twin data in Japan.

These research projects included three residential twin data collection periods from the BRR (1998–2002, 2003–2004, 2009), and cover the Tokyo metropolis and the neighboring prefectures (Kanagawa, Chiba, and Saitama). The 1998–2002 data contain approximately 10,000 pairs, which substantially overlap with the 2003–2004 data set, which contains 46,000 pairs. In addition, data from around 1,000 pairs of twins under 3 years of age were added in 2009. Currently, approximately 48,000 sets of multiple birth families are registered at our center.

Additional recruitment of twin child cohorts was conducted by voluntary participation through a poster campaign in public healthcare centers in the target areas and magazine advertisements in publications distributed nation-wide.

Zygosity Diagnosis and DNA Data

In order to identify twins' zygosity, the KTS project mainly used a three-item questionnaire administered to twins themselves (Ooki et al., 1990), whereas the ToTCoP administered the questionnaire to parents (Ooki & Asaka, 2004). These questionnaires asked for judgments about the twins' physical similarities, and experiences of being mistaken for each other. The items in the ToTCoP questionnaire (and the KTS questionnaire) were as follows: 'Were your twin children (you and your co-twin) as alike as two peas in a pod?' 'Were your twin children (you and your co-twin) mixed up (as children)?' and 'If so, by whom were your twin children (you) mixed up?' This questionnaire has been found to have almost 95% accuracy by comparison with genetic markers (Ooki & Asaka, 2004).

DNA samples were collected from approximately 600 pairs of adult twins (KTS) by analyzing blood (approximately 240 pairs in 1998, partially replicated in 1999), buccal smear (approximately 200 pairs in 2005), nail or hair roots (approximately 100 pairs in 2010), and saliva (approximately 60 pairs in 2011; Table 1). These DNA data were also used to identify zygosity. Agreement rate between the DNA-based diagnoses and the questionnaire-based diagnoses was 93.0% (94.3% for monozygotic (MZ) and 87.5% for dizygotic (DZ); a preliminary result was reported by Shikishima et al., 2007).

In the following sections, the research design and major findings of each of the sub-projects at the KoTReC are introduced.

TABLE 1

Data Collection History of the KTS (KTP)

			N	1998	1999	2000	2001	2002	2003	2004	2005	2006	2007	2008	2009	2010	2011	2012	Publications
Entry	Twin	Wave 1	315 pairs	→	→	→	→	→	→	→	→	→	→	→	→	→	→	→	
		Wave 2	45 pairs		→	→	→	→	→	→	→	→	→	→	→	→	→	→	
		Wave 3	354 pairs			→	→	→	→	→	→	→	→	→	→	→	→	→	
		Wave 4	312 pairs				→	→	→	→	→	→	→	→	→	→	→	→	
		Wave 5	672 pairs										→	→	→	→	→	→	
		Wave 6	548 pairs													→	→	→	
	Parent of	Wave 1 & 2	196 Mothers					X									→	→	
			173 Fathers					X											
		Wave 6	600 Mothers																
Survey			On campus	X	X	X	X	X	X	X	X	X	X			X	X	X	
			On mail				X	X		X	X	X				X	X	X	
			Online												X	X	X		
Biology	DNA[a]			Bl	Bl						Bc	Bl				N & H/Bl		S/Bl	
			N of pairs	238	(70)						218	7				101/11		59/6	
	Hormone (teststerone)											X							Uchida, et al, 2006
Cognition	Nx_full										X	X				X	X		Shikishima et al., 2009
	Nx_sub			X	X			X		X	X								Ando et al., 2001
	BAROCO_full										X	X				X			Shikishima et al., 2009
	BAROCO Short															X	X		Shikishima, et al., 2011a
	WAIS					X	X	X	X										
	ERP/EEG					X	X	X	X										
	Reaction time					X													
	Inspection time					X													
	Working memory			X															Ando et al., 2001
	3D mental rotation			X	X			X		X	X								Suzuki et al., 2011
Decision making	Economic game[b]											PG			PG	PG	D/U		
	Time preference														X		X		
	Allais paradox															X	X		
	Ellsberg paradox															X	X		
Personality	NEO-PI-R			X	X		X	X											Ono et al., 2000/ Yamagata et al., 2006/ Jang et al., 2001/Jang et al., 2008 / McCrae et al., 2008/Ekehammar et al., 2010
	NEO-FFI										X		X				X	X	
	TCI				X		X	X							X	X		X	Ando et al., 2004/ Rushton et al., 2009
	BIS/BAS								X			X					X		Takahashi et al., 2007
	SPSRQ									X									
	EC								X										Yamagata et al., 2005a

TABLE 1
Continued.

		N	1998	1999	2000	2001	2002	2003	2004	2005	2006	2007	2008	2009	2010	2011	2012	Publications
Mental health	HADS		X			X	X					X						Ono et al., 2002
	SUBI			X		X	X								X		X	
	AQ						X	X										
	STAI							X										
	SDS							X										
	QIDS-SR									X								
	QLS									X								
Attitude	Social attitude						X	X							X			Shikishima et al., 2006
	Voting behavior													X	X			
	RSES			X		X	X	X								X	X	Kamakura et al., 2001/Kamakura et al., 2007/Shikishima & Ando, 2004
	General trust			X		X	X									X	X	Shikishima et al., 2006/Hiraishi et al., 2008b
	Empathy			X		X	X											Shikishima et al., 2011b
	Authoritarianism						X	X							X			Shikishima et al., 2008
Gender	PSAI									X								
	Klein Grid									X								Hiraishi et al., 2011
	BSRI			X		X	X											Sasaki et al., 2008
Eating	EAT					X	X											
	EDI								X	X								Kamakura et al., 2003
	TFEQ-R21									X								
Physical	Smoking/drinking			X		X	X									X		
	Height/weight			X		X	X		X	X						X		Hur et al., 2008
	2D4D								X	X								Hiraishi et al., 2011
	Laterality									X						X		
Environment	SIDE						X	X										
	School background															X		
	PBI			X		X	X											Shikishima et al., in press
	FACESIII						X	X							X		X	Shikishima & Ando, 2004
Parental data	FACESIII						X											Shikishima & Ando, 2004
	PBI						X											
	RSES						X											
	SUBI						X											
	HADS						X											
	TCI																X	

Note: [a]Bl = *blood*; S = *saliva*; Bc = *buccal smear*; N = *nail*; H = *hair root*.
[b]PG = public game; D = dictator game; U = ultimatum game.
Abbreviation of instruments not introduced in the text.
SPSRQ (The Sensitivity to Punishment and Sensitivity to Reward Questionnaire; Torrubia et al., 2001); EC (Japanese version of Effortful Control Scale; Yamagata et al., 2005b); Klein Grid (Klein et al., 1985); RSES (Rosenberg Self-Esteem Scale; Rosenberg, 1965); PSAI (Pre-School Activities Inventory; Golombok & Rust, 1993); BSRI (Bem Sex Role Inventory; Bem, 1974); EAT (Eating Attitude Test; Garner et al.,1982); TFEQ-R21 (Three Factor Eating Questionnaire; Stunkard & Messick, 1985); SIDE (The Sibling Inventory of Differential Experience; Daniels & Plomin, 1985); PBI (Parental Bonding Instrument; Parker et al., 1979); FACESIII (Family Adaptability and Cohesion Scale; Olson, 1985).

The KTS

The KTS, originally named the Keio Twin Project (Shikishima et al., 2006), was established in 1998 to conduct behavioral genetic studies in adolescence and early adulthood. Twins entering the study in 1998 were aged between 15 and 30 years of age, and new participants within the same age range were added subsequently. Table 1 shows the major variables and survey administration year by year. As shown in the table, there were six entry time points in 1998, 1999, 2001, 2002, 2007, and 2011, totaling more than 2,000 twin pair data sets, some of which include their parents' data.

The variables investigated include cognition (general and specific cognitive abilities), decision-making tasks, personality traits (two-, five-, and seven-factor models), mental health, attitude and gender, eating, physical traits, and family and school environment.

Cognition and Decision Making

Cognition has been an important phenotype of interest in the history of behavioral genetics. At the KoTReC, the Kyodai Nx15- (Lynn et al., 1987; Osaka & Umemoto, 1973, Shikishima et al., 2009) is used as a full-scale intelligence test to measure individual difference of general cognitive ability in adolescence and adulthood. The Kyodai Nx15- is the most systematic group intelligence test available for this age range in Japan, and consists of 12 sub-scales covering verbal and spatial aspects of reasoning, memory, and processing speed. In situations where the full-scale version is too long to be administered (i.e., in an experimental session with many variables), a four-subscale version with two verbal and two spatial sub-tests is used.

Overall, our results indicate that the cognitive domain is a unitary feature of its genetic structure. Ando et al. (2001) reported that different aspects of working memory, storage, and executive functions of verbal and spatial modalities are mediated by a single latent genetic factor that also explains general cognitive ability, measured by the sub-scale version of the Kyodai Nx-15. Shikishima and colleagues developed a syllogistic reasoning test called BAROCO (Shikishima et al., 2009), named from a mnemonic word to memorize a syllogism form in classical logic, with 100 items, and reported that its genetic component completely overlapped with those of the Kyodai Nx-15. Based on these findings, a shortened five-item version, the BAROCO Short, was developed and validated (Shikishima et al., 2011a).

Researchers in our project recently began investigating the possibility of a 'general intelligence gene' by comparing epigenetic differences of discordant identical twin siblings (Yu et al., 2012).

Gender differences in spatial ability were independently investigated using a mental rotation task (Suzuki et al., 2011; Vandenberg & Kuse, 1978). A sex limitation analysis revealed that there were no gender-specific genetic factors affecting this trait, but that the additive genetic influence was greater in males.

Endophenotypes of cognitive abilities, event-related potential (ERP) indices in a working memory task and electroencephalography under resting conditions with eyes open and closed were measured individually for approximately 150 pairs of twins, together with the full-scale Wechsler Adult Intelligence Scale and specific cognitive abilities, simple reaction time and inspection time, in an international collaborative study (Wright et al., 2001). These data, and data from another endophenotype (structural brain imaging examined using magnetic resonance imaging), will be analyzed and published in the near future.

We recently began to conduct behavioral genetic studies of 'decision-making' tasks, such as economic games (a public goods task, and the dictator and ultimatum games), time preferences, and Allais and Ellsberg paradoxes, which are commonly used tasks in behavioral economics. Collaborative studies with economists are also underway at the center.

Personality and Mental Health

Personality traits have been another important research focus in behavioral genetics, and studies in our project have investigated the genetic structure of personality and related phenotypes. Ono and colleagues reported the results of a univariate genetic analysis of the five-factor model of personality using the NEO Personality Inventory Revised Test (NEO-PI-R, Costa & McCrae, 1992; Yoshimura et al., 1998). The results clearly replicated a very robust finding of this field that there are substantial genetic and non-shared environmental influences on personality traits (Ono et al., 2000). Yamagata conducted an international comparative study of the five-factor model by conducting genetic factor analysis based upon 30 sub-scales of the NEO-PI-R, revealing that the genetic structure is strikingly congruent among Japan, Germany, and Canada (Yamagata et al., 2006). Using the same data set, Jang reported genetic comorbidity between Neuroticism and Agreeableness, and their molecular bases (Jang et al., 2001), and proposed a two-higher-order-genetic-factor structure of the Big Five factors (Jang et al., 2006). Furthermore, McCrae, who originally developed the NEO-PI-R, reported that these higher-order genetic factors contained artifacts as well as substance effects (McCrae et al., 2008). Conversely, Rushton proposed a single general personality factor model and reported its genetic validity using our NEO-PI-R and the Temperament and Character Inventory (TCI) data (Rushton et al., 2009).

The TCI was developed by Cloninger, based upon his theory of personality development (Cloninger et al., 1993), which proposes that four temperamental traits (Novelty Seeking, Harm Avoidance, Reward Dependence, and Persistence) are driven by genetic neurotransmission-related factors, whereas three character traits (Self-Directedness, Cooperativeness, and Self-Transcendence) are determined

by post-natal experience. A study in our project attempted to verify this theory, revealing that Novelty Seeking, Harm Avoidance, and Reward Dependence are genetically independent, as Cloninger et al.'s (1993) theory predicts, but persistence and the three character traits exhibited genetic overlap with the three temperamental traits (Ando et al., 2002). In addition, we found that one facet of Novelty Seeking (Exploratory Excitement) is strongly genetically correlated with Harm Avoidance, so should be rearranged by changing combination of facets to make scales genetically consistent (Ando et al., 2004). Yamagata and colleagues (2005) applied the same methodology to examine the genetic structure of Effortful Control (Rothbart et al., 2000) and confirmed its genetic coherence, supporting the validity of the theory.

Ono and colleagues investigated the genetic and environmental overlap between temperamental TCI traits and depressive symptoms measured by the Hospital Anxiety Depression Scale (Kitamura, 1993; Zigmond & Snaith, 1983), suggesting that there are no independent 'depression-specific genes', but that depressive symptoms are dependent on genetic factors involved in normal temperamental dimensions under specific unique environments (Ono et al., 2002). The twin studies at KoTReC are not hospital-based studies, and no medically diagnosed participants have been identified. However, the data from several scales related to mental health and psychiatry, including the Subjective Well-Being Inventory (SUBI; Sell & Nagpal, 1992), the Autism-Spectrum Questionnaire (AQ; Baron-Cohen et al., 2006), the State and Trait Anxiety Inventory (STAI; Spielberger et al., 1970), the Zung Self-Rating Depression Scale (SDS), the Quick Inventory of Depressive Symptomatology (QIDS-SR), and the Quality of Life Scale (QLS; Rush et al., 2003), are available for our normal twin samples. In addition, a univariate genetic analysis of Eating Disorder Inventory (EDI) data in this sample revealed substantial shared environmental influences on four of five sub-scales of the EDI (Kamakura et al., 2003).

Because the KTS is designed in a longitudinal fashion as shown in Table 1, several cognitive and personality phenotypes were measured at different time points for the same individuals. Developmental changes and the stability of the Behavioral Inhibition System (BIS) and Behavioral Activation System (BAS; Carver & White, 1994) — two measures of temperament based on Gray's reinforcement sensitivity theory — have been investigated (Takahashi et al., 2007). The results indicated that genetic influences contribute only to continuity, whereas environmental influences contribute to both continuity and change in the two traits, and that the degree of genetic influences does not differ across time.

Attitudes

Results similar to those reported by Takahashi et al.'s (2007) BIS/BAS longitudinal study were reported for the self-esteem scale (Kamakura et al., 2007). Developmental stability was affected by genetic and non-shared environmental factors, whereas developmental changes were affected by non-shared environmental factors. However, the degree of genetic influence increased during adolescence and young adulthood.

Self-esteem is a personality trait, and can be considered as a type of attitude. Our twin studies have involved a number of measures of attitudes other than self-esteem, such as general trust, voting behavior, empathy, and authoritarianism (Table 1). Shikishima reported a series of behavioral genetic studies on attitude variables traditionally thought to be transmitted through the family environment. The results revealed a substantial genetic influence on authoritarianism (Shikishima et al., 2008) and trust (Shikishima et al., 2006), with no significant effect of shared environmental factors. However, significant environment × environment interactions were found, indicating that shared family environmental factors significantly affected empathy for individuals exhibiting high or very low parental warmth (Shikishima et al., 2011b). A study using direction of causation (DOC) analysis (Heath et al., 1993), an application of behavioral genetic methodology, revealed that the level of general trust can be predicted by personality factors (Extraversion and Agreeableness; Hiraishi et al., 2008a), indicating that humans adaptively control the activation of domain-specific mental mechanisms in accord with domain-general genetic traits like personality.

Other Variables

As shown in Table 1, a large number of variables have been investigated in previous studies, some of which have been published. These variables include eating disorder symptoms (EDI; Kamakura et al., 2003), gender role personality factors (Sasaki et al., 2009), testosterone (Uchida et al., 2006), the relationship between second to fourth finger ratio (2D4D) and sexual orientation (Hiraishi et al., 2012), and parenting (Shikishima et al., in press).

In 2002, 2010, and 2011, parents of the twin participants in our studies provided information about several additional variables. Since 2009, the Web interface of our project (http://www.futago-labo.net/ in Japanese only) has been available to supplement some experimental and questionnaire data.

The ToTCoP

ToTCoP was established to conduct a longitudinal cohort twin study starting from 2003 (Ando et al., 2006) and continues to conduct studies *of*, *by*, and *for* twins from infancy. This project consists of four data sources; (1) questionnaires (Table 2), (2) cognitive and social investigations in the home (Table 3), (3) cognitive, linguistic, and social investigations in university-based laboratories (Table 4), and (4) brain

TABLE 2

Timeline of Investigation Tools in Questionnaire-Based Research

	Month	Entry	9 months	12 months	15 months	18 months	24–30 months	36 months	42 months	48 months	Preschool	1st grade
		1,639	142	256	376	458	249	585	675	475	392	303
Children's characteristics	Body sizes	x	x	x	x	x	x	x		x		
	Stressful life events		x	x	x		x	x		x		
	Zygosity				x		x	x				
	Laterality		x	x	x		x	x				
	Motor development		DenverII				DenverII					
	Temperament			IBQ-R R-ITQ			ECBQ		CBQ		BIS/BAS	BIS/BAS
	Developmental disorder-related symptoms/social behavior	Excerpt from M-CHAT	M-CHAT		Yale Screener	M-CHAT	Yale Screener				ADHD school refusal behavior (original)	ADHD school refusal behavior (original)
	Sleeping behavior	x			BISQ		BISQMEQ		BISQ	MEQ	MEQ	MEQ
	Problem behavior							SDQ		SDQ	SDQ ODBI KINDL	SDQ ODBI KINDL
	Nutrition	x	x	CFQ		x			x			
Parenting behavior/ environment	Attachment		MAI		MAI							
	Cultural/ educational environment					x			x		x (original)	x (original)
	Parental behavior		x		x				x		x (original)	x (original)
	Home environment		EES			EES					EES (6 items)	EES (6 items)
	Twin situation		x		x		x			x	SIB (18 items)	
Parenting stress	Depressive symptom			SDS		SDS		SDS		SDS		
	Parental stress		PSI		PSI						PSI	PSI
	Social support		x	x	x		x	x		x	x(1 item	x(1 item
	Marital status										RAM Short Marital-Adjustment Scale	RAM Short Marital-Adjustment Scale

Note: ADHD-RS-IV = ADHD Rating Scale — IV, (DuPaul et al., 1998); BISQ = Brief Infant Sleep Questionnaire, (Sadeh, 2004); BIS/BAS = Behavioral Inhibition and Activation Systems Scales (Carver & White, 1994); CBQ = Children's Behavior Questionnaire (Ahadi et al., 1993); CFQ = Child Feeding Questionnaire (Birch, 2001), Denver II (Frankenburg et al., 1992); ECBQ = Early Childhood Behavior Questionnaire (Putman et al., 2002); EES = Evaluation of Environmental Stimulation (Anme, 1997); MAI = Maternal Attachment Inventory (Müller, 1994); IBQ-R = Infant Behavior Questionnaire-Revised (Gartstein & Rothbart, 2003; Nakagawa & Sukigawa, 2005); MEQ = Morningness-Eveningness Questionnaire (Horne & Östberg, 1976); M-CHAT = Modified Checklist for Autism in Toddlers (Baron-Cohen et al., 1992; Robins et al., 2001); ODBI = Oppositional Defiant Behavior Inventory (Harada et al., 2004); PTCI = Preschool Temperament & Character Inventory (Constantino et al., 2002); KINDL = Questionnaire for Measuring Health-Related Quality of Life in Children and Adolescents (Bullinger et al., 1994); RAM = Relationship Attribution Measure (Fincham & Bradbury, 1992); SDQ = Strength and Difficulty Questionnaire (Goodman, 1999); SDS = Self-rated Depression Scale (Zung, 1965); SIB = The Sibling Inventory of Behavior (Volling & Blandon, 2005); Short Marital-Adjustment Scale (Locke & Wallace, 1959).

TABLE 3
Timeline of Home Assessment

Age *N* (pairs)		12 months 127	18 months 236	24 months 277	36 months 279	48 months 188
	Cognitive ability	Bayley II	Bayley II	Bayley II	K-ABC	K-ABC
	Vocabulary	CDI	CDI	CDI		
	Socio-cognitive ability	ESCS	ESCS			
				ToM	ToM	ToM
				EF	EF	EF
Observation	Parent–child relationship	x	x	x	x	x
	Twin sibling relationship				x	x
Questionnaire	Parenting stress	PSI	PSI	PSI	PSI	PSI
	Parenting behavior	x	x	x	x	x
	Depressive symptom			SDS		
	Marital relation				Marital love	Marital love
	Mom's personality				NEO-FFI	
	Problem behavior					SDQ

Note: Bayley = Bayley Scales of Infant Development (Bayley, 1993); ESCS = Early Social Communication Scales (Mundy et al., 2003); K-ABC = Kaufman Assessment Battery for Children (Kaufman & Kaufman, 1983); CDI = MacArthur Communicative Developmental Inventories (Fenson et al., 1993); PSI = Parenting Stress Inventory (Abidin et al., 1995) ; Marital love (Locke & Wallace, 1959).

TABLE 4
Timeline of Laboratory Assessment

Age *N* (pairs)		42 months 245	60 months 175
	Cognitive ability	K-ABC	K-ABC
	Reading	x	x
	Socio-cognitive ability	EF	
		ToM	
Observation	Parent–child relationship	x	x
	Twin sibling relationship	x	x
	Social communication (peer)	x	x
Questionnaire	Parenting Stress	PSI	PSI
	Parenting Behavior	x	x
			PFQ
			PDI
	Marital relation	Marital love	Marital love
	Mom's personality	NEO-FFI	
	Problem behavior	SDQ	SDQ
	Temperament		CBQ-VSF
	Twin sibling relationship		SIB
		MISR	MISR

Note: PFQ = Parental Feelings Questionnaire (Deater-Deckard, 1996; Deater-Deckard, 2000); PDI = Parental Discipline Interview, (Deater-Deckard, 2000); SIB = Sibling Inventory of Behavior (Volling & Blandon, 2005); MISR = Maternal Interview of Sibling Relationships (Stocker et al., 1989).

activity and motor skill experiments in university-based laboratories.

Questionnaire-Based Research

Table 2 shows the timeline of the questionnaire investigation tools used for each specific time point from infancy to childhood when twins enter elementary school. The variables in these questionnaires are related to children's characteristics and parents' characteristics, and both types of questions are given to both mothers and fathers until participants are 36 months old. The versions for fathers are partially shortened or different from the versions for mothers, which contain additional items regarding parenting stress. When participants are aged 42 months or older, the questionnaires are administered only to twins' mothers, because asking twins' fathers to answer questionnaires tended to lower the total response rate, and the reliability of fathers' evaluations of twins' behavior was low.

As Table 2 indicates, the number of participating twin families (over 1,600) was relatively large at the first session, constituting approximately 55% of the total twin births in the target area. Although we observed a high degree of data attrition, we retained substantial numbers of twin pairs that could be investigated longitudinally. For example, Fujisawa and colleagues investigated the relationship between head circumference growth from birth to 10 months of age, and socio-cognitive ability at 19 months. Although no significant phenotypic correlation was found between them, significant genetic and shared environmental correlations in opposite directions (i.e., genetically negative and environmentally positive) were reported (Fujisawa et al., 2012a). In addition, Yamagata examined the longitudinal association between authoritative parenting and children's peer problems at 42 and 48 months using a longitudinal MZ twin difference design. They reported that when genetic and family environmental covariates were controlled, authoritative parenting and children's peer problems concurrently influenced each other, peer problems increased authoritative parenting, and authoritative parenting decreased peer problems, canceling each other out (Yamagata et al., in press).

For preschool and first grade elementary school children, additional twin families were recruited. The main research target of these two age groups is social adaptation to changes in educational environmental conditions from preschool to elementary school. To tap these

TABLE 5
The Variables List of the CROSS Study

		Early childhood	Middle childhood	Late childhood		Adolescence		Adult
		3–5 years	7–9 years	10–12 years		13–18 years		19–26 years
	Mailed	3,291	3,196	3,396		5,279		5,095
	Returned (entry)	859	857	740		960		697
	Response rate	26.10%	26.80%	21.80%		18.90%		13.70%
	Informant	Parent	Parent	Child	Parent	Child	Parent	Child
Family and parents	Family structure	X	X		X		X	X
	Age, sex, zygosity, and sib order	X	X	X	X	X	X	X
	Twinship							X
	Sharing toys, room, clothes, etc.	X	X	X	X	X	X	
	Assisted reproductive technology	X	X		X		X	
	Maternal smoking/drinking in pregnancy				X		X	
	Sib interaction	X	X					
	Child-rearing attitude				X		X	X
	Join twin-mother organization?	X	X					
	Same class in nursery school?	X	X		X		X	
Academic-related variables	Income							X
	Name of schools	X	X	X	X	X	X	X
	Academic achievement		X	X	X	X	X	
	Time spent for academic learning		X	X	X	X	X	X
Academic-related variables	Academic motivation					X		
	Learning strategy					X		
	Contingent cognition of effortful results					X		
	Attribution of failure					X		
	Cognition of competition					X		
	Helplessness					X		
In classroom and out of classroom	Intrinsic/extrinsic motivation			X		X		
	Academic autonomy			X		X		
	Goal structure			X		X		
	Self-efficacy			X		X		
	Class mate			X		X		
	Teacher's explanation length			X		X		
	Skill for integrated learning			X		X		
	Class size			X		X		
	Classroom teacher			X		X		
	After-school activities			X		X		
	Academic rank of high school					X		X
	What is society/learning			X		X		X
	Club activity							X
	Family cultural environment		X	X		X		X
	Learning out of school	X	X	X		X		X
Mental health	Depression							X
	QOL							X
	Loneliness				X			X
	Adaptation to school			X		X		
	Suicide					X		X
	CFS			X				
	Problematic behavior	X	X	X		X		
	Gender orientation/identification	X	X	X	X	X	X	X
Personality and social attitude	Personality		X	X		X		X
	Language development	X						
	Correctivism					X		X
	Authoritarianism					X	X	X
	Value					X		
	Hobby							X
	Job supervisor							X
Body and physics	Height/weight	X	X	X		X		X
	Head/chest circumference	X						X
	Eye sight			X		X		X
	Sleeping	X	X	X		X		X
	Blood pressure/ fever					X		X
	Mense					X		
	Allergy	X	X		X			X
	Health			X		X		X
	Liability					X		
	Decade teeth			X				X
	Athletic ability			X		X		

TABLE 5
Continued.

		Early childhood	Middle childhood	Late childhood		Adolescence		Adult
		3–5 years	7–9 years	10–12 years		13–18 years		19–26 years
	Mailed	3,291	3,196	3,396		5,279		5,095
	Returned (entry)	859	857	740		960		697
	Response rate	26.10%	26.80%	21.80%		18.90%		13.70%
	Informant	Parent	Parent	Child	Parent	Child	Parent	Child
	Exercises	X	X					X
	Smoking/drinking							X
	Birth height/weight	X	X		X		X	
	Nutrition							X
	Eating/walking speed	X	X	X		X		X
	Ideal weight					X		
	Nutrition	X	X	X		X		X
Home environment	Home hygienic status	X	X		X		X	X
	Housing condition	X	X		X		X	X
	N of books	X	X		X		X	X
	Reading	X						
	School commuting			X		X		
	Coming home time							X
	Family cohesion					X	X	X
	Child rearing attitude	X	X		X		X	X
	Parenting		X				X	
	Parental intervention				X		X	
	Media exposure	X	X	X	X	X	X	X
	Cell phone			X		X		X
	TV game	X						
Parent	Parental job	X	X		X		X	X
	Parental educational history	X	X		X		X	X
	Parental income	X	X		X		X	X
	Sleeping time	X	X					
	Religiousness						X	
	Life-long education				X		X	
	Life events			X		X		X
	Parental personality	X	X		X		X	
	Rearing burden	X						
	Social support	X						

time-specific features of environmental change, we conducted preliminary but relatively large-scale studies with approximately 1,000 non-twin individuals to develop appropriate items.

Performance-Based Research at Home and in the University Lab

Two independent performance-based studies (with some overlapping twin pairs) are currently underway, as shown in Table 3 (assessment and observation at home) and Table 4 (assessment in the university laboratory). Both studies involve individual cognitive ability tests (Bayley II for younger and Kaufman Assessment Battery for Children (K-ABC) for older twin children), theory of mind and executive function tasks, questionnaires, and observation of dyadic and triadic interactions between twin siblings and among twin siblings and parents.

One of the main purposes of our studies is to investigate the development of pre-reading skills and the relationship with cognitive abilities during early childhood. The Japanese *kana* writing system is different from alphabetic systems such as English. Our experiments are designed to be comparable with English language experiments, such as Byrne et al.'s (2002) study. We developed a Japanese version of a test battery to measure pre-reading skills such as phonological awareness, non-word repetition, receptive vocabulary, and visual perceptual skills (Kakihana et al., 2009). Preliminary results revealed a significant influence of shared environmental factors on kana pre-reading skills, and no significant effect of genetic influence (Fujisawa et al., 2012b). However, we found that genetic factors had significant and stable effects on cognitive abilities (Fujisawa & Ando, 2010, 2011).

As mentioned above, studies *of* twins typically have another important aim. As such, we compared twin siblings with non-twin siblings to investigate the relationship between sibling relationships and social adjustment among children. We found that the effects of sibling relationships on pro-social behaviors and conduct problems were stronger for twin siblings than for non-twin siblings, and positive relationships between siblings increased peer problems only among MZ twins; this is the opposite effect compared with that reported among DZ twins and non-twin siblings (Nozaki et al., in press).

TABLE 6
Items of Two Anonymous High School Twin Studies

Category		2009	2010
Entry		Anonymous Junior/senior high 570 families (1,062 twins, 553 mothers, & 459 fathers)	Anonymous Senior high 424 families (751 twins, 402 mothers, & 318 fathers)
Physical	Height/weight	X	
Academic	App/av motivation	X	
	Sense of belonging to school	X	X
Cognition	BAROCO Short	X	X
Social attitude	Social attitude	X	
	Party identification	X	
	Attitude to political issues	X	
	RSES	X	X
	Authoritarianism	X	X
Gender	Gender identity	X	
Environment	Class atmosphere	X	X
Environment	Parent party identification	X	
Environment	FACESIII	X	
Parental data	FACESIII	X	
Parental data	BAROCO Short	X	X

Brain Activity and Motor Skills

The stimulation of brain function by social stimuli such as mothers' vocalizations in infancy and early childhood twins was investigated using ERPs and near infrared spectroscopy at 6, 9, 18, and 36 months, and data from a total of 161 pairs of twins are currently being analyzed. Development of laterality, especially handedness, has also been investigated. The results of these studies indicate a non-additive genetic influence on handedness, suggesting that spatial constraint is a crucial factor for the expression of genetic effects on handedness in infants (Suzuki et al., 2009).

Three Independent Anonymous Twin Studies

Longitudinal studies place a heavy burden on participants, sometimes resulting in severe data attrition. To obtain large samples to verify specific research questions, the KoTReC conducted three independent 'anonymous' twin studies (i.e., twins who received questionnaire mails do not have to inform their names to the KoTReC, which lets them know that they are not followed longitudinally and reduces their burdens to collaborate in our research), a large-scale cross-sectional twin study (CROSS) and two high school twin studies.

The CROSS was conducted in 2007 with over 4,000 pairs of twins and their parents, with an age range of 3 to 26 years old. There were five age categories: early childhood from 3 to 5 years old, middle childhood from 6 to 9 years, late childhood from 10 to 12 years, adolescence from 12 to 18 years, and adulthood from 19 to 26 years.

The design and sample size of this study is shown in Table 5. As shown in the table, the item questions in the CROSS were not based upon standardized, well-organized, or internationally used psychological scales like those in our cohort studies. Rather, the CROSS used independent measures focusing on specific questions, even though some were related and can be grouped in categories such as academic performance and parental stress. For example, Strengths and Difficulties Questionnaire (SDQ; Goodman, 1999) data were used to examine genetic and environmental influences on the relationship between negative parenting and conduct problems of children in terms of attention deficit hyperactivity disorder status (Fujisawa et al., 2012c).

Two high school twin studies (Table 6) were conducted to investigate the genetic and environmental relationships between educational attainment, cognitive ability, and family social environment. Murayama and colleagues (2011) applied academic motivation data to verify the performance-approach and performance-avoidance achievement goal theories (Murayama et al., 2011).

Ozaki (2008) challenged methodological limitations using paired comparison analysis applied to biometric modeling (Ozaki, 2008), non-normal structural equation modeling with higher order moments applied to DOC (Ozaki & Ando, 2009), and estimation of four parameters (additive genetic, non-additive genetic, shared, and non-shared environmental factors) at the same time (Ozaki et al., 2011).

Future Perspectives

The KoTReC has collected the largest active twin sample in Japan, with a total of approximately 9,000 twin pairs from infancy to young adulthood. Some of these data (approximately 2,000 pairs) are longitudinal, and data collection is ongoing. This is the largest Japanese twin research database ever developed. However, many aspects of the database are incomplete. We have not yet established a complete DNA sample from all twin participants in our project because of budget limitations, which have also led to difficulties in long-term planning and administration of well-organized