ubiquitin antibody. No ubiquitination was detected in control cells without expression of AR. Although both AR-24Q and AR-97Q were ubiquitinated without coexpression of CHIP, AR-97Q was more strongly ubiquitinated. The levels of ubiquitinated AR were further increased in cells simultaneously expressing mutant AR and CHIP (Fig. 1*F*). CHIP expression significantly enhanced the level of ubiquitinated mutant AR but only slightly enhanced that of the wild-type AR (Fig. 1*F*).

Colocalization of CHIP with mutant AR in the nuclei

Next, we evaluated the colocalization of endogenous CHIP and mutant AR in AR-97Q mice and in SBMA patients. Double-immunofluorescence staining with chicken anti-CHIP and mouse anti-expanded polyQ (1C2) antibodies revealed that the endogenous CHIP (Fig. 2*A*,*D*,*G*,*J*) and mutant AR (Fig. 2*B*,*E*,*H*,*K*) were colocalized diffusely in the nuclei (Fig. 2*C*,*I*) and NIs (Fig. 2*F*,*L*) in the spinal anterior horn neurons of the AR-97Q mice and in the hypoglossal nucleus cells and spinal anterior horn neurons of SBMA patients, suggesting that the endogenous CHIP coexists with mutant AR and exerts its function in both AR-97Q mice and SBMA patients.

Nondeleterious effects of CHIP overexpression and generation of double-transgenic mice

Because CHIP colocalizes with polyQ-expanded AR, we further tested the effects of CHIP overexpression in the SBMA transgenic mouse model to explore a potential strategy for SBMA therapy. We generated transgenic mice expressing full-length human CHIP under the control of a cytomegalovirus enhancer and a chicken β-actin promoter (Fig. 3*A*). From 14 available lines, we established four that express CHIP in the brain and skeletal muscle and examined the effects of overexpressed CHIP on mouse phenotypes. Through 50 weeks of age, none of the hemizygous or homozygous transgenic mice overexpressing CHIP showed any neurological phenotypes assessed using the rotarod task; they did, however, display slightly delayed weaning. Histological examination at 50 weeks did not show any detectable effects on neuronal cell morphology, neuronal cell number, or muscle structure (data not shown). These studies indicated that overexpression of human CHIP alone does not impair neuronal development or motor function.

To determine whether overexpression of human CHIP could ameliorate the disease phenotype of the SBMA transgenic mouse model, we crossed the AR-24Q mice and the AR-97Q mice (Katsuno et al., 2002) with mice overexpressing human CHIP (CHIP2 line). The AR-97Q mice (SBMA model) are small and have short lifespans, progressive muscle atrophy, and weakness, as well as reduced cage activity (Katsuno et al., 2002). Because these phenotypes are markedly pronounced in the males, similar to SBMA patients (Katsuno et al., 2002), we used male transgenic mice in this study. We generated AR-24Q/CHIP$^{(tg/tg)}$ and AR-97Q/CHIP$^{(tg/tg)}$ mice as homozygotes, the AR-24Q/CHIP$^{(tg/-)}$ and AR-97Q/CHIP$^{(tg/-)}$ mice as hemizygotes, and the AR-24Q/CHIP$^{(-/-)}$ and AR-97Q/CHIP$^{(-/-)}$ mice as controls. The AR transgene expression was at the hemizygous level in all AR-24Q/CHIP and AR-97Q/CHIP double transgenics.

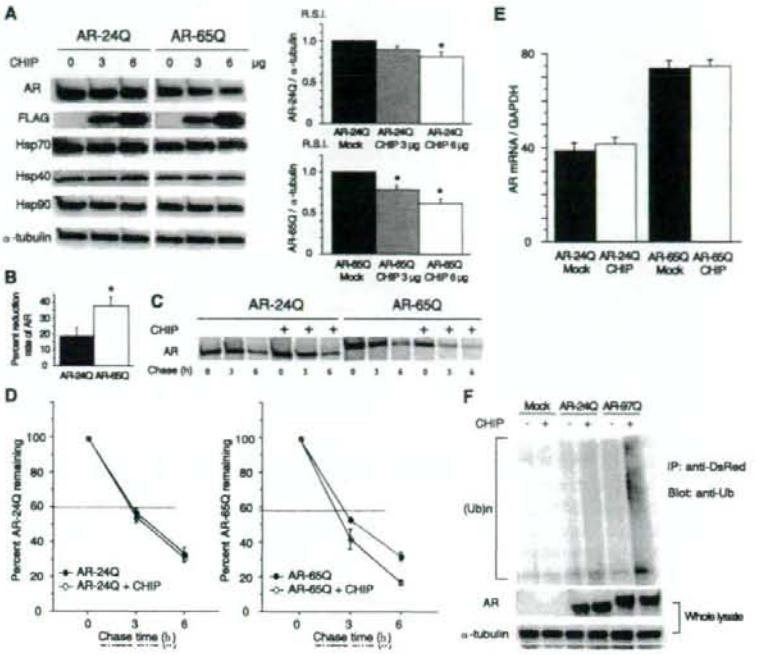

Figure 1. Effect of CHIP on the AR or chaperones in cultured cells. ***A***, Although anti-AR (N20) immunoblotting and densitometry demonstrated a dose-dependent decline of both wild-type (24Q) and mutant (65Q) AR expression in response to CHIP overexpression, the mutant AR decreased more than did the wild type. Mean levels of AR-24Q and AR-65Q expression were relatively compared between CHIP-transfected cells and mock-transfected cells. CHIP overexpression did not increase the expression of Hsp70, Hsp40, and Hsp90. $^*p < 0.005$. ***B***, The decrease in mutant AR in response to CHIP overexpression was much higher than that of the wild type (18.8% vs 38.0%; 6 μg of CHIP). $^*p < 0.05$. ***C***, Pulse-chase analysis of two forms of AR. Data are from one representative experiment for wild-type and mutant AR. ***D***, Pulse-chase assessment of the half-life of wild-type (left) and mutant (right) AR. The percentages of AR-24Q and AR-65Q remaining in the absence (●) and presence (○) of overexpressed CHIP are indicated. Mutant AR was degraded more rapidly than the wild-type AR in the presence of overexpressed CHIP. ***E***, Real-time RT-PCR of wild-type and mutant AR mRNA normalized to GAPDH levels. The wild-type and mutant AR mRNA levels were similar under CHIP overexpression. ***F***, Ubiquitination of AR in control, AR-24Q-, and AR-97Q-transfected cells in the absence (−) or presence (+) of CHIP cotransfection. No ubiquitination was detected in control cells without expression of AR. Although both AR-24Q and AR-97Q were ubiquitinated without coexpression of CHIP, AR-97Q was strongly ubiquitinated. CHIP significantly enhanced the level of mutant AR ubiquitination but only slightly enhanced that of wild-type AR. ***A***, ***B***, ***D***, ***E***, Values represent means ± SEM ($n = 5$). IP, Immunoprecipitation; Ub, ubiquitin.

Expression of CHIP in double-transgenic mice

We examined whether the AR/CHIP double-transgenic mice express increased levels of the CHIP protein in the spinal cord and skeletal muscle. Western blot analysis revealed that CHIP expression in the spinal cords of AR-97Q/CHIP$^{(tg/-)}$ and AR-97Q/CHIP$^{(tg/tg)}$ mice was three and six times as high, respectively, as endogenous CHIP in the AR-97Q/CHIP$^{(-/-)}$ mice. In muscle, it was six times as high in the AR-97Q/CHIP$^{(tg/-)}$ mice and 12 times as high in the AR-97Q/CHIP$^{(tg/tg)}$ mice (Fig. 3*B*). As in transfected cells, the expression levels of Hsp90, Hsp70, and Hsp40 were not changed after CHIP overexpression in the double-transgenic mice (Fig. 3*B*). The increased CHIP was coim-

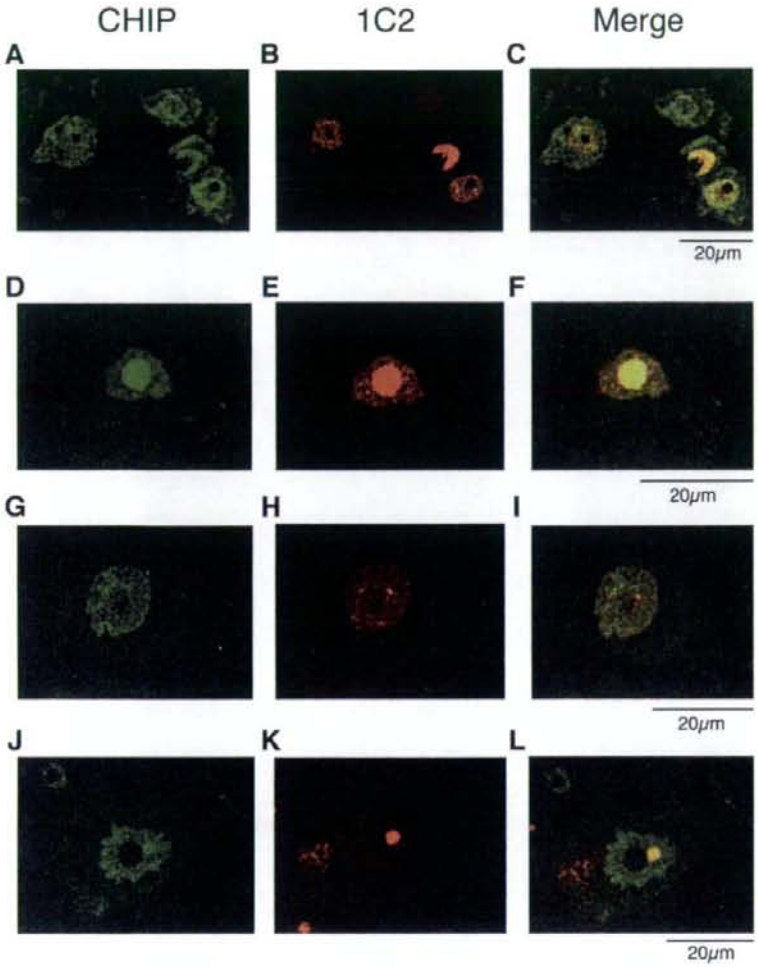

Figure 2. Colocalization of nuclear-localized CHIP with mutant AR. ***A–L***, Anti-CHIP and anti-polyQ immunohistochemistry in spinal cords of 16-week-old AR-97Q mice (***A–F***) and an SBMA patient (***G–L***). ***A–C***, Double-immunofluorescence staining for CHIP (***A***; green), expanded-polyQ (***B***; red), and overlay of the two signals (***C***; yellow) in the spinal anterior horn cells. ***D–F***, CHIP (green; ***D***) and mutant AR (red; ***E***) are colocalized in nuclear inclusions (shown in yellow; ***F***) in the spinal anterior horn cell. ***G–I***, Double-immunofluorescence staining in cells of the hypoglossal nucleus of an SBMA patient revealed diffuse nuclear colocalization of CHIP (***G***) and mutant AR (***H***, ***I***). ***J–L***, CHIP (green; ***J***) and mutant AR (red; ***K***) were also colocalized in NIs (shown in yellow; ***L***) in the spinal anterior horn cell of SBMA patients.

munoprecipitated with polyQ-expanded AR and Hsp70, suggesting that CHIP may recognize AR either directly or indirectly through association with Hsp70 (Fig. 3*C*). We also performed Western blotting analysis using 8-, 16-, 24-, and 32-week-old CHIP$^{(tg/tg)}$ and AR-97Q/CHIP$^{(tg/tg)}$ mice to examine the effect of age on the expression level of CHIP in spinal cord and muscle. We found that the expression levels of CHIP did not change even in the 32-week-old mice (Fig. 3*D*). Immunohistochemical studies of double-transgenic mice tissue stained with the CHIP-specific antibody confirmed that spinal anterior horn neurons and muscle cells expressed the CHIP (Fig. 3*E*,*F*). CHIP protein was diffusely distributed in the nuclei and cytoplasm (Fig. 3*E*,*F*). Glial cells also showed diffuse nuclear staining of CHIP protein (Fig. 3*E*).

Human CHIP overexpression ameliorates phenotypic expression of SBMA mice

To determine whether CHIP overexpression has an ameliorative effect on the motor phenotypes in the double-transgenic mice, we performed the rotarod task and measured locomotor cage activity with an infrared sensor system (Fig. 4*A*,*B*). Motor impairment on the rotarod task was evident in the AR-97Q/CHIP$^{(-/-)}$ mice as early as 8 weeks after birth but was seen, to a lesser degree, in the AR-97Q/CHIP$^{(tg/tg)}$ mice beginning at only 16 weeks (Fig. 4*A*). Although both the AR-97Q/CHIP$^{(tg/tg)}$ and AR-97Q/CHIP$^{(tg/-)}$ mice performed significantly better than the AR-97Q/CHIP$^{(-/-)}$ mice ($p < 0.005$ and $p < 0.025$, respectively) (Fig. 4*A*), the AR-97Q/CHIP$^{(tg/tg)}$ mice were on the rod longer than the AR-97Q/CHIP$^{(tg/-)}$ mice during the trial. The locomotor cage activity of the AR-97Q/CHIP$^{(-/-)}$ mice was also significantly decreased at 32 weeks compared with the other two double transgenics ($p < 0.005$, respectively) (Fig. 4*B*). Although there were no differences in body weight at birth among the various lines, the AR-97Q/CHIP$^{(-/-)}$ mice lost weight significantly earlier than did the AR-97Q/CHIP$^{(tg/-)}$ and AR-97Q/CHIP$^{(tg/tg)}$ mice ($p < 0.005$) (Fig. 4*C*). The survival rate was significantly higher in the AR-97Q/CHIP$^{(tg/-)}$ and AR-97Q/CHIP$^{(tg/tg)}$ mice than in the AR-97Q/CHIP$^{(-/-)}$ mice ($p < 0.0001$) (Fig. 4*D*). Because the decrease in ameliorative effects of CHIP overexpression in the aged mice is not attributable to decreased CHIP expression (Fig. 3*D*), it is probably caused by the progressive nuclear accumulation of toxic mutant AR in the aged mice (Katsuno et al., 2003). The affected AR-97Q/CHIP$^{(-/-)}$ mice exhibited motor weakness, took short steps, or dragged their legs, whereas the AR-97Q/CHIP$^{(tg/tg)}$ mice moved almost normally, and the AR-97Q/CHIP$^{(tg/-)}$ mice only took somewhat shorter steps (Fig. 4*E*). Both the AR-97Q/CHIP$^{(tg/-)}$ and AR-97Q/CHIP$^{(tg/tg)}$ mice took significantly longer steps than the AR-97Q/CHIP$^{(-/-)}$ mice ($p < 0.005$) (Fig. 4*F*). Although the SBMA phenotypes were ameliorated in both the AR-97Q/CHIP$^{(tg/tg)}$ and AR-97Q/CHIP$^{(tg/-)}$ mice, the AR-97Q/CHIP$^{(tg/tg)}$ mice were better than the AR-97Q/CHIP$^{(tg/-)}$ mice in most of the parameters, suggesting that the improved motor phenotype depended on the CHIP expression level rather than the genetic background.

Immunohistochemical staining for mutant AR using the 1C2 antibody showed a marked reduction in diffuse nuclear staining and NIs in spinal cord (Fig. 5*A–C*) and muscle (Fig. 5*D–F*) of the AR-97Q/CHIP$^{(tg/-)}$ and AR-97Q/CHIP$^{(tg/tg)}$ mice compared with the AR-97Q/CHIP$^{(-/-)}$ mice. In the AR-97Q/CHIP$^{(-/-)}$ mice, intense staining was frequently seen in the nuclei (Fig.

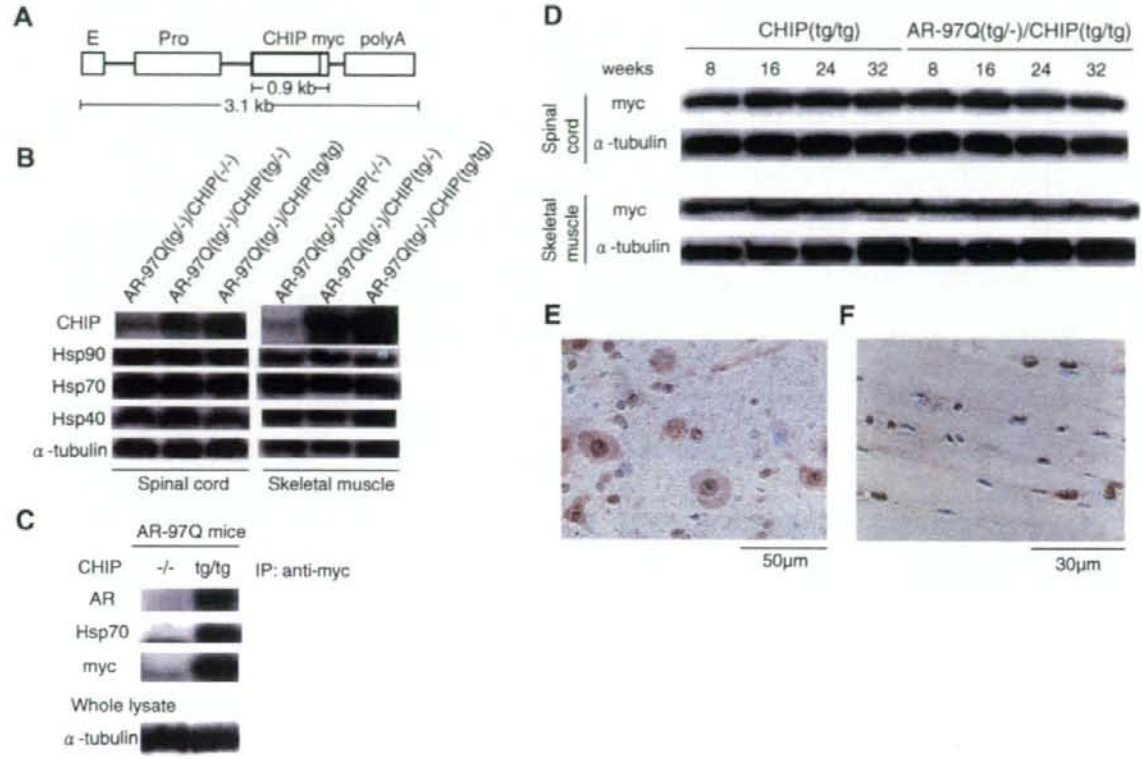

Figure 3. Increased CHIP expression in double-transgenic mice. ***A***, Schematic view of the transgene construct. The microinjected fragment was composed of a cytomegalovirus enhancer (E), a chicken β-actin promoter (Pro), full-length human CHIP with a myc tag, and a rabbit β-globin polyadenylation signal sequence (polyA). ***B***, Western blot analysis of total spinal cord and muscle protein lysates from AR-97Q/CHIP$^{(-/-)}$, AR-97Q/CHIP$^{(tg/-)}$, and AR-97Q/CHIP$^{(tg/tg)}$ mice immunolabeled with antibodies against CHIP, Hsp90, Hsp70, and Hsp40. ***C***, Coimmunoprecipitation Western blots for CHIP. Soluble fractions were collected from the spinal cord of AR-97Q/CHIP$^{(-/-)}$ and AR-97Q/CHIP$^{(tg/tg)}$ mice. Equal amounts of protein were immunoprecipitated with an antibody to myc and immunoblotted for AR and Hsp70. Coimmunoprecipitation of CHIP and the polyQ-expanded mutant AR or the Hsp70 chaperone was detected. ***D***, Western blot analysis of CHIP expression in total spinal cord and muscle protein lysates from CHIP$^{(tg/tg)}$ and AR-97Q/CHIP$^{(tg/tg)}$ mice of the indicated ages, immunolabeled with antibodies against myc. ***E***, ***F***, CHIP immunohistochemistry in spinal anterior horn and skeletal muscle of 16-week-old AR-97Q/CHIP$^{(tg/tg)}$ mice counterstained with Mayer's hematoxylin. ***E***, CHIP immunoreactivity is localized to the nuclei and cytoplasm, with intense and diffuse staining in the anterior horn cells. ***F***, Skeletal muscle showed diffuse nuclear and cytoplasmic staining. IP, Immunoprecipitation.

5*A*,*D*), whereas staining was infrequent in the AR-97Q/CHIP$^{(tg/-)}$ mice (Fig. 5*B*,*E*) and much less frequent in the AR-97Q/CHIP$^{(tg/tg)}$ mice (Fig. 5*C*,*F*). There were significantly more 1C2-positive cells in spinal cord (Fig. 5*G*) and muscle (Fig. 5*H*) of the AR-97Q/CHIP$^{(-/-)}$ mice than in the AR-97Q/CHIP$^{(tg/-)}$ and AR-97Q/CHIP$^{(tg/tg)}$ mice. The 1C2-positive cell populations were not, however, statistically different in the AR-97Q/CHIP$^{(tg/-)}$ and AR-97Q/CHIP$^{(tg/tg)}$ mice. GFAP-specific antibody staining showed an apparent reduction in reactive astrogliosis in the AR-97Q/CHIP$^{(tg/tg)}$ mice compared with the AR-97Q/CHIP$^{(-/-)}$ mice in the spinal anterior horn (Fig. 5*I*). Muscle histology also demonstrated marked amelioration of muscle atrophy in the AR-97Q/CHIP$^{(tg/tg)}$ mice (Fig. 5*J*). The AR-24Q/CHIP mice displayed no altered phenotypes (data not shown). The numbers of neuronal cells in the spinal ventral horns of AR-97Q/CHIP$^{(-/-)}$, AR-97Q/CHIP$^{(tg/-)}$, and AR-97Q/CHIP$^{(tg/tg)}$ mice were not significantly decreased compared with those in the wild-type mice (data not shown).

Overexpression of CHIP decreases the high-molecular-weight mutant AR protein and monomeric mutant AR protein

Because the mutant AR was preferentially degraded compared with the wild-type AR when CHIP was overexpressed *in vitro*, we also examined levels of AR in the SBMA mouse model. Western blot analysis from lysates of the spinal cord and muscle of AR-97Q mice revealed high-molecular-weight mutant AR protein complex retained in the stacking gel as well as a band of monomeric mutant AR, whereas only the band of wild-type monomeric AR was visible in tissues from the AR-24Q mice (Fig. 6*A*,*B*). CHIP overexpression notably diminished both the high-molecular-weight complex and the monomer of mutant AR in the spinal cord and muscle of the AR-97Q mice but only slightly diminished the wild-type monomeric AR in AR-24Q mice (Fig. 6*A*,*B*). CHIP overexpression decreased the amount of the monomeric AR in AR-97Q mice by 50% in the spinal cord and 75% in the skeletal muscle but only by 8% and 5%, respectively, in AR-24Q mice (Fig. 6*A*,*B*). The levels of wild-type and mutant AR mRNA were similar in both AR-24Q and AR-97Q mice under CHIP overexpression (Fig. 6*C*). These observations suggest that overexpression of CHIP markedly decreases not only the monomeric mutant AR protein but also the high-molecular-weight mutant AR protein.

We also performed filter trap assays to quantitatively analyze the effects of CHIP overexpression on levels of both the large-molecular aggregated and soluble forms of mutant AR (Wanker et al., 1999). Only the larger-sized mutant AR protein was re-

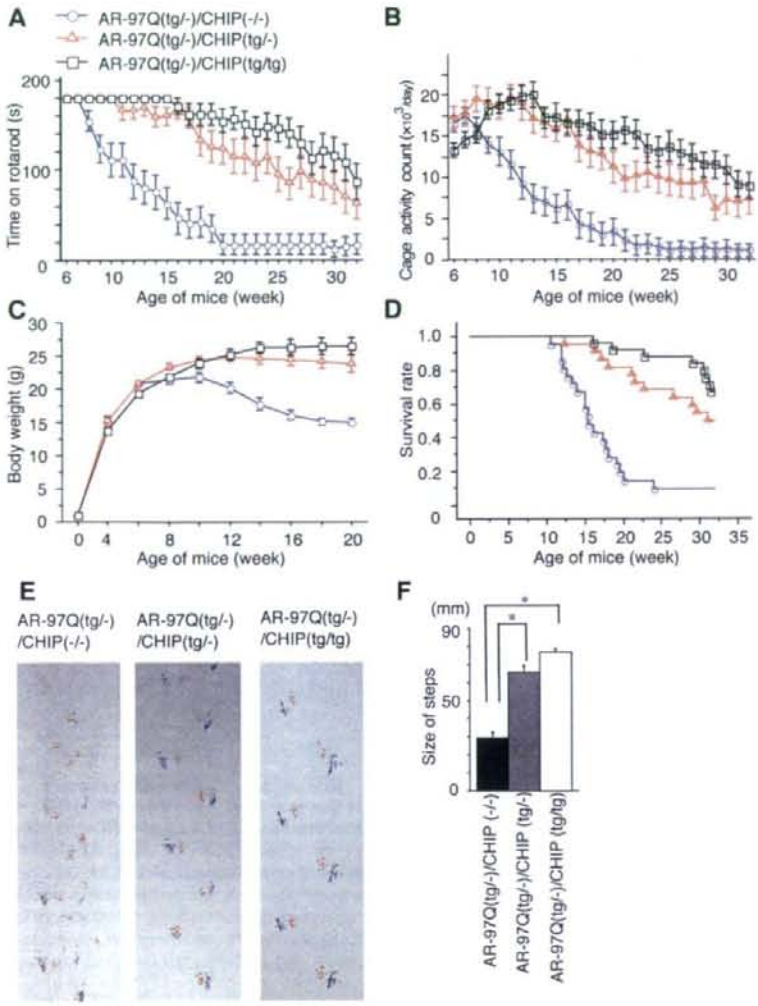

Figure 4. Effects of human CHIP overexpression on the behavioral phenotypes in male AR-97Q mice. ***A–D***, Rotarod task (***A***; $n = 22$), cage activity (***B***; $n = 22$), body weight (***C***; $n = 26$), survival rate (***D***; $n = 26$) of the AR-97Q/CHIP$^{(-/-)}$ (○), AR-97Q/CHIP$^{(tg/-)}$ (□), and AR-97Q/CHIP$^{(tg/tg)}$ mice (△). AR-97Q mice overexpressing human CHIP remained longer on the rotarod and showed higher cage activity than the AR-97Q/CHIP$^{(-/-)}$. The AR-97Q/CHIP$^{(-/-)}$ lost weight earlier than the other two double transgenics. ***D***, A Kaplan-Meier plot shows the prolonged survival of AR-97Q/CHIP$^{(tg/-)}$ and AR-97Q/CHIP$^{(tg/tg)}$ mice compared with the AR-97Q/CHIP$^{(-/-)}$. The AR-97Q/CHIP$^{(-/-)}$ mice were significantly different from either of the other two in all parameters tested. Moreover, the AR-97Q/CHIP$^{(tg/-)}$ mice were worse off than the AR-97Q/CHIP$^{(tg/tg)}$ in all parameters tested. ***E***, Footprints of representative 16-week-old AR-97Q/CHIP$^{(-/-)}$, AR-97Q/CHIP$^{(tg/-)}$, and AR-97Q/CHIP$^{(tg/tg)}$ mice. Front paws are indicated in red, and hindpaws are indicated in blue. AR-97Q/CHIP$^{(-/-)}$ mice exhibit motor weakness with dragging of the legs, AR-97Q/CHIP$^{(tg/tg)}$ mice walk almost normally, and AR-97Q/CHIP$^{(tg/-)}$ mice walk with somewhat shorter steps. ***F***, The average length of hindpaw steps in 16-week-old AR-97Q/CHIP$^{(-/-)}$, AR-97Q/CHIP$^{(tg/-)}$, and AR-97Q/CHIP$^{(tg/tg)}$ mice. Values are expressed as means ± SEM ($n = 6$). *$p < 0.005$.

tained on the upper, cellulose acetate membrane (pores 0.2 μm in diameter), whereas the lower nitrocellulose membrane captured all proteins that passed through the upper membrane (Fig. 6*D*). Values were normalized to endogenous α-tubulin trapped on the nitrocellulose membrane. Both forms of trapped AR-97Q protein were markedly reduced in the spinal cord and muscle of mice overexpressing CHIP, whereas levels of soluble monomeric AR protein from the AR-24Q mice were only slightly reduced (Fig. 6*D*). The endogenous AR protein was not retained on the cellulose acetate membrane in wild-type mice (data not shown). These results strongly indicate that CHIP markedly reduces not only the monomeric mutant AR protein but also the high-molecular-weight mutant AR complex, by preferentially degrading the mutant AR. These observations also suggest that overexpression of CHIP enhanced the function of the ubiquitin-proteasome pathway and subsequently accelerated degradation of monomeric mutant AR protein.

Discussion

CHIP is a U-box-dependent E3 ubiquitin ligase that associates with the Hsp70 and Hsp90 molecular chaperones and targets folded or toxic misfolded proteins for degradation (McDonough and Patterson, 2003). A wide range of different proteins have been identified as CHIP substrates, including members of the steroid hormone receptor family (Connell et al., 2001; Tateishi et al., 2004; Wang and DeFranco, 2005), the cystic-fibrosis transmembrane-conductance regulator (Meacham et al., 2001; Younger et al., 2006), E2A transcription factors (Huang et al., 2004), raf-1 protein kinase (Demand et al., 2001), ErbB2 (Zhou et al., 2003), nucleophosmin-anaplastic lymphoma kinase (Bonvini et al., 2004), dual leucine zipper-bearing kinase (Daviau et al., 2006), caytaxin (Grelle et al., 2006), αB-crystallin (Chavez Zobel et al., 2003), tau (Hatakeyama et al., 2004; Petrucelli et al., 2004; Sahara et al., 2005; Dickey et al., 2006), α-synuclein (Shin et al., 2005), the p53 tumor suppressor (Esser et al., 2005), apoptosis signal-regulating kinase 1 (Hwang et al., 2005), and polyQ-disease causative proteins (Jana et al., 2005; Miller et al., 2005; Al-Ramahi et al., 2006). CHIP can directly interact with and degrade the wild-type AR in a phosphorylation-dependent or -independent manner (Cardozo et al., 2003; Rees et al., 2006) and can repress AR transcriptional activity, suggesting that CHIP may play a role in regulating AR function in the cell (He et al., 2004). CHIP also has been shown to associate with the polyQ-expanded AR (Thomas et al., 2004). In this study, we addressed the question of whether CHIP overexpression promotes the degradation of mutant AR and exerts therapeutic effects on the SBMA phenotype. In a cultured neuronal cell model of SBMA, we demonstrated that increasing levels of CHIP more effectively ubiquitinated and degraded the monomeric mutant AR than the wild-type AR, suggesting that the mutant AR is more sensitive to CHIP than is the wild type. Overexpression of CHIP strongly inhibited nuclear accumulation of the mutant AR and markedly ameliorated motor impairments in SBMA transgenic mice in a dose-dependent manner. Mutant AR and CHIP were co-localized diffusely in the nuclei and in the NIs in neurons of the

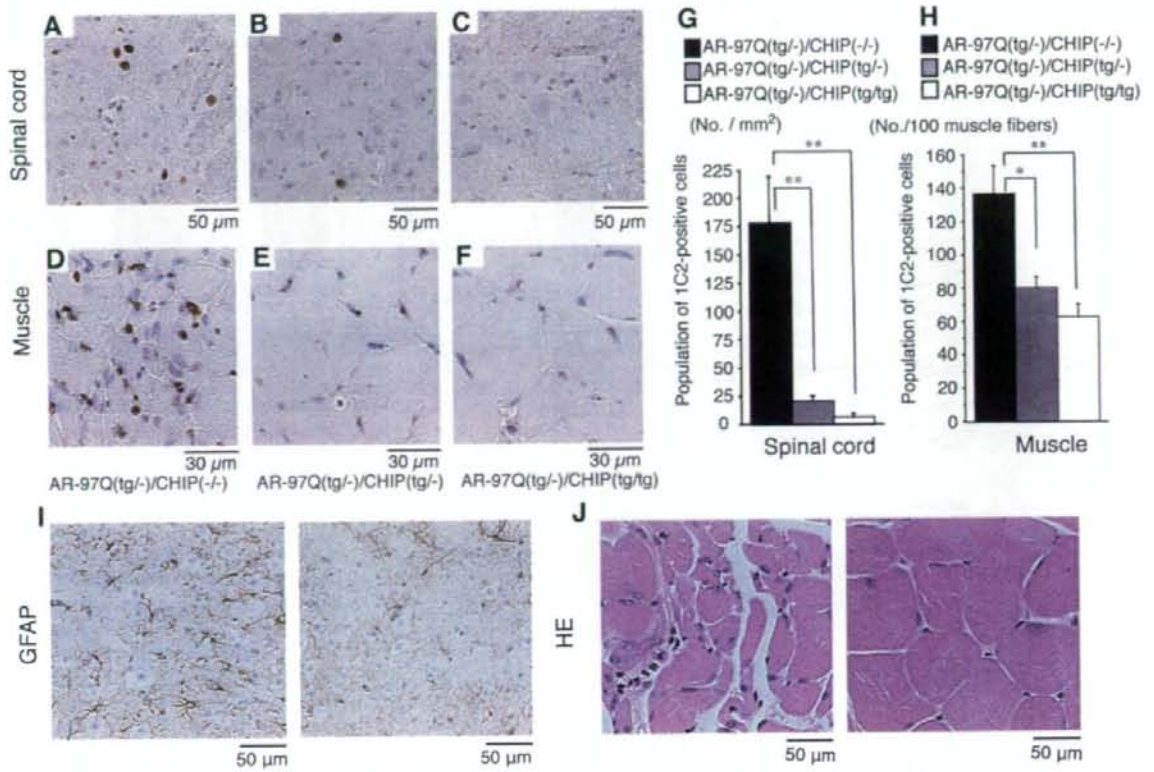

Figure 5. CHIP decreases nuclear-localized mutant AR in double-transgenic mice. ***A–F***, PolyQ immunohistochemistry (1C2) in the spinal anterior horn (***A–C***) and muscle (***D–F***) of 16-week-old AR-97Q/CHIP$^{(-/-)}$ and AR-97Q/CHIP double-transgenic mice. AR-97Q/CHIP$^{(-/-)}$ mice have intense and frequent staining for 1C2 in the nucleus (***A***, ***D***). ***B***, ***C***, ***E***, ***F***, AR-97Q/CHIP$^{(tg/-)}$ (***B***, ***E***) and AR-97Q/CHIP$^{(tg/tg)}$ (***C***, ***F***) mice exhibit low levels of 1C2 staining in the nucleus. ***G***, ***H***, Quantitative assessment of diffuse nuclear staining for 1C2 in the spinal ventral horn (***G***) and muscle (***H***). Bars represent the density of 1C2-positive cells in the AR-97Q/CHIP$^{(-/-)}$, AR-97Q/CHIP$^{(tg/-)}$, and AR-97Q/CHIP$^{(tg/tg)}$ mice. There are significantly more 1C2-positive cells in AR-97Q/CHIP$^{(-/-)}$ mice than in AR-97Q/CHIP$^{(tg/-)}$ mice or AR-97Q/CHIP$^{(tg/tg)}$ mice in both tissues. Results are expressed as mean ± SEM for six mice. $*p < 0.025$; $**p < 0.005$. ***I***, Immunohistochemical staining with GFAP-specific antibody also showed an obvious reduction in reactive astrogliosis in the spinal anterior horn of AR-97Q/CHIP$^{(tg/tg)}$ mice. ***J***, Hematoxylin and eosin (HE) staining of muscle tissue in AR-97Q/CHIP$^{(-/-)}$ mice revealed obvious atrophy and small-angulated fibers, which were not seen in AR-97Q/CHIP$^{(tg/tg)}$ mice. No., Number.

AR-97Q/CHIP double-transgenic mice. More importantly, CHIP also colocalized with mutant AR aggregates present in the anterior horn cells from postmortem tissues of SBMA patients. Western blot analysis showed that both a band of monomeric mutant AR and the high-molecular-weight form of mutant AR protein complexes retained in the stacking gel were diminished in the spinal cord and muscle of the double-transgenic mice, suggesting that the degradation of mutant AR may have been accelerated by overexpression of CHIP.

Our AR-97Q transgenic mice display progressive muscular atrophy and weakness, as well as diffuse nuclear staining and NIs of the mutant AR (Katsuno et al., 2002). These phenotypes are very pronounced in male transgenic mice, similar to human SBMA patients. The fact that AR has a specific ligand (i.e., testosterone), renders the pathogenesis of SBMA unique among polyQ diseases (Poletti et al., 2005). There is increasing evidence that the AR ligand (Katsuno et al., 2003; Chevalier-Larsen et al., 2004; Sopher et al., 2004; Katsuno et al., 2006; Yu et al., 2006) and molecular chaperones (Kobayashi et al., 2000; Bailey et al., 2002; Adachi et al., 2003) play a crucial role in the pathogenesis of SBMA. The success of androgen deprivation therapy in SBMA mouse models has been translated into clinical trials (Banno et al., 2006). In addition, elucidation of its pathophysiology using SBMA animal models has led to the development of other chaperone-related disease-modifying drugs, an Hsp90 inhibitor (Waza et al., 2005) and a heat shock protein inducer (Katsuno et al., 2005), which inhibit the pathogenic process of neuronal degeneration. Recent studies suggested that soluble causative protein species, not insoluble protein aggregates, might be toxic and thus targets in treatments of neurodegenerative disorders (Slow et al., 2006). Here, we demonstrated that overexpression of human CHIP exerts therapeutic effects on motor dysfunction in the AR-97Q mouse. Overexpression of CHIP served to decrease monomeric mutant AR in the double-transgenic mice. The large aggregated mutant AR protein complexes were also significantly reduced by CHIP overexpression, suggesting that CHIP accelerated the turnover of mutant AR. Together, these data suggest that targeting mutant AR for proteasomal degradation by overexpression of CHIP could be a disease-modifying therapeutic strategy in SBMA neuropathology. These findings are consistent with previous studies showing that CHIP serves as a protective factor in other polyQ diseases by promoting reduced aggregation of disease proteins (Jana et al., 2005; Miller et al., 2005) and proteasomal degradation (Al-Ramahi et al., 2006). In contrast to

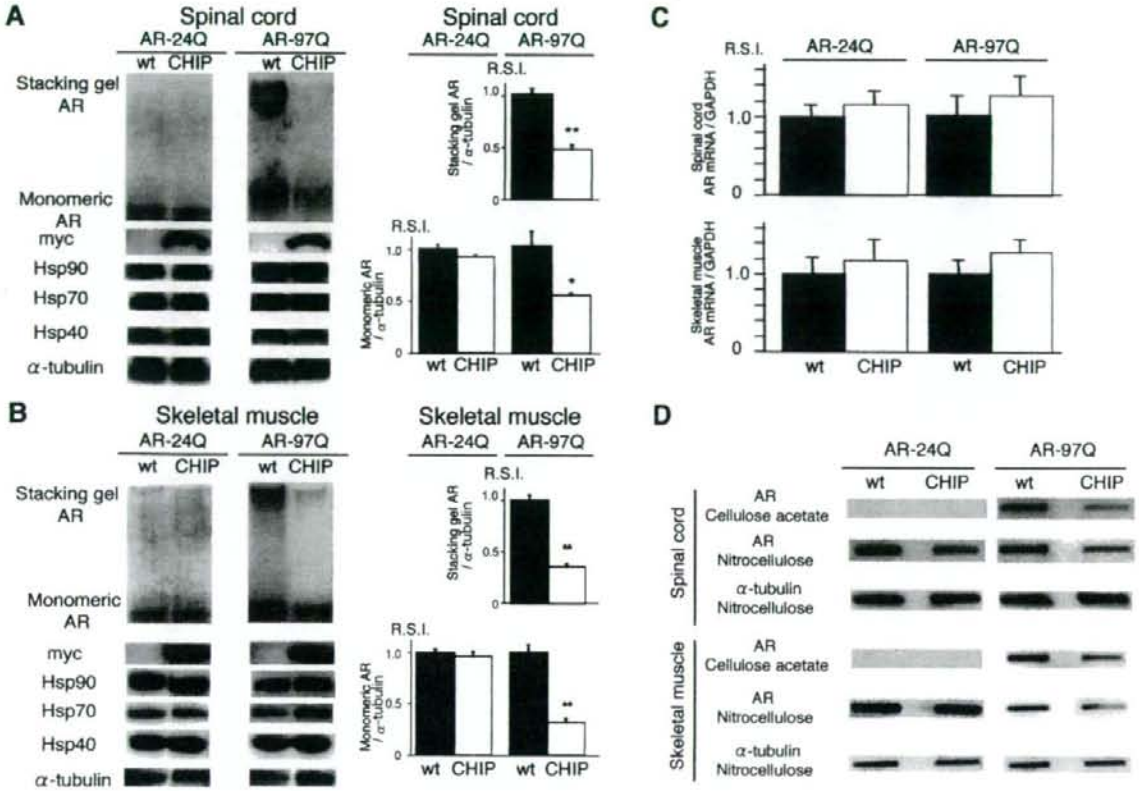

Figure 6. CHIP decreases mutant AR protein complexes as well as monomeric mutant AR. ***A***, ***B***, Western blot analysis of total tissue homogenates from the spinal cord (***A***) and muscle (***B***) of AR-24Q/CHIP$^{(-/-)}$, AR-24Q/CHIP$^{(tg/tg)}$, AR-97Q/CHIP$^{(-/-)}$, and AR-97Q/CHIP$^{(tg/tg)}$ mice (16-week-old) probed with an AR-specific antibody (H280). The mutant AR complex appears in the stacking gel, and the monomeric mutant AR appears in the separating gel. Values are expressed as mean ± SEM for six mice. $*p < 0.001$; $**p < 0.0001$. ***C***, Real-time RT-PCR of wild-type (AR-24Q) and mutant AR (AR-97Q) mRNA in transgenic mouse spinal cord and skeletal muscle in the absence (wt) and presence (CHIP) of CHIP overexpression. Values are expressed as means ± SE ($n = 6$). ***D***, Filter trap assay of total tissue homogenates from the spinal cord and muscle of AR-97Q/CHIP$^{(-/-)}$ and AR-97Q/CHIP$^{(tg/tg)}$ mice (16 weeks of age), in the absence and presence of CHIP overexpression. Homogenates were filtrated and immunolabeled with an antibody against AR (H280). Large aggregated mutant AR complexes were trapped by the cellulose acetate membrane; soluble monomeric mutant AR passed through the cellulose acetate membrane and was trapped by the nitrocellulose membrane. Endogenous α-tubulin was used as a loading control.

symptom-relief therapies, such as L-DOPA for Parkinson's disease, these disease-modifying therapies inhibit or slow down the pathogenic processes of neuronal degeneration.

CHIP interacts with Hsp90 or Hsp70, ubiquitylates unfolded proteins trapped by molecular chaperones, and degrades them, thus acting as a quality control E3 ubiquitin ligase (Murata et al., 2001). The remarkable reduction of monomeric mutant AR in the AR-97Q/CHIP mice may reflect accelerated degradation of mutant AR through the CHIP-mediated E3-proteasome system. CHIP also ubiquitinated the AR protein in a polyQ length-dependent manner, suggesting that overexpression of CHIP enhances the degradation of monomeric mutant AR by activating the Hsp70-interacting, quality control E3 system. This subsequently reduces the amount of nuclear-localized mutant AR, resulting in amelioration of phenotypic expression induced by mutant AR. Interaction between Hsp70 and CHIP, detected by coimmunoprecipitation and Western blot analysis, in the double-transgenic mice supports this view. Increased activity of CHIP was reported to modify a neurodegenerative phenotype caused by expanded ataxin-1 and huntingtin in a chaperone-dependent manner in cellular and *Drosophila* models (Jana et al., 2005; Al-Ramahi et al., 2006). CHIP lacking the normal TPR domain did not ubiquitinate the ataxin-1 protein (Al-Ramahi et al., 2006). These results suggest that chaperone interaction is essential for CHIP-dependent ubiquitination. Hsp70 overexpression in cell culture and mouse models of SBMA enhanced degradation of mutant AR-polyQ protein via its interaction with the ubiquitin-proteasome system (Bailey et al., 2002; Adachi et al., 2003). CHIP might be one such coupling factor between the Hsp70 chaperone system and the machinery responsible for degrading mutant AR. Another possibility is that overexpression of CHIP may accelerate chaperone-independent interaction with mutant AR and its degradation through the proteasome pathway. CHIP directly interacts with and degrades the AR protein (He et al., 2004; Rees et al., 2006). Thus, the interactions we demonstrated between CHIP and AR may be either direct or mediated by chaperones that are known to interact with CHIP. This direct interaction of CHIP with the AR protein might promote mutant AR degradation through the proteasome system.

Accumulation of misfolded proteins is causally related to

many age-related neurodegenerative diseases (Muchowski and Wacker, 2005; Bates, 2006). Prompt removal and/or refolding may be required more in aged or damaged cells than in young healthy cells in which appropriate protein quality control systems function (Wickner et al., 1999). In SBMA patients, diffuse nuclear accumulation of mutant AR is frequent and extensive, being distributed in a wide array of CNS nuclei and in visceral organs (Adachi et al., 2005). In this study, we demonstrated that overexpression of CHIP significantly ameliorates the phenotypes of SBMA transgenic mice, by reducing the amount of both the monomeric and large aggregated forms of nuclear-accumulated mutant AR protein, suggesting that CHIP may change the triage of mutant AR and promote its degradation by the proteasome system (Marques et al., 2006). Thus, CHIP overexpression might provide a potential therapeutic avenue for SBMA and other polyQ diseases.

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Pathogenesis and molecular targeted therapy of spinal and bulbar muscular atrophy

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H. Adachi, M. Waza, M. Katsuno, F. Tanaka, M. Doyu and G. Sobue (2007) *Neuropathology and Applied Neurobiology* **33**, 135–151
Pathogenesis and molecular targeted therapy of spinal and bulbar muscular atrophy

Spinal and bulbar muscular atrophy (SBMA) or Kennedy's disease is a motor neurone disease characterized by muscle atrophy, weakness, contraction fasciculations and bulbar involvement. SBMA mainly affects males, while females are usually asymptomatic. SBMA is caused by expansion of a polyglutamine (polyQ)-encoding CAG trinucleotide repeat in the androgen receptor (AR) gene. AR belongs to the heat shock protein 90 (Hsp90) client protein family. The histopathologic hallmarks of SBMA are diffuse nuclear accumulation and nuclear inclusions of the mutant AR with expanded polyQ in residual motor neurones in the brainstem and spinal cord as well as in some other visceral organs. There is increasing evidence that the ligand of AR and molecular chaperones play a crucial role in the pathogenesis of SBMA. The success of androgen deprivation therapy in SBMA mouse models has been translated into clinical trials. In addition, elucidation of its pathophysiology using animal models has led to the development of disease-modifying drugs, that is, Hsp90 inhibitor and Hsp inducer, which inhibit the pathogenic process of neuronal degeneration. SBMA is a slowly progressive disease by nature. The degree of nuclear accumulation of mutant AR in scrotal skin epithelial cells was correlated with that in spinal motor neurones in autopsy specimens; therefore, the results of scrotal skin biopsy may be used to assess the efficacy of therapeutic trials. Clinical and pathological parameters that reflect the pathogenic process of SBMA should be extensively investigated.

Keywords: 17-allylamino-17-demethoxygeldanamycin, androgen receptor, heat shock protein, luteinizing hormone-releasing hormone analogue, polyglutamine, spinal and bulbar muscular atrophy

Introduction

Spinal and bulbar muscular atrophy (SBMA) was first described in a paper entitled, 'Progressive bulbar palsy', in 1897 by Kawahara in Japan [1,2]. The author reported the clinical characteristics of two brothers and their uncle on the maternal side with progressive atrophy of the tongue, dysarthria, dysphagia and gait disturbance. SBMA is also known as Kennedy's disease, named after William R. Kennedy, whose study on 11 patients from two families depicted the clinical, genetic and pathological features of this disorder [3]. As SBMA mainly affects males and SBMA is frequently associated with gynaecomastia, testicular failure and other feminized signs, this disease had been thought to be caused by abnormality of the androgen receptor (AR). The underlying genetic abnormality was determined to be abnormal expansion of a CAG repeat in the *AR* gene in 1991 [4]. This was the first discovery of a polyglutamine (polyQ)-mediated neurodegenerative disease caused by expansion of a trinucleotide CAG repeat encoding glutamine in the causative gene. To date, nine polyQ-mediated neurodegenerative disorders

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have been identified [5], and many molecular biological studies have been undertaken to elucidate the pathogenesis of these diseases and to develop treatment methods. Therapies for SBMA can be broadly classified into two categories: (i) disease-modifying therapies and (ii) symptom-relief therapies. The ideal therapy appears to be a combination of these two potential therapeutic strategies. As to disease-modifying therapies, long-term clinical trials are required to verify that these potential therapies can delay the clinical onset and progression of SBMA by targeting certain clinical events. On the other hand, for symptom-relief therapies such as L-dihydroxyphenylalanine (L-DOPA) for Parkinson's disease, the duration of clinical trials tends to be short. Here we highlight the research findings from which the concept of the ligand- and chaperone-dependent pathophysiology of SBMA has emerged, and discuss disease-modifying therapeutic approaches.

Clinical features of SBMA

Spinal and bulbar muscular atrophy is characterized by premature muscular exhaustion, slowly progressive muscular weakness, atrophy, and fasciculation in bulbar and limb muscles [6]. For instance, bilateral facial and masseter muscle weakness, poor uvula and soft palatal movements, and atrophy of the tongue with fasciculation are observed. The muscle weakness and atrophy in the limbs are either generalized or prominent in the proximal muscles, and are usually symmetrical. Patients also present occasional painful muscle cramps mainly in the lower legs and trunk, and hand tremor [7,8]. The motor involvement is slowly progressive and eventually confines some patients to a wheelchair. SBMA patients may also have mild sensory impairment, although it usually remains subclinical. In most cases, the vibration sense is slightly diminished in the distal lower extremities, but occasionally all of the sensory modalities are slightly disturbed [6]. Deep tendon reflex is diminished or absent with no pathological reflex. Patients with SBMA do not have cerebellar symptoms, dysautonomia, or cognitive impairment. Patients occasionally show signs of androgen insensitivity such as gynaecomastia, impaired spermatogenesis, testicular atrophy, impotence and decreased fertility, some of which are detected before the onset of motor symptoms [7,9–12]. Serum testosterone levels are usually normal or elevated. Abdominal obesity is common, whereas male pattern baldness is rare. Testosterone treatment has been administered to some patients, although it does not affect the progression of SBMA [13–15].

In patients with SBMA, needle electromyography shows neurogenic abnormalities, and distal motor latencies are often prolonged in nerve conduction studies. Both the sensory nerve action potential and sensory evoked potential are reduced or absent in some cases [16]. The serum creatine kinase level is elevated in the majority of patients [17]. Hyperlipidemia, slight hepatic dysfunction, and impaired glucose tolerance or diabetes mellitus are also detected in some patients [18]. Female heterozygous and homozygous carriers are usually asymptomatic, although some have subclinical phenotypes such as high amplitude motor unit potentials or an elevated serum creatine kinase level [19–22]. The two major components of muscle weakness in patients with SBMA are motor neurone degeneration and myopathic degeneration. SBMA patients display myopathic symptoms including elevated serum creatine kinase level and frequent muscle cramps prior to the onset of muscle weakness [7,8,23]. Elucidation of the basis of the neuronal or muscle-specific pathogenesis will help clarify the origin of muscle weakness in patients with SBMA.

Molecular genetics

Androgen receptor, the causative protein of SBMA, is a 110-kDa nuclear receptor that belongs to the steroid/thyroid hormone receptor family [24]. Upon binding of an androgen, that is, testosterone or dihydrotestosterone, with AR, the AR binds to an androgen response element in the target gene to regulate its expression. AR is essential for the major effects of androgens including normal male sexual differentiation and pubertal sexual development, although AR-independent, nongenomic functions of androgens have been reported [25–27]. AR is expressed not only in primary and secondary sexual organs, but also in nonreproductive organs including the kidney, skeletal muscle, adrenal gland, skin and nervous system, suggesting the far-reaching influence of androgens on a variety of mammalian tissues. In the central nervous system, AR is expressed at relatively high levels in spinal and brainstem motor neurones, which are the same cells that are vulnerable in SBMA. The *AR* gene is located on chromosome Xq11-12. This approximately 90-kb-long gene contains eight exons coding for the functional domains specific to the nuclear receptor

family. The first exon encodes the N-terminal transactivating domain. Exons 2 and 3 encode the DNA-binding domain, whereas exons 4 through 8 encode the ligand-binding domain. The N-terminal transactivating domain, which contains the polyQ region, possesses a major transactivation function (AF-1) that is maintained by interaction with general transcriptional coactivators such as the c-AMP response element binding protein, TAFII130 and steroid receptor coactivator-1. The CAG repeat begins at codon 58 in the first exon of *AR*. The number of CAG repeats is highly variable due to slippage of DNA polymerase upon DNA replication. Whereas abnormal elongation of CAG repeats causes SBMA, an abnormally low number of CAG repeats increases the risk of prostate cancer [28]. The number of polymorphic CAG repeats in the *AR* gene normally ranges between 14 and 32, but it ranges between 40 and 62 in SBMA patients [29]. It also shows somatic mosaicism [30]. An inverse correlation has been reported between the CAG repeat size and the age at onset of SBMA [22,31–33]. There was also an inverse correlation between the CAG repeat size and the degree of muscular weakness adjusted by the age of the patient at examination [8]. Intergenerational CAG repeat expansion is observed via predominantly paternal transmission rather than maternal transmission, suggesting that particular instability of the CAG repeat occurs during spermatogenesis [31,34]. The severity of the disease differs in each male member of the same family.

Spinal and bulbar muscular atrophy has been considered to be an X-linked disease, whereas other polyQ diseases show autosomal dominant inheritance. In fact, female SBMA patients have few, if any, clinical manifestations, even though they possess a similar number of CAG repeats in the disease allele as their siblings with SBMA. Reduced mutant AR expression due to X-inactivation may prevent disease manifestation in females. However, hormonal intervention studies in mouse and fly models strongly suggest that a reduced testosterone level prevents nuclear accumulation of the mutant AR protein, resulting in absence of a neurological phenotype in females [35–37]. This view is strongly supported by the observation that homozygous female carriers manifest few symptoms [19]. Therefore, it seems to be inappropriate to regard SBMA as an X-linked recessive inherited disease, but rather its neurological phenotype likely depends on the serum testosterone concentration.

Histopathology

In patients with SBMA, lower motor neurones are markedly depleted through all spinal segments and in brainstem motor nuclei except for the third, fourth and sixth cranial nerves (Figure 1A) [6,38]. The number of nerve fibres in the ventral spinal nerve root is reduced, reflecting motor neuronopathy. Sensory neurones in the dorsal root ganglia were less severely affected, and large myelinated fibres demonstrate a distally accentuated sensory axonopathy in the peripheral nervous system [39,40]. The neurones in Onufrowicz nuclei, intermediolateral columns and Clarke's columns of the spinal cord are generally well preserved. Muscle histopathology shows both neurogenic and myogenic findings: there are groups of atrophic fibres with small angular fibres, fibre type grouping and clumps of pyknotic nuclei as well as variability in fibre size, hypertrophic fibres, scattered basophilic regenerating fibres and central nuclei (Figure 1B,C) [40].

A pathologic hallmark of most polyQ diseases is the presence of nuclear inclusions (NIs). In SBMA patients, NIs containing the mutant AR are detected in the residual motor neurones in the brainstem and spinal cord [41] as well as in the skin, testis and some other visceral organs [42]. These NIs are detectable by antibodies that recognize (i) a small portion of the N-terminus of the AR protein; (ii) the expanded polyQ (1C2); (iii) many components of the ubiquitin–proteasome system (UPS); and (iv) molecular chaperone pathways, but not by antibodies against the C-terminus of the protein. This observation suggests that the C-terminus of AR is truncated or masked upon formation of NIs [43–46]. Although NIs are important histopathological findings, their role in the pathogenesis of polyQ diseases has been debated [47–51]. Several studies suggested that inclusion formation might be a cellular response against the toxicity of abnormal polyQ proteins [52–54]. On the other hand, nuclear localization or accumulation of the abnormal proteins has been considered to be decisive for inducing neuronal cell dysfunction and degeneration in polyQ diseases including SBMA [55–59]. Immunohistochemical studies on autopsied SBMA patients and scrotal skin biopsy specimens from SBMA patients using 1C2 antibody showed that diffuse nuclear accumulation of the mutant AR was far more frequently observed than NIs, being distributed in a wide array of central nervous system nuclei and in a greater number of visceral organs than thus far believed (Figure 1D–F) [58]. In neural systems, diffuse nuclear

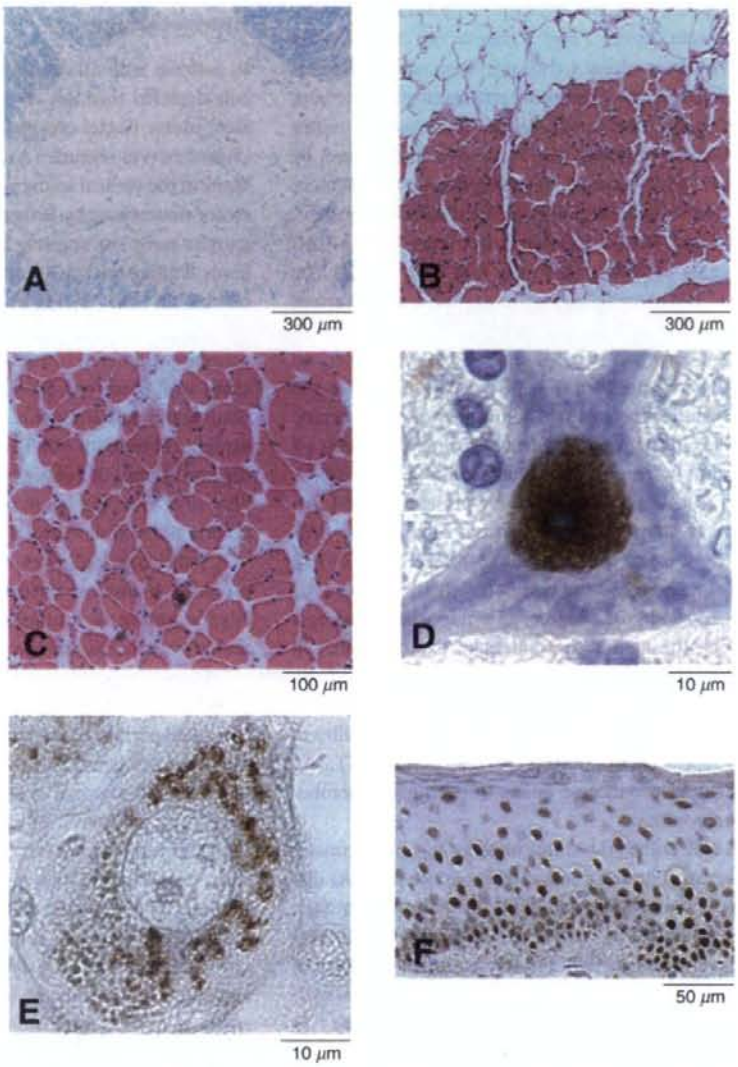

Figure 1. Histochemical analysis of neural and nonneuronal tissues from spinal and bulbar muscular atrophy patients. (A–E) Autopsy specimens. (F) biopsy specimen. (A) Kluver-Barrera's staining of a transverse section of the spinal cord demonstrates marked depletion of motor neurones in the anterior horn. Original magnification ×20. (B) Haematoxylin and eosin (HE) staining of the tongue muscle shows various degrees of grouped atrophy of muscle fibres and replacement with adipose tissue. Original magnification ×400. (C) HE staining of the iliopsoas muscle shows atrophic fibres interspersed between hypertrophic fibres, and rounded fibres with central nuclei. Original magnification ×400. (D) A residual motor neurone in the lumbar anterior horn shows diffuse nuclear accumulation of mutant androgen receptor (AR) detected by 1C2 antibody. Original magnification ×1000. (E) In addition to nuclear inclusions, large and small cytoplasmic inclusions immunoreactive for 1C2 are frequently observed in the cytoplasm of neurones in the spinal dorsal root ganglia. Original magnification ×1000. (F) Nuclear accumulation of mutant AR is also detected in epithelial cells of the scrotal skin, a nonneuronal tissue. Original magnification ×1000.

mutant AR accumulation also occurs in unaffected tissues including the basal ganglia, thalamus, hypothalamus, various midbrain, pontine and medullary nuclei, posterior horn, intermediolateral and Clarke's nuclei of the spinal cord, and sensory and sympathetic ganglion neurones, as well as in the affected brainstem and spinal cord motor neurones. The distribution of NIs was similar to the distribution of diffuse nuclear accumulation of mutant AR among neural and nonneural tissues, although the frequency of NIs in each tissue was far less than the frequency of diffuse nuclear accumulation (Figure 1**d**) [58]. It is of note that the extent of diffuse nuclear accumulation of mutant AR in motor and sensory neurones of the spinal cord was strongly correlated with the CAG repeat length, but not with the number of NIs in the spinal motor and sensory neurones [58]. Accumulating evidence suggests that NIs are not the toxic polyQ species, but that an oligomeric form of mutant AR may be the major pathogenic species. In view of the time course of the disease, diffuse nuclear accumulation of mutant proteins with an expanded polyQ tract might be an early event prior to NI formation, which is closely related to manifestation of neuronal dysfunction [35,60–62]. However, the molecular pathogenetic process by which diffuse nuclear mutant AR accumulation induces neuronal dysfunction and death still remains unclear. One possibility is that the interaction of transcriptional regulatory proteins with polyQ-expanded proteins results in aberrant transcriptional regulation which may result in neuronal dysfunction and cell death [63,64].

Another important observation is the presence of cytoplasmic mutant AR inclusions in neural and nonneural tissues [58]. In neural tissues, cytoplasmic accumulation is restricted to certain neuronal populations including dorsal root ganglia neurones, the mammillary body, hypothalamus, facial motor nucleus, and anterior and posterior horn neurones (Figure 1**e**). Among nonneural tissues, cytoplasmic inclusions occur in certain organs. Cytoplasmic inclusions are detectable by antibodies that recognize the Golgi apparatus [58]. Colocalization of a polyQ-expanded mutant protein with the Golgi apparatus has also been reported for ataxin-2 [65], although the significance of this colocalization remains unclear. Expression of polyQ-expanded mutant ataxin-2 disrupted the normal morphology of the Golgi complex and increased cell death [65]. On the other hand, lysosomal occurrence of other mutant proteins with an expanded polyQ tract in neurones has been reported in dentatorubral-pallidoluysian atrophy [66] and Huntington's disease (HD) [59]. Lysosomal localization of polyQ-expanded mutant proteins suggests a lysosomal autophagic degradation process acting independently of the ubiquitin–proteasome pathway in polyQ diseases [59]. Additionally, the reason why particular tissues have predominantly nuclear or cytoplasmic accumulation of polyQ-expanded mutant AR is not known. Differences in the predominant pathway of degradation of the mutant AR could influence the intracellular site of accumulation of mutant AR and eventual cell toxicity. One important question is whether cytoplasmic accumulation of mutant AR exerts cytotoxicity in neural and nonneural tissues. Cytoplasmic accumulation of mutant AR [67] as well as the accumulation of other mutant proteins involving an expanded polyQ tract [65,68,69] in Golgi apparatus and lysosomes indeed has been found to induce cytotoxicity. The accumulation of mutant protein with expanded polyQ tract in Golgi apparatus or lysosomes increases death of cultured cells via activation of apoptosis-related effectors such as caspase-3 [65,69,70]. It should be noted that histologically or immunohistochemically evident accumulation of a mutant protein is not necessarily cytotoxic, while microaggregates at the molecular level that are histologically undetectable may exert cytotoxicity. Indeed, excessive accumulation of mutant AR in aggresomes was found to protect cells from a cytotoxic form of mutant AR [67]. However, an immunohistochemical study on autopsied SBMA patients strongly suggested that cytoplasmic accumulation of mutant AR is related to mutant AR-mediated cytotoxicity and eventual symptom manifestation [58]. For instance, the pancreas showed cytoplasmic accumulation of mutant AR without obvious nuclear accumulation [58]. SBMA patients have an elevated serum glucose level and impaired glucose tolerance, suggesting islet cell dysfunction in the pancreas [18]. Although further studies on the significance of cytoplasmic accumulation of mutant AR are needed, nuclear accumulation of the mutant AR protein in motor neurones appears to cause motor neurone dysfunction, while cytoplasmic accumulation may underlie some visceral and possibly some neuronal dysfunction in patients with SBMA. The pathologic process is likely to differ in different tissues, being more prominent in the nuclei of motor neurones, but mainly cytoplasmic in certain neuronal populations and visceral organs. This cytoplasmic mutant AR is not ubiquitinated in contrast to nuclear-accumulated mutant AR, particularly the heavily ubiquitinated NIs.

suggesting that modification of mutant AR differs in the nucleus and cytoplasm. We also need to further clarify which degradation process affecting mutant AR is most active in a given tissue, for example, lysosomal in certain viscera *vs.* via the ubiquitination pathway in most neural tissues. Taken together, diffuse nuclear accumulation of mutant AR is apparently a cardinal pathogenetic process underlying neuronal dysfunction and eventual cell death, while cytoplasmic accumulation may also contribute to the pathophysiology of SBMA.

Molecular pathogenesis

Two mechanisms of the involvement of polyQ-expanded mutant AR in the pathogenesis of SBMA have been proposed: mutant AR acquires a toxic property, damaging motor neurones; or loss of normal AR function induces neuronal degeneration [71]. As androgens have trophic effects on neuronal cells, one can assume that loss of AR function may play a role in the pathogenesis of SBMA. Expansion of the polyQ tract mildly suppresses the transcriptional activities of AR, probably because it disrupts the interaction between the N-terminal transactivating domain of AR and transcriptional coactivators. Although this loss of function of AR may contribute to androgen insensitivity, a gain of toxic function of the mutant AR due to the expanded polyQ tract has been believed to play a pivotal role in the pathogenesis of SBMA. This hypothesis is supported by the observations that patients with severe testicular feminization lacking AR function and AR knockout mice do not have motor impairment [72]. Moreover, a transgenic mouse model expressing a protein composed of expanded polyQ driven by the human AR promoter demonstrated motor impairment, suggesting that the expanded polyQ protein is sufficient to induce the pathogenic process [73], whereas the male AR knock-in mouse model of SBMA has signs of androgen insensitivity such as decreased fertility, progressive abnormalities of germ cell maturation and the Sertoli cell cytoskeleton, and testicular atrophy [74]. However, a recent study revealed that the absence of endogenous normal AR protein in SBMA transgenic mice had deteriorative effects on neuromuscular and endocrine-reproductive features of these mice, although this mouse model expressing AR with expanded polyQ tract does not display signs of androgen insensitivity in the presence of the normal endogenous mouse *AR* gene [75]. Collectively, both the gain-of-function mutant protein toxicity and loss of normal AR protein function are the basis for motor neurone degeneration in SBMA, whereas impairment of AR function possibly causes signs of androgen insensitivity.

The fact that AR has a specific ligand, that is, testosterone, renders the pathogenesis of SBMA unique among polyQ diseases. An *in vitro* study using transfected COS cells showed that AR localized in the nucleus in the presence of testosterone, while AR remained largely in the cytoplasm in the absence of the hormone [76]. The AR is normally confined to a multiheteromeric inactive complex in the cell cytoplasm, and translocates into the nucleus in a ligand-dependent manner. Moreover, the half-life of AR is prolonged in the presence of its ligand, suggesting ligand-dependent stabilization of AR [76,77]. This intracellular trafficking and stabilization of AR appear to play important roles in the pathogenesis of SBMA.

We previously generated transgenic mice expressing the full-length human *AR* gene containing either 24 or 97 CAG repeats under the control of a cytomegalovirus enhancer and a chicken β-actin promoter [35]. This model recapitulated not only the neurologic disorder but also the phenotypic difference with gender which is a specific feature of SBMA. Mice that expressed *AR* with 97 CAG repeats (AR-97Q) exhibited progressive motor impairment, while none of the mice that expressed *AR* with 24 CAG repeats (AR-24Q) showed abnormal phenotypes [35]. The AR-97Q mice demonstrated small body size, short lifespan, progressive muscle atrophy, and weakness as well as reduced cage activity, all of which were markedly pronounced and accelerated in the male AR-97Q mice, but were either not observed or far less severe in the female AR-97Q mice. Western blot analysis revealed the transgenic AR protein smearing from the top of the gel in proteins isolated from the spinal cord, cerebrum, heart, muscle and pancreas. Although the male AR-97Q mice had greater amounts of smearing protein than their female counterparts, the female AR-97Q mice had a greater amount of monomeric AR protein. Diffuse nuclear staining of AR-97Q and less frequent NIs as detected by 1C2, were demonstrated in neurones of the spinal cord, cerebrum, cerebellum, brainstem, and dorsal root ganglia as well as in nonneuronal tissues such as the heart, muscles and pancreas. Male AR-97Q mice showed markedly more abundant diffuse nuclear staining and NIs than females, in agreement with the gender differences in symptoms and Western blot profile. Despite the profound gender difference in pathogenic AR protein expression, there was no significant difference in the mRNA level of

transgene expression between the male and female AR-97Q mice. Other laboratories have also generated various animal models expressing the full-length human AR with expanded polyQ tract, almost all of which display phenotypic expression of motor dysfunction with gender effects [78]. Ligand-dependent neurodegeneration has also been observed in a fruit fly model of SBMA [36]. These observations indicate that the ligand plays important roles in the gender difference of phenotypes, especially with regard to its interactions with mutant AR in the posttranscriptional stage.

Hormonal therapies

The dramatic gender difference of phenotypes led us to attempt hormonal interventions as a treatment for SBMA. First, we castrated male AR-97Q mice in order to reduce their testosterone level [35]. Next, leuprorelin, a potent luteinizing hormone-releasing hormone (LHRH) analogue, was administered subcutaneously to noncastrated AR-97Q mice [37]. Leuprorelin suppresses the release of the gonadotrophins, luteinizing hormone and follicle-stimulating hormone. Two to 4 weeks after the start of leuprorelin administration, the serum testosterone level decreased to the level achieved by surgical castration. Leuprorelin-treated male AR-97Q mice showed profound improvement of symptoms and histopathologic findings, and reduced nuclear localization of the mutant AR compared with the untreated, noncastrated male AR-97Q mice [35,37]. The body weight, motor function, and lifespan of male AR-97Q mice significantly improved by castration or leuprorelin administration. Western blot analysis and histopathologic studies revealed diminished nuclear accumulation of mutant AR in the male AR-97Q mice that had undergone castration or leuprorelin administration compared with that in untreated, noncastrated male AR-97Q mice. These results suggest that testosterone has toxic effects in AR-97Q mice by accelerating nuclear translocation of the mutant AR and promoting stabilization of the mutant AR protein. On the contrary, castration and leuprorelin administration each prevented nuclear localization and stabilization of the mutant AR by reducing the testosterone level. Nuclear localization of the mutant protein with expanded polyQ tract is likely to be important for inducing neuronal cell dysfunction and degeneration in the majority of polyQ diseases. It thus appears logical that a reduction in testosterone level improved the phenotypic expression of SBMA by preventing nuclear localization of the mutant AR. Testosterone deprivation reversed motor dysfunction in another transgenic mouse model of SBMA [79].

When leuprorelin was subcutaneously administered to male AR-97Q mice every 2 weeks starting at 5 weeks of age, leuprorelin initially increased the serum testosterone level by acting as an agonist at the LHRH receptor, but subsequently reduced it to an undetectable level. Leuprorelin-treated AR-97Q mice showed deterioration of body weight, gait and performance of the rotarod task at 8–9 weeks of age, during the time when the serum testosterone level initially increased through the agonistic effect of leuprorelin [37]. This elevation in testosterone level was transient and was followed by suppression of testosterone production and sustained reversibility of polyQ pathogenesis. Intriguingly, immunostaining of tail specimens, sampled from the same individual mouse, demonstrated an increase in the number of muscle fibres with nuclear 1C2 staining at 4 weeks of leuprorelin administration, although the number of muscle fibres with nuclear 1C2 staining decreased after another 4 weeks of treatment [37]. These results indicate that testosterone deprivation is sufficient to reverse both the symptomatic and pathologic phenotypes in AR-97Q mice.

Successful treatment of AR-97Q mice with leuprorelin led us to perform testosterone blockade therapies in SBMA patients [80]. In a preliminary open trial, treatment with leuprorelin for 6 months significantly reduced nuclear accumulation of mutant AR in the scrotal skin of SBMA patients, suggesting that androgen deprivation interrupts the pathogenic process of human SBMA [80]. Another trial on a larger scale is currently underway to verify the clinical benefits of leuprorelin in SBMA patients.

Hsp90-dependent pathogenesis

Heat shock protein (Hsp) 90, a molecular chaperone, is essential for the function and stability of the AR, the C-terminus of which has a high affinity for Hsp90. Hsp90 induces a conformational change in AR that is required for its nuclear translocation after ligand activation [24,81,82]. Hsp90 functions in multicomponent complexes of chaperone proteins including Hsp70, Hop, Cdc37 and p23, leading to the folding, activation and assembly of Hsp90 client proteins [83]. Two main Hsp90 complexes are thought to exist: one complex is a stabilizing form with Cdc37 and p23 and this complex stabilizes Hsp90 client proteins including AR, while the other

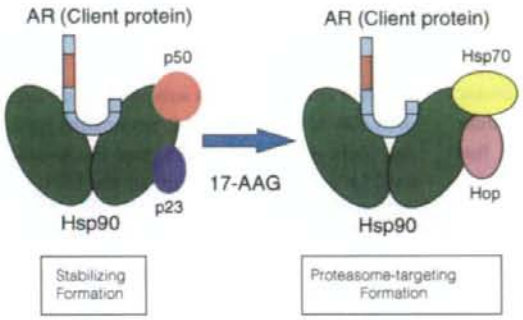

Figure 2. Diagram showing the change in the Hsp90 complex induced by an Hsp90 inhibitor. Hsp90 is required for nuclear translocation of androgen receptor (AR). Hsp90 functions in multicomponent complexes of cochaperone proteins including Hsp70, Hop, Cdc37 and p23. Two main Hsp90 complexes exist. One complex is a stabilizing form with Cdc37 and p23 and stabilizes Hsp90 client proteins, while the other complex is a proteasome-targeting form with Hsp70 and Hop and directs Hsp90 client proteins to proteasome degradation. Hsp90 inhibitor (17-AAG) specifically binds the ATP-binding site of Hsp90, resulting in a shift of the Hsp90 complex from the stabilizing form towards the proteasome-targeting form.

complex is a proteasome-targeting form with Hsp70 and Hop and it directs Hsp90 client proteins to proteasome degradation (Figure 2) [84–87]. p23 is thought to modulate Hsp90 activity during the last stages of the chaperoning pathway, leading to stabilization of Hsp90 client proteins in an ATP-dependent manner [88]. Hop is known to independently bind with both Hsp90 and Hsp70, thereby promoting the Hsp90/Hsp70 linkage, and is thought to direct the triage decision for client proteins by bridging the Hsp90–Hsp70 interaction [87]. Hsp90 inhibitors inhibit the ATP-dependent progression of the Hsp90 complex towards the stabilizing form and shift it to the proteasome-targeting form, resulting in proteasomal degradation of the Hsp90 client protein (Figure 2) [89,90]. Steroid receptors, including the progesterone receptor and the glucocorticoid receptor, were the first Hsp90 client proteins to be identified [91,92]. As for AR, Hsp90 is essential for maintaining its high ligand-binding affinity and for its stabilization [81,93]. In practice, Hsp90 inhibitors reduce the binding affinity of AR for androgens, and induce the degradation of AR [93,94]. Numerous oncoproteins belonging to the Hsp90 client protein family are selectively degraded in the UPS by Hsp90 inhibitors, and 17-allylamino-17-demethoxygeldanamycin (17-AAG), a first-in-class Hsp90 inhibitor, is now under clinical trials as a novel molecular-targeted agent for a wide range of malignancies [95]. In addition, Hsp90 inhibitors have been shown to have some neuroprotective effects against various stresses such as drug-induced toxicity, oxidative stress, and oxygen glucose deprivation [96–99]. AR also belongs to the Hsp90 client protein family, and is degraded in the presence of Hsp90 inhibitors [85,93,94]. Therefore, we explored the possibility of using 17-AAG as a therapeutic agent for neurodegenerative diseases by examining its effects on mutant AR in cultured cells and in a mouse model of SBMA. We found that 17-AAG inhibits nuclear accumulation of this protein in cultured cells, leading to marked amelioration of the motor phenotype of AR-97Q mice without detectable toxicity [100]. Of interest is the finding that 17-AAG preferentially targeted mutant AR rather than wild-type AR to proteasomal degradation. A high association between p23 and AR containing expanded polyQ tract renders the mutant AR more sensitive to Hsp90 inhibitors than wild-type AR [100]. Western blot and filter trap analyses in AR-97Q mice both showed that 17-AAG significantly reduced the amount of the insoluble high-molecular-weight complex of mutant AR as well as the amount of soluble monomer of mutant AR in the spinal cord and skeletal muscle. Moreover, in an immunostaining study of nervous tissue from AR-97Q mice, 17-AAG significantly reduced the amount of diffuse nuclear-accumulated AR, suggesting that 17-AAG had a curative effect on SBMA by reducing the amounts of oligomers and the soluble monomeric form of the mutant AR. Alternatively, 17-AAG may inhibit aggregation of mutant AR by inducing Hsp70 and Hsp40 expression. Hsp90 inhibitors cause disassociation of heat shock factor-1 (HSF-1) from the Hsp90 complex and trimerization of the HSF-1, thereby resulting in HSP activation [100]. The Hsp90 inhibitor, geldanamycin, induced Hsp70 and Hsp40 expression in transfected COS-1 cells, thereby inhibiting polyQ-induced abnormal aggregation of huntingtin protein [101]. However, as 17-AAG displayed only a limited ability to induce Hsp70 and Hsp40 expression in mouse tissue [100], the large decrease in the amount of AR seen in the insoluble fraction *in vivo*, rather than being a result of HSP induction, may be due to the potent ability of 17-AAG to degrade the soluble monomeric form of the mutant protein, thereby preventing its aggregation [100]. Furthermore, assembly of Hsp90 and its cofactors into complexes is required for retrograde, dynein-dependent movement of several steroid receptors [102,103]. Thus, in cells that do not express HSF-1, Hsp90 inhibitors inhibit the translocation of AR to the

nucleus and prevent ligand-dependent aggregation of the polyQ-expanded AR by inhibiting dynein-dependent AR trafficking [104].

Among the other proposed therapeutic approaches we previously studied [35,37,105,106], the efficacy of 17-AAG most closely approximated the successful hormonal therapy using the LH-RH analogue, leuprorelin [100]. However, unlike leuprorelin, the Hsp90 inhibitor 17-AAG holds enormous potential for application to a wide range of neurodegenerative diseases in addition to SBMA as previously reported [107–109]. We regard this general versatility as very important for the development of Hsp90 inhibitors as a treatment for neurological disorders. The strategy behind Hsp90 inhibitors differs from previous strategies employed against polyQ diseases, which unavoidably allowed abnormal protein to remain and placed much value mainly on inhibition of protein aggregation. We consider that the ability to facilitate degradation of disease-causing proteins by modulation of Hsp90 function would be of value when applied to SBMA and other neurodegenerative diseases. There is no doubt that reduction of the amount of the main culprit protein would have a curative effect against various neurodegenerative diseases. In fact, one therapeutic approach that directly reduces the level of abnormal protein by RNA interference has already proven beneficial in various mouse models of polyQ diseases and amyotrophic lateral sclerosis [110–112].

17-AAG-induced degradation requires a well-preserved proteasome function [89,90,100,113]. However, a question as to whether the UPS is impaired or not in patients with SBMA has been raised concerning this UPS-dependent therapy [113]. It is generally accepted that the UPS is involved in the pathology of polyQ diseases, as many components of the UPS and molecular chaperones are known to colocalize with polyQ-containing NIs [114,115]. Previous studies performed in cultured cell models suggested that the UPS is impaired in patients with polyQ diseases [54,116–118]. If this hypothesis were true, 17-AAG would not be able to exert its pharmacological effect on polyQ diseases. In this regard, recent studies using *in vivo* proteasome assays have raised important questions as to whether patients with polyQ diseases have an impaired UPS [53,119,120]. It has been reported that neuronal dysfunction developed without significant impairment of the UPS in a mouse model of SCA7 [53]. Consistent with this, it was also demonstrated that proteasome impairment did not contribute to the pathogenesis of HD in a mouse model [120]. Furthermore, in conditional mouse models of polyQ disease, genetic loss of the abnormal gene product led to rapid clearance of pre-existing polyQ-mediated NIs and reversible improvement of the abnormal phenotypes [121,122]. If the UPS were irreversibly damaged in patients and animal models of polyQ diseases, then the amount of pre-existing NIs would not decrease. We therefore consider that treatment with 17-AAG, which takes advantage of a self-clearing system to target disease-causing proteins, is a reasonable therapeutic strategy against polyQ-related and other neurodegenerative diseases.

Induction of heat shock proteins

Many components of the UPS and molecular chaperones are known to colocalize with polyQ-containing NIs, implying that these proteins are involved in neurodegeneration in polyQ diseases. HSPs are classified into different families according to molecular size: Hsp100, Hsp90, Hsp70, Hsp60, Hsp40 and small HSPs [123]. These HSPs are either constitutively expressed or inducibly synthesized after cellular stress. HSPs play important roles in maintaining correct folding, assembly, and intracellular transport of newly synthesized proteins. For example, Hsp70 and Hsp90, which are essential components of the AR–chaperone complex in the cell cytoplasm, regulate the function, nuclear translocation, and degradation of AR [124]. Under toxic conditions, the synthesis of HSPs is rapidly up-regulated, and nonnative proteins are refolded as a consequence. Therefore, forced overexpression of HSPs resulted in acquisition of tolerance against various types of stresses, and protection against apoptosis in various disease models [125]. In various polyQ disease models, both genetic and pharmacological overexpression of HSPs have been shown to suppress aggregate formation and cellular toxicity [109,114,126–128]. Hsp70 cooperates with Hsp40 in functioning as a molecular chaperone. These HSPs have been proposed to prevent the initial conformation conversion of mutant polyQ-containing protein from a random coil to a β-sheet, leading to attenuation of toxic oligomer formation [128]. Overexpression of Hsp70, together with Hsp40, inhibited the toxic accumulation of abnormal polyQ-containing protein and suppressed cell death in a variety of cellular models of polyQ diseases including SBMA [114,129]. Hsp70 has also been shown to facilitate proteasomal degradation of abnormal AR protein in a cell culture model of SBMA [130]. The favour-

able effects of Hsp70 have been verified in studies using mouse models of polyQ diseases. Overexpression of the inducible form of human Hsp70 markedly ameliorated the symptomatic and histopathological phenotypes of the SCA1 mouse model [131] and AR-97Q mice [105]. These beneficial effects were dependent on the dose of the Hsp70 gene and correlated with the reduction in the amount of nuclear-accumulated mutant AR protein [105]. It should be noted that Hsp70 overexpression also significantly reduced the amount of the soluble form of mutant AR, suggesting that Hsp70 overexpression accelerated the degradation of mutant AR in AR-97Q mice.

Favourable effects obtained by genetic modulation of HSPs suggest that pharmacological induction of molecular chaperones might be a promising approach for the treatment of SBMA and other polyQ diseases. Many studies have shown that HSP induction by Hsp90 inhibitors exerted potentially neuroprotective effects in models of HD [101,132,133], tauopathies [134–136], Parkinson's disease [137–139], stroke [140,141] and autoimmune encephalomyelitis [142]. As for polyQ diseases, Sittler *et al.* [101] first showed that geldanamycin significantly suppressed aggregation of mutant huntingtin in a cultured cell model of HD via induction of Hsp70 and Hsp40 heat shock response. Geranylgeranylacetone (GGA), an acyclic isoprenoid compound with a retinoid skeleton, has been shown to strongly induce HSP expression in various tissues [143]. This compound has been used as an oral anti-ulcer drug. Oral administration of GGA up-regulated the levels of Hsp70, Hsp90 and Hsp105 via activation of HSF-1 in the central nervous system and inhibited nuclear accumulation of the pathogenic AR protein, resulting in amelioration of polyQ-dependent neuromuscular phenotypes of the AR-97Q mice [144]. Thus, enhancement of cellular defences using Hsp90 inhibitors and GGA is a reasonable clinical approach for the treatment of neurodegenerative diseases. The ability of Hsp90 inhibitors to significantly induce the expression of HSPs has been demonstrated in cultured cells and a fly model, but not in mammals. As 17-AAG had only a limited ability to induce Hsp70 expression in mouse tissue [100], further studies should be performed to address to what extent Hsp90 inhibitors can induce the expression of HSPs in mouse models of neurodegenerative disorders other than SBMA.

On the other hand, several studies suggest that polyQ elongation interferes with the protective cellular responses against cytotoxic stress [128]. Transfected cells expressing truncated AR composed of the first N-terminal 127 amino acids of human AR with an expanded polyQ tract showed delayed induction of Hsp70 after heat shock compared with that in transfected cells expressing the full-length AR [145]. Bates and colleagues reported progressive decreases in the expression of Hsp70 and Hsp40 in the brain lesion of an animal model of HD [132], and such decreases were also observed in AR-97Q mice [144]. The threshold of HSP induction is known to be relatively high in spinal motor neurones [146]. Taken together, impairment of the capability of HSP induction is implicated in the pathogenesis of motor neurone degeneration in SBMA as HSPs are potent suppressors of polyQ toxicity.

Biomarkers for clinical trials

As SBMA is a slowly progressive disease and its precise natural history has not been well elucidated, long-term clinical trials are necessary to assess whether certain drugs can alter the natural progression of the disease. Suitable biomarkers that reflect the pathogenesis and severity of SBMA, are necessary to be able to assess the therapeutic efficacy and to improve the power and cost-effectiveness of longitudinal drug trials. Punch biopsy of the scrotal skin is safe and easy to perform on patients, whereas it is not practical to obtain a biopsy specimen from the central nervous system. We studied a biomarker of SBMA, that is, the degree of nuclear accumulation of mutant AR in epithelial cells of scrotal skin biopsy samples, which can be used as a surrogate endpoint in therapeutic trials [80]. In that study, the degree of nuclear accumulation of mutant AR in scrotal skin biopsy samples from 13 SBMA patients was assessed by 1C2 staining (Figure 1f). The percentage of cells with nuclear 1C2 staining among the scrotal skin epithelial cells tended to be correlated with that in spinal motor neurones among five autopsied SBMA cases, and it was positively correlated with the CAG repeat length and inversely correlated with the functional Activities of Daily Living scale as assessed by the Norris score on limbs among the 13 SBMA patients [80]. The results demonstrate that the percentage of cells with nuclear 1C2 staining in scrotal skin biopsy samples could predict the pathogenic process in the motor neurones of patients with SBMA. Moreover, subcutaneous injections of leuprorelin in SBMA patients reduced both the intensity and frequency of diffuse nuclear 1C2 staining in scrotal skin epithelial cells during the first 4 weeks of therapy and this effect was markedly enhanced after

12-week treatment, suggesting that 1C2 immunostaining of scrotal skin biopsy samples for nuclear mutant AR is a practical procedure for estimating the severity of SBMA pathogenesis in the nervous system [80]. Based on the observations described above, the degree of 1C2-stained nuclear mutant AR accumulation in biopsied scrotal skin is likely to be a potent biomarker reflecting the pathogenic process of SBMA. Since the degree of nuclear accumulation of mutant AR in biopsied scrotal skin appears to be a promising surrogate endpoint, further trials are necessary to evaluate this biomarker by confirming that the change in scrotal skin findings correctly predicts the true clinical outcome event such as becoming wheelchair-bound, aspiration pneumonia or death.

Conclusions

Studies using cellular and animal models provide insight into mechanisms involved in neurodegeneration in SBMA, and reveal promising approaches to treatment of this disease, among which LHRH analogues and 17-AAG appear to be potent agents for treating patients. The results of animal studies should be verified in carefully designed clinical trials. Clinical studies on SBMA patients, however, are challenging because of the slowly progressive nature of SBMA and the low sensitivity of clinical examination to detect changes over short periods of time. Furthermore, these treatments for SBMA are disease-modifying therapies that inhibit the pathogenic process of motor neurone degeneration rather than symptom-relief therapies (Figure 3). Therefore, large-scale clinical trials of long duration are necessary, and establishment of objective biomarkers is of utmost importance in order to improve the power and cost-effectiveness of longitudinal clinical treatment trials (Figure 3). For this purpose, clinical and pathological parameters representing the pathogenic process of SBMA should be extensively investigated.

The ideal treatment for polyQ diseases appears to be a combination of several potential therapeutic strategies, as each approach has adverse effects and long-term treatment is unavoidable in the therapy of polyQ diseases. Elucidation of the pathophysiology, high-throughput drug screening and intensive clinical trials are necessary for establishing human therapeutics for this disease.

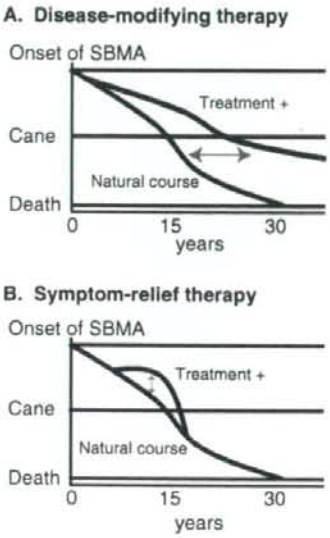

Figure 3. Disease-modifying and symptom-relief therapies for spinal and bulbar muscular atrophy (SBMA). (A) Long-term clinical trials are necessary to evaluate the effects of disease-modifying therapies, as SBMA is a slowly progressive disease. (B) Symptom-relief therapy ameliorates symptoms of SBMA, and the duration of these clinical trials is short. The *y*-axis shows the general clinical course of SBMA patients. 'Cane' indicates the requirement of a cane for walking. Arrows indicate the study duration that is required for each therapy.

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