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ORIGINAL PAPER

Androgen-dependent impairment of myogenesis in spinal and bulbar muscular atrophy

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Abstract Spinal and bulbar muscular atrophy (SBMA) is an inherited neuromuscular disease caused by expansion of a polyglutamine (polyQ) tract in the androgen receptor (AR). SBMA is triggered by the interaction between polyQ-AR and its natural ligands, testosterone and dihydrotestosterone (DHT). SBMA is characterized by the loss of lower motor neurons and skeletal muscle fasciculations, weakness, and atrophy. To test the hypothesis that the interaction between polyQ-AR and androgens exerts cell-autonomous toxicity in skeletal muscle, we characterized the process of myogenesis and polyQ-AR expression in DHT-treated satellite cells obtained from SBMA patients and age-matched healthy control subjects. Treatment with androgens increased the size and number of myonuclei in myotubes from control subjects, but not from SBMA patients. Myotubes from SBMA patients had a reduced number of nuclei, suggesting impaired myotube fusion and altered contractile structures. The lack of anabolic effects of androgens on myotubes from SBMA patients was not due to defects in myoblast proliferation, differentiation or apoptosis. DHT treatment of myotubes from SBMA patients increased nuclear accumulation of polyQ-AR and decreased the expression of interleukin-4 (IL-4) when compared to myotubes from control subjects. Following DHT treatment, exposure of myotubes from SBMA patients with IL-4 treatment rescued myonuclear number and size to control levels. This supports the hypothesis that androgens alter the fusion process in SBMA myogenesis. In conclusion, these results provide evidence of an androgen-dependent impairment of myogenesis in SBMA that could contribute to disease pathogenesis.

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Introduction

Spinal and bulbar muscular atrophy (SBMA), also known as Kennedy's disease, is an X-linked adult motor neuron disorder caused by expansion of a polyglutamine (polyQ)-encoding CAG trinucleotide repeat in the first exon of the gene coding for the androgen receptor (AR) [19]. CAG trinucleotide repeat number ranges from 10 to 36 in healthy individuals and from 37 to 62 in SBMA patients. The main clinical features of SBMA are proximal limb and bulbar muscle weakness and atrophy [18]. Patients occasionally show signs of androgen insensitivity. SBMA belongs to the family of polyQ disorders that also includes Huntington's disease, dentatorubral-pallidoluysian atrophy, and six types of spinocerebellar ataxia [27]. PolyQ diseases share several features, including accumulation of misfolded polyQ protein in the form of oligomers/micro-aggregates and inclusions, late-onset appearance of disease symptoms, and the phenomenon of genetic anticipation. A unique feature of SBMA among polyQ diseases is sex-specificity, with males showing full disease manifestations, and females showing only mild symptoms even if homozygous for the mutation [39, 42]. SBMA is triggered by the interaction between polyQ-AR and the natural ligands, testosterone and its more potent derivative dihydrotestosterone (DHT) [31, 32]. The sex-dependent nature of SBMA is also observed in animal models of SBMA [8, 16, 48]. Hormone binding results in a substantial change in AR biology that leads to dissociation from heat shock proteins in the cytosol, dimerization, and nuclear translocation. In the nucleus, the AR binds to specific sequences on the genome known as androgen-responsive-elements and interacts with transcriptional co-activators and co-repressors to regulate the expression of androgen-responsive genes. These ligand-induced events have all been implicated in SBMA pathogenesis [25, 26].

There is evidence that muscle atrophy in SBMA is the result of motor neuron dysfunction and degeneration with consequent denervation. However, findings also support the idea that polyQ-AR may exert cell-autonomous toxicity in skeletal muscle [reviewed in 36]. SBMA patients have unusually high levels of circulating creatine kinase [12], even before disease onset [44], suggesting skeletal muscle damage. Moreover, SBMA patients present with signs of both denervation (e.g., sprouting, fiber atrophy and grouping) and myopathy (e.g., fiber splitting and the presence of large fibers with central nuclei) [43]. Interestingly, these features positively correlate with the degree of disability [43]. Signs of muscle denervation and myopathy are also present in rodent models of SBMA [48]. In a knock-in mouse model of SBMA, muscle atrophy precedes spinal cord pathology, suggesting a causative role of muscle [48]. Also, the muscle-specific overexpression of an AR with a non-pathogenic polyQ tract resulted in a phenotype that resembles SBMA, indicating that dysregulation of androgen signaling in skeletal muscle is sufficient to produce an SBMA-like phenotype in mouse [24].

AR is highly expressed in skeletal muscle and in particular in satellite cells, where AR expression is regulated by androgens [41]. Satellite cells are adult stem cells positioned under the basal lamina and are direct targets for androgen action [9]. Mechanical, hormonal, and growth factor signaling stimulates satellite cell proliferation, differentiation into myotubes and incorporation into the growing muscle fibers to promote muscle regeneration. Satellite cell activation and differentiation are controlled by a specific gene expression program that includes upregulation of myogenic regulatory factors, such as myogenin (MyoG) and MyoD [30]. The AR-mediated action of androgens in skeletal muscle stimulates satellite cell commitment into the myogenic lineage [reviewed in 14]. Whether androgens affect the myogenic program in SBMA remains to be established.

The focus of this study was to investigate whether muscle damage triggered by the interaction between androgens and polyQ-AR in skeletal muscle has a primary pathogenetic role in SBMA. We report that myoblasts from SBMA patients grown in a culture medium enriched with androgens undergo normal proliferation and differentiation. However, these SBMA-derived myoblasts have fusion defects, as they generate hypotrophic myotubes that pathologically accumulate AR within the nucleus. Overall, our findings suggest intrinsic abnormalities in skeletal muscle of SBMA patients. Based on these observations, we propose that direct toxicity of polyQ-AR in skeletal muscle plays a central role in disease pathogenesis.

Materials and methods

Cell cultures

Primary myoblast cells were extracted from quadriceps femoris muscle biopsies taken from seven SBMA patients (referred to as SBMA myoblasts) and six healthy age-matched male control subjects (referred to as control myoblasts) (Tables 1, 2), as previously described [21]. In all SBMA patients, muscle biopsy specimens showed both neurogenic and myopathic alterations. Cells were cultured in Ham's F14 medium (Euroclone) plus 30 % fetal bovine serum (FBS, Gibco, Invitrogen) and 10 mg/ml insulin (Sigma). Only myoblasts at similar passage number (from

Table 1 Clinical and molecular data of SBMA patients in the study

Patient	Age at onset (years)	Age at biopsy (years)	CAG repeats number	Presenting symptoms	Biopsied muscle	MRC score of the biopsied muscle	CK (U/L)
1	54	54	44	Cramps	Quadriceps femoris	5/5	1,000
2	50	56	46	Limb-girdle weakness	Quadriceps femoris	4/5	2,040
3	52	61	49	Cramps, limb-girdle weakness	Quadriceps femoris	5/5	1,000
4	56	57	49	Limb-girdle weakness	Quadriceps femoris	4/5	2,196
5	27	29	44	Cramps	Quadriceps femoris	5/5	112
6	44	54	49	Cramps, limb-girdle weakness	Quadriceps femoris	5/5	798
7	44	64	>38	Cramps	Quadriceps femoris	5/5	481

MRC Medical Research Council, *CK* creatine kinase

Table 2 Clinical data of male control subjects

Pt	Diagnosis	Age at biopsy (years)	Biopsied muscle	Muscle pathology
1	Cramps	52	Quadriceps femoris	Rare scattered atrophic fibers
2	Cramps	56	Quadriceps femoris	Normal
3	Muscle rigidity	19	Quadriceps femoris	Very mild myopathic changes
4	Exercise intolerance	45	Quadriceps femoris	Very mild myopathic changes
5	Cramps	23	Quadriceps femoris	Rare scattered atrophic fibers
6	Muscle rigidity	62	Quadriceps femoris	Rare scattered atrophic fibers

2 through 7) were used for analysis. Cells at 70 % confluence were differentiated by lowering FBS to 2 %. All the proliferating and differentiating cell lines were seeded at a density of 7,000 cells/cm^2 and cultured in parallel with and without DHT (10 nM, Sigma), and, when indicated, with IL-4 (5 ng/ml, Prepotech). Samples were collected at 0 (T0), 10 (T10), and 15 (T15) days of differentiation. For each experiment, samples from at least three different SBMA patients were used.

Growth curve

Myoblast proliferation was determined as previously described [45]. In brief, 10^5 cells were seeded at a density of 4,000 cells/cm^2 then collected and counted after 2, 4, and 7 days in duplicate.

Morphological analysis and immunofluorescence

Bright-field images of living myotubes were collected using a Zeiss IM35 microscope equipped with a standard camera. Fusion index was quantified by counting the nuclei in at least 100 myotubes per cell line using contrast phase microscopy. Average myotube width at T10 and T15 of differentiation was measured using ImageJ ProPlus 6 software. Briefly, once the two borders of each myotubes were drawn, the software gave the average width between the two lines. Immunofluorescence analyses were performed in myoblasts and myotubes fixed in 4 % paraformaldehyde (20 min), permeabilized with 0.2 % Triton X-100 and incubated for 30 min in 1 % bovine serum albumin (BSA). The AR (1:100, clone H280, Santa Cruz) and fetal myosin (1:100, Abcam) primary antibodies were diluted in

phosphate buffered saline (PBS) plus 2 % BSA and incubated overnight at 4 °C. After washing, secondary Alexa 488 antibody (Invitrogen) was incubated for 1 h at room temperature. Samples were then washed and mounted on Vectashield medium containing 40-6-diamidino-2-phenylindole (DAPI, Vector Laboratories) and analyzed with an Olympus BX60 fluorescence microscope.

Electron microscopy (EM)

T10 differentiated myotubes were first fixed in 2.5 % glutaraldehyde in cacodylate buffer for 3 h and then in 1 % osmium tetroxide in cacodylate buffer. All samples were dehydrated and detached from the plastic dish using propylene oxide, centrifuged, and embedded in Araldite. Ultrathin sections were stained with uranyl acetate and lead citrate and analyzed with a Philips 400T transmission electron microscope.

Western blotting analysis

Total cell lysates were prepared by maintaining the cells on ice for 30 min with 200 µl of RIPA buffer (65 mM Tris, 150 mM NaCl, 1 % NP-40, 0.25 % Na-DOC, 1 mM EDTA, pH 7.4) and a cocktail of protease inhibitors (Sigma). To obtain nuclear and cytosolic fractions, the cells were scraped in 500 µl 1× PBS and centrifuged for 5 min at 4,000 rpm. Cell pellets were separated into nuclear and cytosolic fractions using the NE−PER Nuclear and Cytoplasmic Extraction kit (Thermo Scientific), supplemented with protease inhibitor cocktail (Sigma). After centrifugation at 14,000*g* for 15 min at 4 °C, an equal amount of protein (30 µg) from each sample was separated into 7.5 or 10 % Criterion precast gels (Bio-Rad Laboratories) and transferred onto a nitrocellulose membrane. Membranes were blocked in 5 % (w/v) fat-free milk in 0.02 M Tris/HCl pH 7.5, 137 mM NaCl, and 0.1 % (v/v) Tween-20 for 1 h at room temperature. Membranes were then incubated overnight at 4 °C with the primary antibodies for AR (N-20, 1:1000, Santa Cruz), Lamin B (M20, 1:1000, Santa Cruz), total Akt, phosphorylated Akt (1:1000, Cell Signaling), myogenin (1:400, Millipore) and β-actin (1:10,000, Chemicon). After 1 h incubation with secondary HRP-conjugated antibodies, signals were visualized by chemiluminescence (GE HealthCare). Integrated optical density of each band was calculated with commercial software (Gel Pro Analyzer).

Real-time PCR

Total RNA was isolated from myoblasts and myotubes using Trizol (Invitrogen). In all samples, 1 µg of total RNA was reverse-transcribed to cDNA (SuperScript III First-Strand Synthesis System, Invitrogen) and transcript levels were measured using SYBR Green Real-Time PCR (DyNAmo HS SYBR GREEN, Finzyme) using the ABI PRISM 7000 sequence detection system. Primer sequences are listed in Table 3 of supplementary material.

TUNEL assay

TUNEL assay (DeadEnd Fluorometric TUNEL System, Promega) was used to detect apoptosis. This method measures nuclear DNA fragmentation of apoptotic cells by catalytically incorporating fluorescein-12-dUTP at 3′-OH DNA ends using the enzyme terminal deoxynucleotidyl transferase (TdT). The fluorescein-12-dUTP-labeled DNA was visualized directly by fluorescence microscopy (Olympus BX60). Cells were mounted with Vectashield medium containing DAPI. At least 50 myotubes per cell line were analyzed.

Statistical analysis

Quantitative data were presented as mean ± SD. Statistical comparisons were performed using either Student's *t* test or ANOVA.

Results

Androgens promote hypertrophy in control, but not SBMA myotubes

To determine whether androgens exert toxic effects in skeletal muscle cells expressing polyQ-AR, we established primary muscle cell cultures obtained from seven SBMA patients and six age-matched healthy male subjects. Myoblasts were cultured in differentiation medium for either 10 (T10) or 15 days (T15) in the presence or absence of DHT, followed by the analysis of myotube morphology and width as described in the "Materials and methods" section (Fig. 1). In the absence of DHT, the width of SBMA myotubes was similar to that of control myotubes at both T10 and T15 (Fig. 1a, b). In the presence of DHT, the average width of control myotubes increased by 20 % ($p < 0.05$) at T10 and T15, indicating that androgens exert hypertrophic effects on control myotubes. Importantly, the effect of androgens on myotube width was absent in the cells obtained from SBMA patients (Fig. 1a, b).

The insulin-like growth factor 1 (IGF-1)/Akt signaling plays a critical role in muscle hypertrophy [37]. Moreover, selective activation of Akt by IGF-1 in skeletal muscle has been shown to be protective in SBMA mice [29]. Activation of Akt occurs through phosphorylation at serine 473 [3]. To determine whether the lack of androgens' hypertrophic

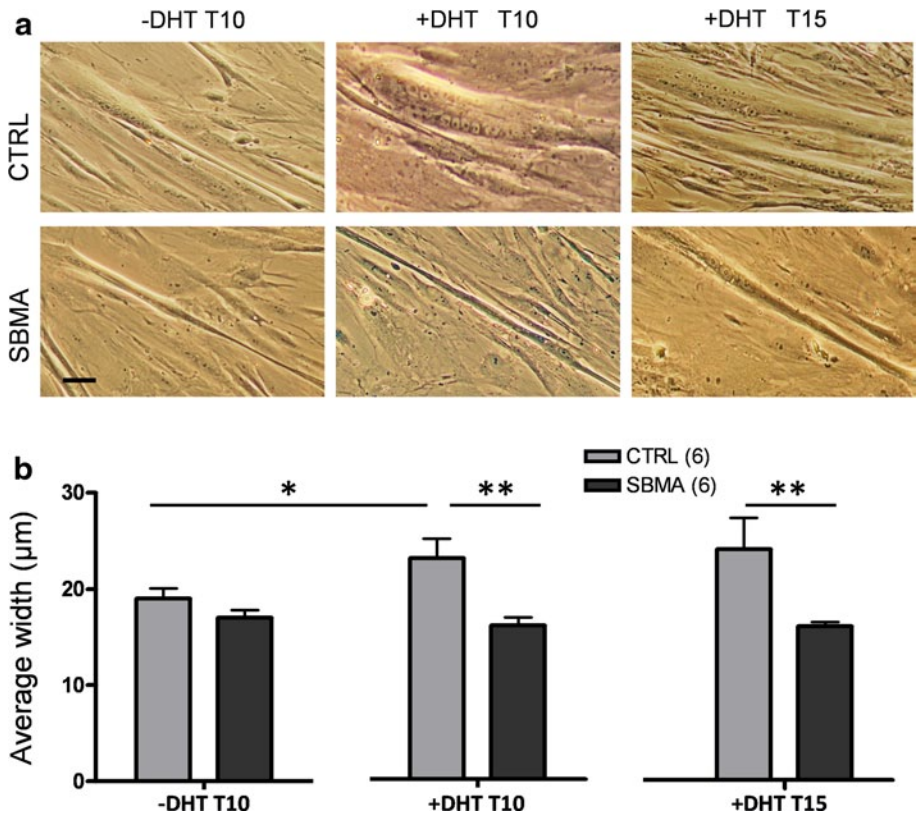

Fig. 1 Lack of androgens' hypertrophic effect on SBMA myotubes. **a** Representative bright-field images of control and SBMA myotube population at 10 (T10) and 15 (T15) days of differentiation. *Scale bar* 40 µm. **b** DHT treatment induced a significant increase in myotube width in control (CTRL), but not in SBMA myotubes at T10 and T15 ($n = 100$ myotubes/cell line). *Graph* mean ± SD. *$p < 0.05$; **$p < 0.01$ by ANOVA, while post hoc multiple comparisons were done by Tukey test. The number of single lines studied is given in *brackets*

effect was due to altered Akt signaling in SBMA muscle cells, the levels of phosphorylated Akt were measured by Western blotting analysis in SBMA and control muscle cell cultures treated with DHT (Supplementary Fig. 1). We did not observe any difference in the ratio between phosphorylated and total Akt in SBMA myoblasts and myotubes when compared with control cells. These findings indicate that Akt activation is not altered in cultured SBMA muscle cells.

Androgens exert anabolic effects on muscle also by enhancing contractile protein synthesis [14]. To examine whether androgens influence the status of contractile proteins in T10-differentiated myotubes, we performed immunofluorescence analysis of fetal myosin. In the absence of DHT, the immunofluorescence signal of the anti-fetal myosin antibody was similar in SBMA and control myotubes (Fig. 2a1, b1). Treatment with DHT enhanced the fetal myosin signal in control myotubes (Fig. 2c1, c2). This increase was absent in SBMA myotubes (Fig. 2d1, d2). Ultrastructural analysis confirmed a difference in myofibrillar organization, which was well defined in DHT-treated control myotubes (Fig. 2c3). In contrast, DHT-treated SBMA myotubes presented poor and disorganized myofibrillar structures (Fig. 2d3). In line with this morphological analysis, both fetal myosin (MYH3) mRNA (Fig. 2g) (SBMA: 4.3 ± 1.7 vs. control: 13.8 ± 2.0; $p < 0.01$) and protein levels (Fig. 2e, f) (SBMA: 2.52 ± 0.97 vs. control: 19.38 ± 3.56; $p < 0.01$) were significantly decreased in DHT-treated SBMA myotubes. These results indicate that androgens exert hypertrophic effects on control myotubes, but they have reduced anabolic effects on SBMA myotubes.

Proliferation and differentiation of SBMA myoblasts are not affected by polyQ-AR

We hypothesized that the lack of androgens' effect on SBMA myotubes results from reduced myoblast proliferation or altered differentiation. To test this hypothesis, we assessed the duplication time of SBMA and control myoblasts. We observed that the proliferation rate of SBMA myoblasts is similar to that of control myoblasts and is independent of DHT treatment (data not shown). MyoD is required for myoblast determination [15]. Using RT-PCR, we examined MyoD expression levels in SBMA and control myoblasts and observed no difference between groups (Fig. 3a, left panel). Taken together, these data indicate that polyQ-AR does not alter the proliferation of SBMA myoblasts.

To test whether androgens affect the differentiation of myoblasts to myotubes, we performed RT-PCR analysis of the expression levels of MyoD (Fig. 3a, right panel), MyoG (Fig. 3c, right panel), and muscle-specific creatine kinase (CK-M), a marker of muscle differentiation (Fig. 3b, right panel). In the presence and absence of DHT, the levels

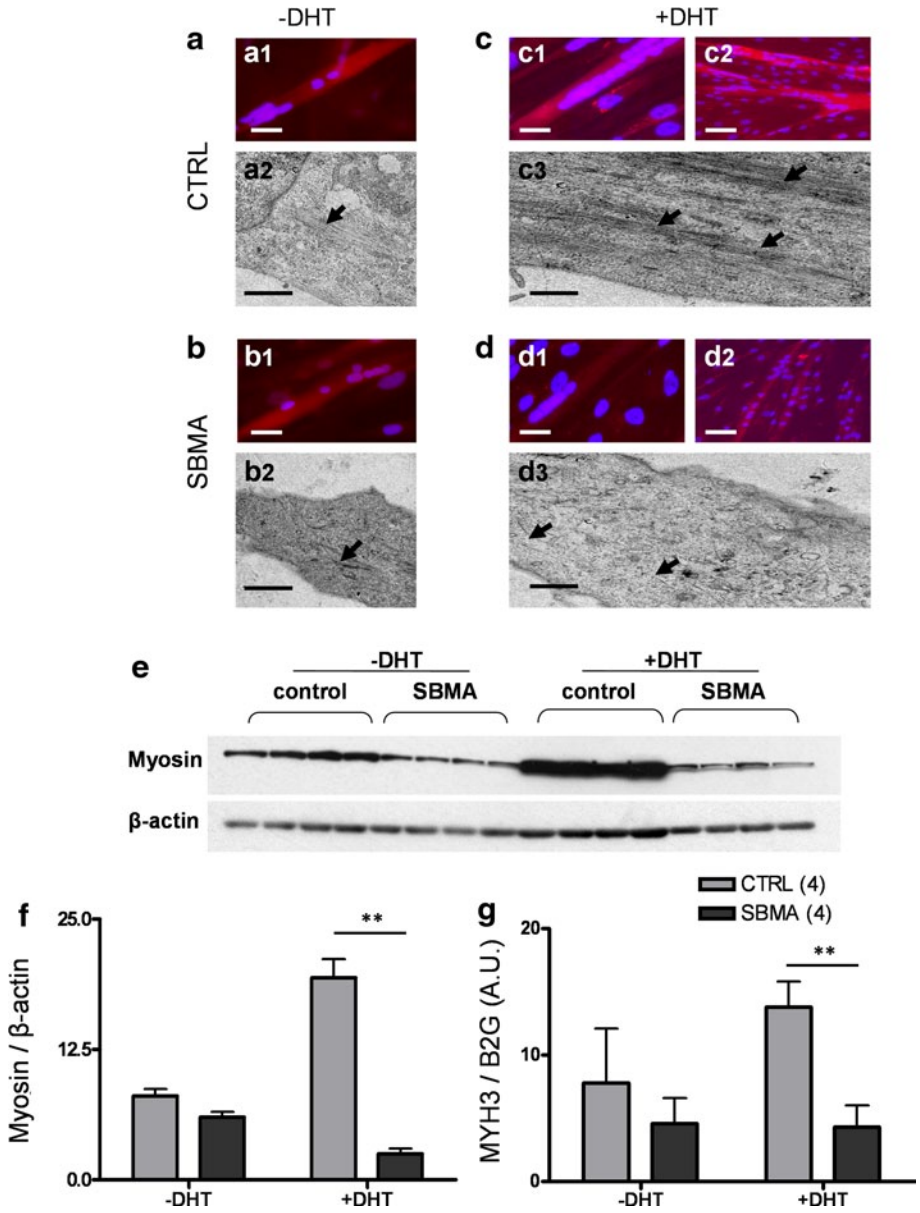

Fig. 2 DHT treatment alters the cytoskeleton in SBMA myotubes. Representative images of T10 myotubes from DHT-untreated controls (*a1*, high magnification) and SBMA (*b1*, high magnification) and DHT-treated controls (*c1*, high magnification; *c2*, low magnification) and SBMA (*d1*, high magnification; *d2*, low magnification) stained with anti-fetal myosin (nuclei labeled with DAPI). *Scale bars* 10 μm (high magnification), and 40 μm (low magnification). Electron microscopy representative images of DHT-untreated controls (*a2*) and SBMA (*b2*), and DHT-treated controls (*c3*) and SBMA (*d3*). *Scale bar* 1 μm. In both the analyses, SBMA DHT-treated myotubes showed a reduced cytoskeletal organization (*arrows*) compared to control. DHT treatment reduced the fetal myosin (MYH3) protein (**e**–**f**) and mRNA transcript levels (**g**) in SBMA myotubes. The values of fetal myosin mRNA and protein levels are given as arbitrary units (AU) of the ratio between β_2-microglobulin and β-actin, respectively. The data are expressed as mean ± SD of two independent experiments of RT-PCR (carried out in triplicate) and a single WB analysis. $^{**}p < 0.01$ by Student *t* test. The number of single lines studied is given in *brackets*

of MyoD, MyoG, and CK-M were similar in SBMA and control myotubes. Using Western blotting analysis, we confirmed that MyoG levels did not change in response to DHT treatment (Fig. 3d, e). Taken together, these results indicate that expansion of polyQ in AR does not interfere with the proliferation and differentiation capabilities of skeletal muscle cells.

DHT treatment increases AR nuclear accumulation in SBMA myotubes

Androgens regulate the expression of the AR at the transcriptional level [46]. Therefore, we asked whether androgen treatment affects AR expression in myoblasts and myotubes derived from SBMA patients. To address this question, we analyzed the amount of AR mRNA by RT-PCR (Fig. 4a, b). We found that treatment of the cells with DHT results in a significant increase of AR mRNA levels in control (1.16–1.49, $p < 0.05$), but not in SBMA myoblasts (0.94–0.92). However, DHT treatment increased AR mRNA levels in both control and SBMA myotubes. In addition to regulating AR mRNA, androgens increase AR stability through reduced protein degradation [17, 28]. Using Western blotting analysis, we investigated the effect of androgens on AR accumulation in myoblasts and myotubes. We observed a DHT-dependent accumulation of AR in total lysates of control (170 %) and SBMA (245 %) myoblasts (Fig. 4c, e), as well as control (500 %) and SBMA (400 %) myotubes (Fig. 4d, f). These results indicate that androgens positively regulate AR levels in SBMA muscle cells.

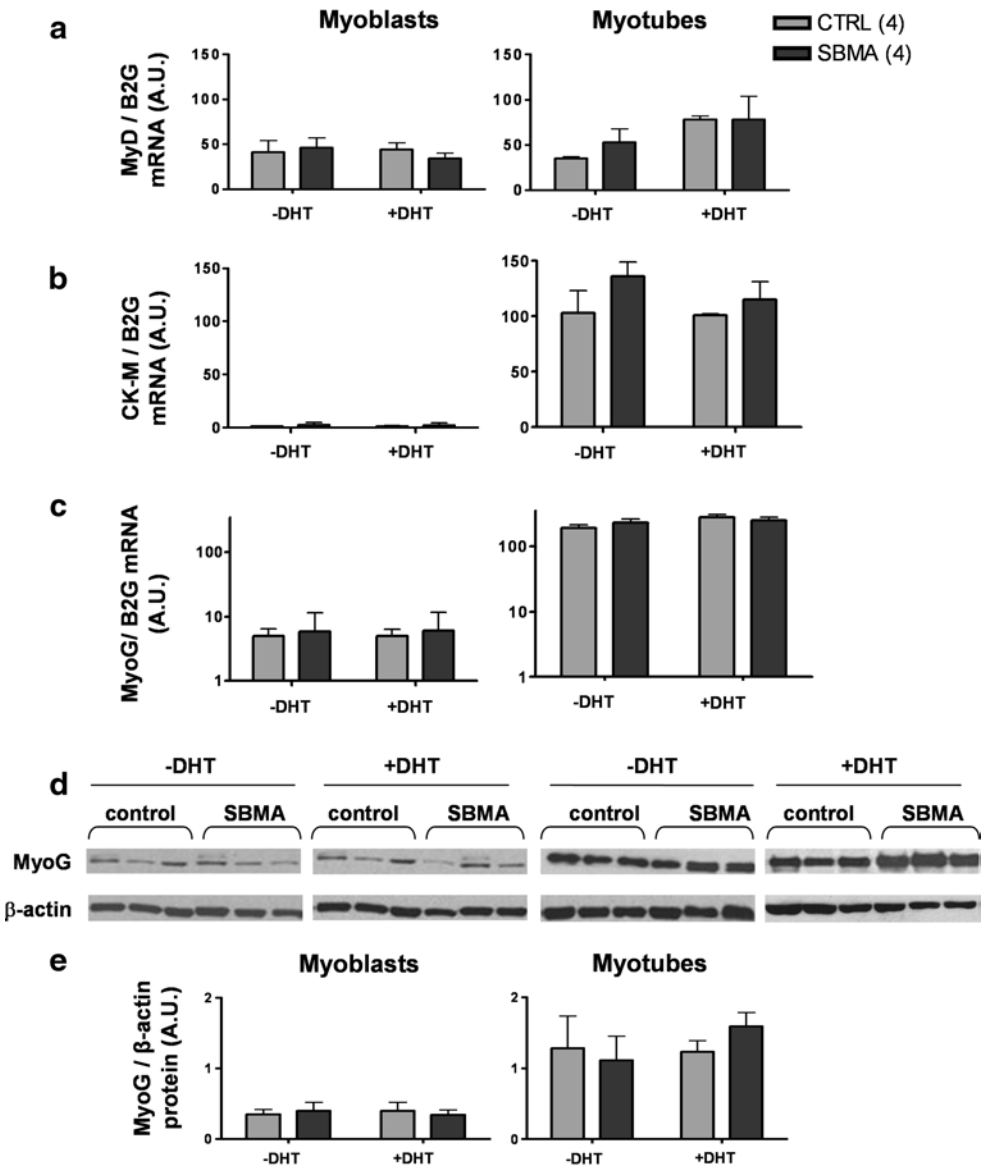

Fig. 3 The normal myogenic potential of SBMA myoblasts is unaffected by DHT. Expression of mRNA, analyzed by RT-PCR, of MyoD (**a**), CK-M (**b**) in control and SBMA myoblasts and in T10 differentiated myotubes was unaffected by 10-nM DHT treatment. Analysis of mRNA transcript **(c)** and protein (**d**, **e**) levels of myogenin (MyoG) in the presence or absence of DHT. The values of MyoG mRNA and protein levels are given as arbitrary units (AU) of the ratio with β_2-microglobulin and β-actin, respectively. The data are expressed as mean ± SD of two independent experiments of RT-PCR (carried out in triplicate) and a double WB analysis. The number of single lines studied is given in *brackets*

As translocation of polyQ-AR to the nucleus is a crucial event in SBMA pathogenesis [25, 26], we analyzed the subcellular distribution of AR in DHT-treated SBMA and control myoblasts and myotubes. Immunofluorescence analysis showed that the AR has a similar diffuse localization in the nucleus and cytosol of DHT-treated myoblasts from SBMA and control subjects (Fig. 5a). In the presence of DHT, SBMA myotubes showed an increased nuclear signal of the AR compared to control myotubes (Fig. 5b, inset). Western blotting analysis of the AR in nuclear fractionations confirmed that SBMA myoblasts do not show any difference in ligand-dependent nuclear accumulation of AR relative to control myoblasts (Fig. 5c, e). Moreover, differentiation of myoblasts to myotubes was associated with a twofold increase in nuclear accumulation of AR in control myotubes (1.73–3.31) and a tenfold increase in SBMA myotubes (1.27–13.01, $p < 0.01$) (Fig. 5c–f). In line with these findings, we observed a fourfold higher ($p < 0.01$) nuclear accumulation of mutant AR induced by ligand stimulation in SBMA myotubes relative to control myotubes (Fig. 5d, f). Together, these results indicate that the process of differentiation of SBMA myoblasts to myotubes is associated with an abnormal accumulation of polyQ-AR in the nucleus.

The nuclear AR accumulation in differentiated SBMA myotubes treated with DHT, but not in SBMA myoblasts, correlated with AR expression. There was an increase in the AR mRNA transcript levels upon DHT treatment in control and SBMA myotubes and in control myoblasts, but not in SBMA myoblasts (Fig. 4 a, b). In addition, we found that the differentiation of myocytes significantly increases AR expression of 31 % in DHT-treated control myotubes

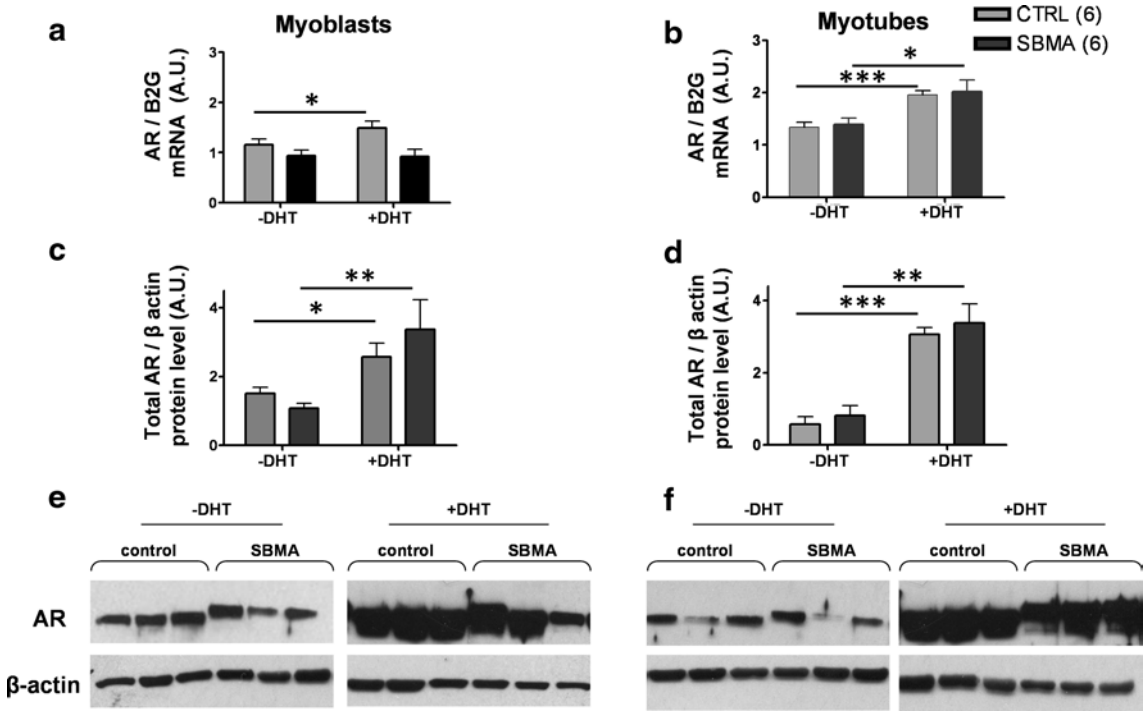

Fig. 4 DHT effects on AR transcript and protein levels in myoblasts and T10 differentiated myotubes from control and SBMA patients. **a**, **b** DHT induced an increase in AR mRNA transcript levels. **c**, **d** In total cell lysates from control and SBMA muscle cells, DHT induced an increase in the amount of AR. Data are given in arbitrary units (AU) of the ratio with β_2-microglobulin and β-actin, respectively, and are expressed as mean ± SD of three independent RT-PCR experiments (carried out in triplicate) and WB analysis performed in duplicate. **e–f** Representative WB analysis of AR in total cell lysates of myoblasts and T10 myotubes from control and SBMA patients. $*p < 0.05$, $**p < 0.01$, $***p < 0.001$ by Student's *t* test. The number of single lines studied is given in *brackets*

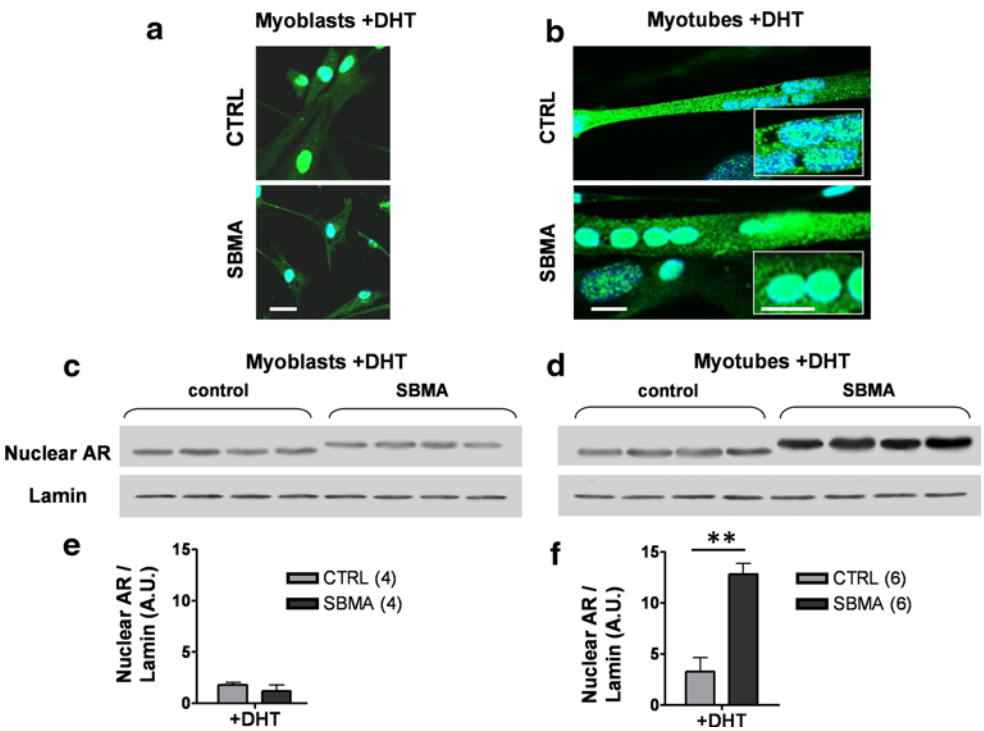

Fig. 5 DHT increases the AR nuclear localization in differentiated T10 SBMA myotubes. Representative epifluorescent images of DHT-treated myoblasts (**a**) and of DHT-treated T10 differentiated myotubes (**b**) from control and SBMA patients. The nuclei were labeled with DAPI (*blue*) coupled to immunodetection of the AR (*green*). Insert high magnification of nuclei. *Scale bar* 10 μm. Insert *scale bar* 5 μm. Representative WB analysis of AR in the nuclear fraction of DHT-treated myoblasts (**c**) and DHT-treated T10 myotubes (**d**) from control and SBMA patients. Lamin protein levels were measured for normalization. (**e–f**) The *graphs* represent the mean ± SD of at least three independent experiments. $**p < 0.01$ by Student's *t* test. The number of single lines studied is given in *brackets*

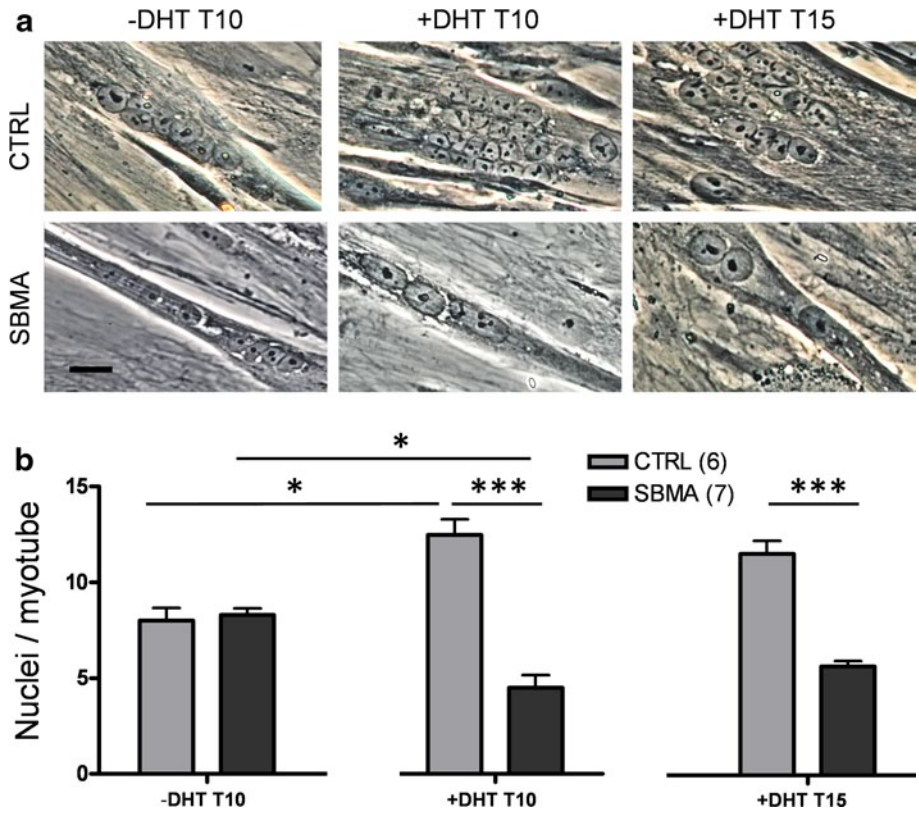

Fig. 6 DHT reduces the syncytial nuclei number in SBMA T10 and T15 differentiated myotubes. **a** Representative bright-field images of control and SBMA myotube populations at T10 and T15 of differentiation. *Scale bar* 5 μm. **b** The nuclei/myotubes values were obtained from at least 100 myotubes/cell line and are expressed as mean ± SD. *$p < 0.03$, ***$p < 0.005$ by ANOVA, while post hoc multiple comparisons were done by Tukey test. The number of single lines studied is given in *brackets*

versus myoblast (1.96 ± 0.21 vs. 1.49 ± 0.31; $p < 0.01$) and 219 % in DHT-treated SBMA myotubes versus myoblasts (2.02 ± 0.55 vs. 0.92 ± 0.36; $p < 0.002$).

Androgens perturb SBMA myotube maturation

During the process of myoblast-to-myotube differentiation, muscle cells fuse to form new multinucleated myotubes. To test whether androgens alter SBMA myotube fusion, we analyzed the fusion index by quantifying the number of nuclei per myotube (Fig. 6). In the absence of androgens, fusion index was similar in control and SBMA myotubes. Treatment with DHT increased the fusion index in control myotubes by about 150 % (8.0 ± 1.4 vs. 12.5 ± 1.4 at T10; and 11.6 ± 1.1 at T15, relative to untreated control myotubes; $p < 0.03$). Notably, treatment of the SBMA myotubes with DHT significantly reduced the fusion index at each time of differentiation tested (8.3 ± 0.6 vs. 4.5 ± 1.1 at T10; and 5.7 ± 0.5 at T15, relative to untreated SBMA myotubes; $p < 0.03$ and $p < 0.005$, respectively) (Fig. 6b).

IL-4 treatment reduces fusion defects in SBMA myotubes

A decrease in the number of nuclei can be caused by apoptosis [38]. To determine whether the decreased number of syncytial nuclei in SBMA myotubes was caused by apoptosis, we measured programmed cell death by analyzing chromatin fragmentation by terminal deoxynucleotidyl transferase-mediated dUTP nick end labeling (TUNEL). We did not detect any significant difference in the number of positive apoptotic nuclei in SBMA myotubes relative to control myotubes (Supplementary Fig. 2).

The fusion of myoblasts into myotubes occurs in two distinct phases, the first phase being characterized by the formation of small nascent myotubes, and the second phase in which mononucleate myoblasts fuse with the nascent myotubes to form large, mature myotubes with multiple nuclei [reviewed in 1]. The second phase of the fusion process is largely dependent on IL-4 [13]. We tested whether IL-4 levels are altered in SBMA cells. We found that the expression of IL-4 in SBMA myotubes treated with DHT is significantly reduced by 56 % ($p < 0.001$) at 4 days (SBMA: 0.66 ± 0.25 vs. Control: 1.51 ± 0.74) and by 70 % ($p < 0.001$) at 7 days (SBMA: 1.57 ± 1.13 vs. Control: 5.15 ± 3.29) of differentiation compared to control myotubes (Fig. 7a). To determine whether this reduction plays a role in SBMA myotube fusion, we added IL-4 to the DHT-treated SBMA cell cultures. In the DHT-treated SBMA myotubes, IL-4 treatment for 7 days (T7) rescued both the myonuclei number (9.60 ± 1.14 vs. 4.99 ± 1.21 relative to untreated IL-4 SBMA myotubes, $p < 0.02$; Fig. 7b, c) and size (26.77 ± 0.38 vs. 16.18 ± 1.59, $p < 0.01$; Fig. 7b, d) to levels observed in control myotubes. In the DHT-treated control myotubes, IL-4 treatment had a slight, but not significant effect on both the myonuclei

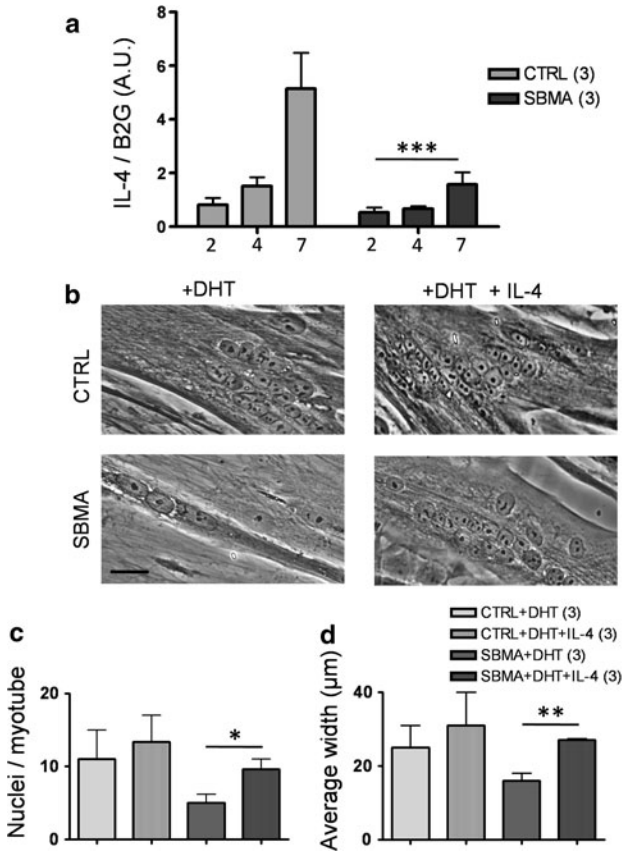

Fig. 7 IL-4 rescues the DHT hypertrophic effect in SBMA myotubes. **a** Reduced mRNA expression of IL-4 in 4 and 7 days differentiated SBMA myotubes. Data are given in arbitrary units (AU) of the ratio between IL-4 and β_2-microglobulin and expressed as mean ± SD of two independent RT-PCR experiments. **b** Representative bright-field images of DHT-treated SBMA myotubes at 7 days of differentiation, with or without IL-4 (5 ng/ml). *Scale bar* 10 µm. IL-4 rescued the nuclei/myotube number in DHT-treated myotubes from SBMA to normal control levels. **c** The nuclei/myotube values were obtained from 100 myotubes/cell line and are expressed as mean ± SD. **d** IL-4 rescued the average width in DHT-treated myotubes compared with the untreated SBMA myotubes. Average width was measured in at least 100 myotubes/cell line and is expressed as mean ± SD. ***$p < 0.001$ by ANOVA; *$p < 0.02$; **$p < 0.01$ by repeated measures ANOVA. The number of single lines studied is given in *brackets*

number (13.41 ± 3.64 vs. 11.24 ± 3.78, relative to untreated IL-4 control myotubes, Fig. 7b, c) and size (30.61 ± 9.06 vs. 25.08 ± 6.38, Fig. 7b, d). While confirming an impairment of SBMA myoblasts, these findings indicate that poly-Q AR accumulation interferes with IL-4 production during myotube maturation.

Discussion

We investigated the effect of polyQ-AR expression in the context of muscle cell biology and function. We show that treatment of cell cultures with androgens increases fiber width in control myotubes, but not in SBMA myotubes. This lack of androgens' effect was due to neither defects in myoblast proliferation and differentiation nor to apoptosis. Rather, it was associated with an increased accumulation of polyQ-AR in the nucleus of SBMA myotubes. Importantly, we found impaired myoblast fusion and reduced expression of IL-4. This fusion defect was rescued by IL-4 treatment. These data show that the toxic effects of polyQ-AR in skeletal muscle interfere with IL-4 expression during muscle maturation.

SBMA is characterized by the loss of lower motor neurons, an event that leads to fasciculation, weakness, and atrophy of limb and face muscles. In the last decade, several pieces of evidence have been provided in support of the idea that, in addition to muscle denervation, there is cell-autonomous skeletal muscle degeneration in SBMA [reviewed in 36]. We have previously shown that muscle biopsies derived from SBMA patients present not only with signs of muscle denervation, but also of muscle degeneration [43]. In addition, SBMA patients have abnormal levels of creatine kinase found in the serum, which are higher than expected for a purely neurogenic disease [4, 11, 12, 22]. Although the levels of creatine kinase can be altered in both myopathic and neurogenic conditions, their elevation in SBMA suggests primary muscle damage. Evidence of myopathic changes has also been observed in mouse models of SBMA. In knock-in SBMA mice, muscle damage occurs before spinal cord degeneration [48], further providing support to the concept that skeletal muscle is a primary target of polyQ-AR toxicity. Importantly, overexpression of normal AR selectively in mouse skeletal muscle resulted in a phenotype that resembles SBMA. These findings indicate that dysregulation of androgen signaling in skeletal muscle is critical for the maintenance of muscle homeostasis [24]. The AR is a transcription factor and polyQ expansion alters AR transactivation [20, 28]. Consistent with this observation, gene expression is altered in the muscle of knock-in and transgenic SBMA mice, as well as in mice overexpressing normal AR in muscle, further implicating altered AR function in skeletal muscle [23]. Importantly, not only gene expression is altered, but also RNA processing is defective in SBMA muscle, providing insights into the mechanism through which the interaction between androgens and polyQ-AR causes muscle damage [47].

In muscle, androgens have anabolic effects. Administration of androgens to hypogonadal men results in increased muscle strength and size, together with enhanced lean body mass and performance [6, 7, 40]. The anabolic effects of androgens on muscle fibers are important for muscle homeostasis. This becomes critical during aging, when skeletal muscle undergoes profound changes in biology, a process known as sarcopenia [reviewed in 10, 35]. Our findings

provide evidence that, although SBMA myoblasts cultured with DHT regularly proliferate and differentiate, they form small myotubes with reduced number of nuclei and altered contractile structures. Importantly, we show that while androgens stimulate an increase in myofibre size in normal myotubes, they do not exert similar effects in the SBMA myotubes. The finding that expression of polyQ-AR in myotubes attenuates the anabolic effects of androgens on skeletal muscle suggests that the expansion of polyQ in AR causes a toxic effect that alters normal androgen signaling in muscle. As SBMA is a late-onset disease, this process may have a major negative impact on the aging muscle, as androgen signaling is critical for the maintenance of muscle homeostasis.

In absence of DHT, SBMA and control myotubes are indistinguishable in terms of size and fusion index, whereas DHT treatment triggers an abnormal myogenic response in SBMA muscle cells. Myoblast fusion is a highly regulated process that involves cell migration, adhesion, and signal transduction pathways [reviewed in 1]. Multinucleated myotubes form in two phases. First, a small nascent myotube containing 4–5 nuclei originates, and then the nascent myotube fuses with additional myoblasts or other myotubes to form a mature myotube, leading to increased myotube size. These two phases are regulated by a different gene expression program. We found a decrease in the number of syncytial nuclei in DHT-treated SBMA cells. In myotonic dystrophy type-1 myotubes, there is a progressive decrease in syncytial nuclei from T10 through T15 of differentiation, which is associated with apoptosis [21]. We did not observe apoptosis in SBMA primary myotubes. Rather, we found a significant reduction in IL-4 expression in DHT-treated SBMA muscle cells compared to control cells. Our data strongly suggest that SBMA myoblasts do not fuse normally because there is an impairment in the second stage of fusion, which requires IL-4 signaling, in parallel with nuclear AR accumulation. Consistent with this idea, treatment with IL-4 rescued the myogenic growth of SBMA myotubes, indicating that polyQ-AR alters IL-4 production, thereby blocking the normal maturation of SBMA myotubes.

Actin cytoskeleton and actin polymerizing proteins play a crucial role during the fusion process. Interestingly, we noticed that SBMA myotubes treated with DHT present with defects in cytoskeleton organization. A recent microarray analysis of gene expression profile identified several genes whose expression is altered in SBMA muscle, including some involved in actin cytoskeleton modulation and muscle contraction [23]. Fusion defects have been reported in other degenerative diseases, such as amyotrophic lateral sclerosis (ALS) and spinal muscular atrophy (SMA) [5, 33]. Different from ALS and SMA, the fusion defects in SBMA are androgen-dependent. Moreover, the lack of fusion of SMA myotubes was not associated with altered actin–myosin expression [5], which further reinforces the peculiarity of muscle pathology in SBMA relative to that occurring in other motor neuron diseases.

As a result of impaired myoblast fusion, satellite cells may fail to regenerate muscle during chronic denervation, which may contribute to muscle deterioration in SBMA. The growth of skeletal muscle depends on both protein and cell turnover [reviewed in 37]. It may thus be argued that an abortive myogenesis can lead to compensatory increases in protein synthesis to maintain muscle mass. In fact, an abnormal protein turnover was suggested in muscle from SBMA patients, where we observed frequent scattered hypertrophic muscle fibers [43] and found a higher fiber hypertrophy index relative to other neurogenic diseases (unpublished data).

Nuclear localization of pathogenic proteins is essential for inducing neuronal cell dysfunction and degeneration in the majority of polyglutamine diseases [34]. Translocation to the nucleus is a critical event in SBMA pathogenesis [25, 26]. DHT-treated SBMA myotubes showed a massive accumulation of the expanded polyQ-AR inside their nucleus, much higher than that of control myotubes. The nuclear AR appears diffuse, and no nuclear inclusions were observed. Accumulation of toxic diffused AR was also reported in the nuclei of motor neurons of SBMA patients [2]. Furthermore, toxicity of the expanded polyQ-AR principally requires species that are produced in the early stages of the aggregation cascade [25]. Finally, the frequency of diffuse nuclear accumulation of polyQ-AR in spinal motor neurons correlates with the CAG repeat in the AR gene [48].

Further studies are necessary to determine whether the observed detrimental effects of DHT on SBMA myogenesis are caused by a loss of function or a toxic gain of function mechanism, and if SBMA myoblasts are affected by abnormalities in motor neuron function. Our results support a gain of function model. Indeed the normal maturation of SBMA muscle cells without DHT suggests that polyQ-AR is per se working in our system. Moreover, DHT treatment of SBMA cells showed a normal up-regulation of myogenic genes, which points to a preserved action of androgens in the myogenic gene expression program. We reported that in the absence of DHT, SBMA and control myotubes are indistinguishable in terms of size and fusion index, whereas DHT treatment triggers abnormal myogenesis of SBMA muscle cells. Our results suggest that SBMA muscle cells might be not defective per se for an ongoing pathological process related to abnormal motor neuron function and support cell-autonomous toxicity in SBMA myoblasts triggered by androgens.

In summary, we report here the first study of myoblast–myotube function obtained from SBMA patients. Our results suggest an impairment in androgen-dependent

fusion leading to atrophic myotubes. We also show that pathological SBMA myotubes accumulate polyQ-AR within the nucleus. Our results indicate cell-autonomous muscle damage as a possible contributor to SBMA pathogenesis.

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