Protein kinase A activation inhibits DUX4 gene expression in myotubes from patients with facioscapulohumeral muscular dystrophy

Facioscapulohumeral muscular dystrophy (FSHD) is among the most prevalent of the adult-onset muscular dystrophies. FSHD causes a loss of muscle mass and function, resulting in severe debilitation and reduction in quality of life. Currently, only the symptoms of FSHD can be treated, and such treatments have minimal benefit. The available options are not curative, and none of the treatments address the underlying cause of FSHD. The genetic, epigenetic, and molecular mechanisms triggering FSHD are now quite well-understood, and it has been shown that expression of the transcriptional regulator double homeobox 4 (DUX4) is necessary for disease onset and is largely thought to be the causative factor in FSHD. Therefore, we sought to identify compounds suppressing DUX4 expression in a phenotypic screen using FSHD patient–derived muscle cells, a zinc finger and SCAN domain–containing 4 (ZSCAN4)-based reporter gene assay for measuring DUX4 activity, and ~3,000 small molecules. This effort identified molecules that reduce DUX4 gene expression and hence DUX4 activity. Among those, β2-adrenergic receptor agonists and phosphodiesterase inhibitors, both leading to increased cellular cAMP, effectively decreased DUX4 expression by >75% in cells from individuals with FSHD. Of note, we found that cAMP production reduces DUX4 expression through a protein kinase A–dependent mode of action in FSHD patient myotubes. These findings increase our understanding of how DUX4 expression is regulated in FSHD and point to potential areas of therapeutic intervention.

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The authors declare that they have no conflicts of interest with the contents of this article.

This article contains Figs. S1–S5.

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3 The abbreviations used are: FSHD, facioscapulohumeral muscular dystrophy; GM, growth medium; qPCR, quantitative PCR; CMV, cytomegalovirus; SEAP, secreted alkaline phosphatase; AU, arbitrary units; PDE, phosphodiesterase; 8-Br-cAMP, 8-bromoadenosine 3′,5′-cyclic monophosphate; PKA, protein kinase A; CREB, cAMP-response element–binding protein; caPKA, constitutively active PKA catalytic domain; dnPKA, dominant-negative PKA regulatory domain; β2AR, β2-adrenergic receptor; RGA, reporter gene assay; DAPI, 4′,6-diamidino-2-phenylindole; BisTris, 2-[bis(2-hydroxyethyl)aminol]-2-(hydroxymethyl)propane-1,3-diol; HA, hemagglutinin; GPCR, G protein–coupled receptor.

Estimations of incidence were initially reported to be in the range of 1 in 14,000 to 1 in 20,000 (1–4), and a more recent study has calculated an occurrence as great as 1 in 8,500 worldwide (5). FSHD results in gradual yet ultimately severe atrophy and degeneration of specific muscle groups. Patients often first present in the second decade of life with weakness of the face and periscapular muscles, eventually resulting in the inability to raise their arms above shoulder height, make facial expressions, or even close their eyes. As the disease progresses, muscles of the upper arms, the legs, and the postural muscles in the back lose mass and strength. Loss of strength in the paraspinal muscles is particularly debilitating and often results in patients becoming wheelchair-bound (6–8). Whereas physical therapy, pain management, and surgery can alleviate some of the disabilities associated with FSHD, these treatments are not curative, and none of them address the underlying cause of FSHD (9, 10). A pharmacologic intervention addressing the root cause of the degenerative muscle pathology seen in FSHD patients has yet to be developed.

The genetic, epigenetic, and molecular mechanisms underlying FSHD are now increasingly understood. FSHD comprises the clinically recognized FSHD1 and FSHD2. FSHD1, which accounts for 95% of FSHD cases, is an autosomal dominant disease caused mainly by a DNA contraction of the D4Z4 macrosatellite repeat elements found on human chromosome 4, specifically 4q35 (8, 11, 12). This D4Z4 repeat reduction results in epigenetic changes at the locus, such as DNA hypomethylation within the D4Z4 repeats and the surrounding locus (13–15) as well as the loss of repressive histone methyl marks (16). It was postulated that the change in chromosomal conformation from heterochromatin to euchromatin facilitates access to DUX4 by as of yet unidentified transcriptional activators. Moreover, additional genetic elements have been shown to drive DUX4 expression in skeletal muscle (17). DUX4 expression has been demonstrated to be necessary for disease onset and is largely thought to be the causative factor in FSHD (18). Therefore, treatments that inhibit DUX4 expression or block its activity may prove to slow the disease progression and ultimately provide therapy for FSHD patients.
PKA controls DUX4 expression in FSHD

Toward finding a cure for FSHD, we have established a screening platform to identify targets that modulate Dux4 activity. To this end, we use FSHD patient primary myotubes, which naturally express DUX4 from the endogenous locus, combined with a reporter gene assay to measure Dux4 activity. To eliminate false positives and undesired modes of action, such as inhibition of myotube differentiation, we have employed counterscreens for nonspecific reporter activity and myotube morphology. Using the methodology just described, we have screened a collection of ~3,000 potent and selective low-molecular weight tool compounds against various human targets and pathways (19) (also known as the Novartis MoA box (20)) and have been able to identify compounds capable of silencing or further activating DUX4 expression as well as inhibiting or activating Dux4 activity. We particularly focused on those compounds displaying the ability to reduce Dux4-dependent gene transactivation and identified some with exceptional efficacy and potency worthy of follow-up as potential treatments. Furthermore, our analysis of the mechanisms by which many of these compounds reduce DUX4 expression and activity has identified clear pathways and biochemical mechanisms where intervention is possible.

Results

FSHD cell characterization

We established our assays with two previously uncharacterized myoblast isolates from FSHD type 1 patients obtained from the Coriell Institute, namely GM17940 (FSHD940) and GM17869 (FSHD869). To ensure suitability of these FSHD type 1 myoblasts, they were sent to the University of Iowa Diagnostic Laboratories and were confirmed to have short 4q35 EcoRI fragments (21 and 27 kb, respectively) on a 4qA background by Southern blotting, indicating that these myoblasts are from FSHD type 1 patients. Human primary myoblasts (Skmdc) from a healthy donor were also sent to University of Iowa Diagnostic Laboratories for diagnostic testing. No short 4q35 EcoRI fragments were found, and these myoblasts were used as disease-free controls. To identify suitable growth and differentiation conditions, we first tested a few different medium formulations on those compounds displaying the ability to reduce Dux4 expression and activity has identified clear pathways and biochemical mechanisms where intervention is possible.

Screening platform and results

Given the robust differentiation and expression of Dux4-dependent genes, we focused further assay development on the FSHD940 myoblasts and compared them with the Skmdc myoblasts, which exhibited similar kinetics and extent of differentiation (Fig. 1B). As a more sensitive and high-throughput readout for Dux4 activity in the FSHD patient primary cells, we generated a reporter gene assay utilizing the ZSCAN4 promoter to express the highly active Nano luciferase (Fig. S2). We chose the short-lived Nano luciferase containing a PEST domain (NLucP) to detect Dux4 activity loss more rapidly. We packaged the Dux4-responsive ZSCAN4-NLucP transgene (ZSCAN4 reporter gene assay (RGA)) as well as a constitutively expressed cytomegalovirus (CMV) promoter–driven secreted alkaline phosphatase (SEAP) transgene into a bipartite adenovirus. The dual reporter gene assay adenovirus was transduced into Skmdc and FSHD940 myoblasts, and the cells were subjected to proliferation or differentiation conditions. After 3 days in growth medium, very little luciferase activity was detected in either Skmdc or FSHD940 myoblasts; however, there was a >1,000-fold induction of luciferase activity in the differentiating FSHD904 cells compared with undifferentiated FSHD940 cells in growth medium (Fig. 2A), consistent with DUX4 expression in FSHD940 myotubes as detected by immunostaining and Dux4-dependent genes detected by qPCR (Fig. 1, D and E). In comparison, there was less than a 2-fold change in luciferase expression in Skmdc myoblasts compared with Skmdc myotubes (0.0007 ± 0.00004 AU versus 0.0011 ± 0.00008 AU, mean ± S.E.) (Fig. 2A). To confirm equal trans-
duction among the treatment groups, we analyzed SEAP activity in the supernatant from both Skmdc and FSHD940 under growth and differentiation conditions. There was no difference in SEAP activity between Skmdc and FSHD940 during either growth or differentiation (Fig. S2B), indicating that the induction of the ZSCAN4 RGA in FSHD940 during differentiation is not due to an artifact of transduction. We next analyzed DUX4 expression in Skmdc and FSHD940 under growth and differentiation conditions by qPCR. Skmdc myoblasts (day 0) and myotubes (day 5) as well as FSHD940 myoblasts (day 0) expressed undetectable levels of DUX4 mRNA (Ct > 35), whereas FSHD940 myotubes (day 5) expressed vastly greater and detectable levels (Ct = 31.9 ± 0.7, mean ± S.E.). Finally, to determine the dependence of the ZSCAN4 RGA on Dux4, FSHD940 myotubes expressing the ZSCAN4 RGA were transfected with short interfering RNA (siRNA) against Dux4 (siDUX4) or a nontargeting control siRNA (siCON) on day 3 post-differentiation, and ZSCAN4 RGA activity was measured 48 h later. Myotubes transfected with siDUX4 showed a nearly 90% reduction in luciferase activity compared with the siCON-transfected myotubes (Fig. 2C). qPCR of myotubes treated as in the ZSCAN4 RGA experiment confirmed > 85% knockdown of DUX4 mRNA by this siRNA duplex (1.0 ± 0.04 AU versus 0.14 ± 0.019 AU, mean ± S.E., p < 0.01) (Fig. 2D). The combined results indicate that FSHD940 myotubes express functional Dux4 protein capable of transactivating the ZSCAN4 RGA and thus represent a very relevant and useful system to screen for small molecule inhibitors of Dux4 activity. A compound collection of

Figure 1. DUX4- and Dux4-dependent gene expression is induced in FSHD but not healthy myoblasts upon differentiation. A, immunofluorescence of MYHC (red) and nuclei (blue) in healthy (Skmdc) and FSHD1 (FSHD940 and FSHD869) human primary myotubes during 7 days of differentiation. Each individual image is nine 10× fields. B, quantification of MYHC-positive nuclei by high-content imaging (percentage of total nuclei). C, qPCR for MYOG (left) and CKM (right). D, immunofluorescence of Dux4 (green), MYHC (red), and nuclei (blue) in FSHD940 and FSHD869 myotubes after 5 and 7 days of differentiation, respectively. E, qPCR for ZSCAN4 (left) and TRIM43 (right). ND, not detected. Error bars, S.E.
3,000 molecules with known modes of action was tested at 10 M. We selected compounds that reduced ZSCAN4 activity below 3 S.D. values of the neutral control DMSO (75% reduction) as primary hits. These were then further characterized as a dose response again in the ZSCAN4 RGA. To exclude compounds that are direct inhibitors of nanoluciferase, the hits with confirmed activity were then tested for activity against NLucP expressed off a CMV promoter in FSHD940 myotubes. Finally, the remaining compounds were tested for their effects on differentiation, toxicity, and morphology by MYHC/DAPI immunostaining and high-content imaging. Compounds that resulted in a loss of nuclei, a reduction in MYHC area, or abnormal morphology were eliminated. The final validated hit list contained 173 compounds (6% hit rate). Interestingly, 26 of these compounds (>15% of the hits) were annotated to be β-adrenergic receptor agonists or phosphodiesterase (PDE) inhibitors, two compound classes that signal via cAMP, which we studied further. Additional putative GPCR agonists were also identified as hits but have not been thoroughly characterized at the time of publication.

β2-Adrenergic receptor agonists induce cAMP and reduce Dux4 expression

Given that β2-adrenergic receptor (β2AR) agonists are approved drugs and also well-established to cause skeletal muscle growth and block skeletal muscle atrophy, we further explored β2AR agonists and studied formoterol and albuterol for their ability to lower Dux4-dependent gene expression in FSHD1 primary myotubes. In these experiments FSHD940 myotubes were treated 5 days after inducing differentiation for an additional 48 h. Both formoterol and albuterol lowered the Dux4-dependent ZSCAN4 RGA by up to 85 and 80%, respectively. Formoterol reduced the RGA activity with an EC50 of 1 nM, whereas albuterol exhibited a 6 nM EC50 (Fig. 3A). We further tested formoterol and albuterol for their ability to reduce Dux4-dependent gene expression by qPCR. Formoterol reduced ZSCAN4 and TRIM43 expression up to 78 and 83%, respectively, and showed activity as low as 10 nM in this experiment. Albuterol dose-dependently reduced ZSCAN4 and TRIM43 expression with a maximal effect of 72 and 71%, respectively (Fig. 3B). Formoterol and albuterol treatment were also able to significantly reduce DUX4 mRNA in FSHD940 myotubes by 81 and 78%, respectively (1.0 ± 0.15 AU versus 0.19 ± 0.04 AU and 1.0 ± 0.15 AU versus 0.22 ± 0.05 AU, mean ± S.E., p < 0.05) (Fig. 3C). To rule out the possibility that the effect of lowering DUX4 expression was an artifact due to an effect on differentiation, we measured the percentage of MYHC-positive nuclei in β2 agonist–treated myotubes and compared it with vehicle-treated cells. Both formoterol and albuterol caused an increase in the number of MYHC-positive cells compared with DMSO-treated control myotubes, indicating that the effect was not caused by a reduction in differentiation (Fig. 3D). To confirm that formoterol can induce cAMP in FSHD940 myotubes, we measured cAMP levels in FSHD940 myotubes treated with formoterol. Formoterol dose-dependently increased cAMP >150-fold maximally compared with DMSO-treated myotubes and exhibited a 2.5 nM EC50 (Fig. 4A). This potency is in line with formoterol induced reduction of the ZSCAN4 RGA– and Dux4–dependent gene expression, suggesting that it does so through the same mechanism of action. Finally, we tested the ability of the well-described β1-adrenergic receptor antagonist, CGP20712, and β2-adrenergic receptor antagonist, ICI118,551, for their ability to block formoterol-
induced cAMP production. ICI118,551 but not CGP20712 was able to completely block formoterol-induced cAMP production in patient primary myotubes, indicating that the mechanism of action is in fact through the β2AR (Fig. 4B).

**PDE inhibitors and a cAMP analog reduce DUX4 expression**

Similarly to β2AR agonists, PDE inhibitors were identified in our screen as modulators of Dux4 activity. Inhibition of PDEs results in the stabilization and accumulation of cAMP. We therefore tested ibudilast and crisaborole, both approved PDE inhibitor drugs, to determine whether they too could block Dux4-dependent gene expression. Both PDE inhibitors dose dependently reduced the expression of ZSCAN4 and TRIM43 in FSHD940 myotubes (Fig. 5A). Additionally, ibudilast and crisaborole significantly lowered DUX4 mRNA expression by 83 and 76% compared with DMSO-treated FSHD myotubes (1.0 ±
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0.14 AU versus 0.17 ± 0.03 AU and 1.0 ± 0.14 AU versus 0.24 ± 0.037 AU, mean ± S.E., p < 0.01) (Fig. 5B). We further explored whether PDE inhibitors have a negative effect on differentiation that could explain the reduction in DUX4 expression. Neither crisaborole nor ibudilast reduced the number of MYHC-positive nuclei compared with DMSO-treated myotubes (Fig. 5C). This shows that the reduction in DUX4 expression is not an artifact of differentiation.

The effect of these two pathways on lowering DUX4 and Dux4-dependent gene expression implicated cAMP-mediated signaling in the process. We therefore tested the stabilized and cell-permeable cAMP analog, 8-Br-cAMP, for its ability to turn off Dux4-dependent gene expression. 8-Br-cAMP dose-dependently reduced the expression of the ZSCAN4 RGA by >95% compared with vehicle with an EC\textsubscript{50} of 99 μM (Fig. 6A). Additionally 8-Br-cAMP dose-dependently reduced the Dux4-dependent genes ZSCAN4 and TRIM43 with a maximal effect of 86 and 94%, respectively (Fig. 6B). Last, 8-Br-cAMP significantly reduced DUX4 expression by 79% (Fig. 6B). These findings confirm that cAMP leads to a reduction in DUX4 expression in FSHD patient myotubes.

Protein kinase A is necessary and sufficient to reduce Dux4 expression

We next sought to determine how cAMP regulates DUX4 expression. cAMP is known to regulate several signaling pathways, including those mediated by protein kinase A (PKA) as well as exchange protein directly activated by cAMP (EPAC) (23). We first tested the commercially available PKA inhibitors KT720 and H-89 for their ability to block formoterol-induced phosphorylation of the well-described PKA substrate CREB or various PKA substrates detected by a PKA phosphorylation motif antibody. FSHD940 myotubes at day 5 of differentiation were treated with various concentrations of KT720 or H-89 for 60 min, followed by the addition of formoterol (100 nM) for an additional 60 min. Cell extracts were generated and analyzed by
SDS-PAGE and immunoblotting. Neither KT720 nor H-89 was able to reduce formoterol-induced phosphorylation events when used at up to 10 μM (Fig. S3), and given the generally limited selectivity of kinase inhibitors (24), we chose to characterize the involvement of PKA using genetic tools. We generated adenoviruses containing the previously described constitutively active PKA catalytic domain (caPKA) and dominant-negative regulatory domain (dnPKA) (25). Expression of caPKA in FSHD940 myotubes induced a viral concentration-dependent phosphorylation of CREB and additional PKA substrates, as detected by immunoblotting, compared with myotubes transduced by a control adenovirus (CON) (Fig. 7A). Additionally, caPKA expression significantly reduced ZSCAN4 and TRIM43 mRNA expression compared with the control virus (Fig. 7B), indicating that PKA activity is sufficient to reduce Dux4-dependent gene expression in FSHD1 patient primary myotubes and to a similar extent achieved by formoterol. We then expressed dnPKA in FSHD940 myotubes, followed by treatment with formoterol. Formoterol induced PKA substrate phosphorylation in control adenovirus-transduced myotubes, but this induction was blocked by expression of dnPKA (Fig. 8A), showing that the dominant-negative construct blocks PKA activity. Additionally, the reduction in ZSCAN4 and TRIM43 expression by formoterol was completely reversed in myotubes expressing dnPKA (Fig. 8B), highlighting the necessity of PKA-dependent activity in β2AR agonist-induced reduction of Dux4-dependent gene expression. Interestingly, blocking basal PKA activity with higher expression levels of dnPKA was sufficient to increase Dux4-dependent gene expression (Fig. S4). Taken together, these results identify a new role for PKA in the regulation of Dux4 expression in FSHD patient muscle cells.

Discussion

DUX4 misexpression in skeletal muscle is currently the most widely accepted etiology for FSHD (18). The gene program that is transactivated by Dux4 is atypical of adult somatic cells (26) and has been shown to induce cell death when ectopically expressed in numerous cell lines (27, 28). Furthermore, an increased percentage of apoptotic nuclei have been detected in FSHD muscle biopsies compared with healthy controls (29). Additional support for a causative role of Dux4 comes from the requirement of FSHD patients to have at least one copy of D4Z4 to develop the disease (30). We therefore sought to identify compound inhibitors of Dux4 activity in a phenotypic screen using FSHD patient–derived myoblasts differentiated into myotubes. The screen was designed to identify modulators of Dux4 activity that could work by any of the following mechanisms: reducing DUX4 mRNA expression, altering DUX4 mRNA splicing, reducing Dux4 protein stability, or blocking DUX4 transactivation directly. Using this methodology, we have found that both β2AR agonists and PDE inhibitors can lead to a cAMP- and PKA-dependent mode of action regulating DUX4 mRNA expression levels in FSHD patient cells. This finding increases our understanding of how DUX4 expression is regulated and identifies pathways that can be manipulated by therapeutic intervention. An additional publication has recently found evidence that corroborates our findings (31). Campbell et al. (31) have identified β2AR agonists and cAMP as modulators of DUX4 mRNA expression; however, contrary to our results, a PKA-independent mechanism was postulated. We believe this might be due to the poor efficacy (Fig. S3) and selectivity of the commercially available PKA inhibitors (24, 32). Interestingly, PDE inhibitors did not score positive in the
screen run by Campbell et al. (31) despite several being present in the compound library that was used. Additional putative GPCR agonists were also identified in our screen and include the prostaglandin receptor agonists, 11-deoxyprostaglandin E1 and bimatoprost (reviewed in Ref. 33). Both 11-deoxyprostaglandin E1 and bimatoprost were able to induce phosphorylation of PKA substrates in FSHD940-treated myotubes (Fig. S5A); however, they only had a minor effect on reducing Trim43 expression (Fig. S5B). Examination of myotube differentiation by MYHC immunostaining and high-content imaging revealed that prostaglandin receptor agonist treatment resulted in a strong increase in differentiation (Fig. S5C). We surmise that this effect on increased differentiation may have led to an increase in Dux4 expression and a muted response in lowering Trim43 expression. Further study of these molecules is needed to understand their effects on this system, and it highlights the importance of monitoring myoblast differentiation in assays aimed at measuring Dux4 activity.

Our genetic findings that constitutively active PKA can mimic β2AR agonists and PDE inhibitors in reducing $DUX4$ mRNA as well as the finding that dominant-negative PKA can block β2AR agonist-induced reduction of $DUX4$ expression rather support a model that $DUX4$ mRNA is controlled in a PKA-dependent manner. Identification of the direct targets that mediate this effect downstream of PKA requires further investigation even though it is enticing to reason that the pathways affected by β2AR agonists controlling $DUX4$ expression are in common with the pathways that control the anabolic and anti-atrophic affects exhibited by these molecules. Whereas it has been extensively documented that β2AR agonists induce skeletal muscle hypertrophy and can block the atrophic effects of disuse and denervation atrophy (34, 35), the molecular events that control this process are less well-defined. The cAMP-inducing GaS pathway has, however, been shown to be sufficient to induce skeletal muscle hypertrophy (36). A proposed mechanism by which β2AR agonists block skeletal muscle atrophy is by reducing the expression of the E3 ligases MuRF1 and MaFBx, whose activity is responsible for degradation of muscle proteins (37) and the loss of muscle mass during atrophy (38). Interestingly, Dux4 has some commonalities with MuRF1 and MaFBx. Within adult somatic tissues, the expression of all three genes is highest in skeletal muscle (17, 38) and in cell culture expression has been shown to be induced during differentiation of myoblasts into myotubes (Fig. 2B; see Ref. 39). In the case of $DUX4$, this expression pattern is attributable to myogenic enhancers (17); however, the trans-acting machinery that drives $DUX4$ expression in skeletal muscle is not known. Presumably, these factors are induced during differentiation or aging coincident with $DUX4$ expression. Additionally, the DME1 enhancer region of $DUX4$ has multiple myogenic transcription factor–binding consensus sequences, including a putative E-box (17). Several myogenic transcription factors, such as myogenin and MyoD, have been shown to be induced during denervation (40) as well as aging (41), and myogenin has been shown to be required for induction of muscle-specific transcripts, such as MuRF1 and MaFBx, in addition to full skeletal muscle atrophy (42). Finally, myogenin and MyoD expression levels have been shown to be reduced by clenbuterol dur-

**Figure 7.** Active PKA is sufficient to reduce $DUX4$ activity in FSHD myotubes. FSHD940 myotubes were treated at day 5 for 48 h followed by protein extraction, SDS-PAGE, and Western blotting. A, immunoblot analysis of phosphorylated PKA substrates (p-PKA), phosphorylated CREB (p-CREB), total CREB, and HA-tagged (HA) caPKA from FSHD940 myotubes transduced with varying concentrations of caPKA and empty (CON) adenovirus. B, qPCR for ZSCAN4 (top) and TRIM43 (bottom) in FSHD940 myotubes transduced with varying concentrations of caPKA or CON adenovirus or treated with 100 nM forskolin (FOR) for 48 h; **, $p < 0.01$; *, $p < 0.05$. Error bars, S.E.
ing denervation-induced skeletal muscle atrophy in vivo (40). Hence, it is plausible that the reduction in DUX4 expression effected by β2AR agonists is controlled by signaling pathways that lead to a reduction in myogenic transcription factors, some of which may play additional roles in skeletal muscle wasting, thus sharing a common regulatory pathway.

The finding that β2AR agonists can lower Dux4 expression in FSHD patient myotubes is surprising because there have been three randomized, double-blind, placebo-controlled clinical trials that investigated the effect of the approved β2AR agonist drug albuterol (salbutamol) and did not support its use in FSHD patients (43–45). These studies examined muscle strength by maximum voluntary isometric contraction testing (43, 44) or quantitative muscle testing, manual muscle testing, and timed motor tests (45) as the primary end points; however, none reported a significant effect by albuterol. One study combined albuterol with resistance exercise training to address preclinical findings that the effects of β2AR agonists are augmented by exercise (43). Additionally, one study used an intermittent dosing regimen to avoid reduced effects due to β2AR down-regulation and desensitization, which has been shown in preclinical models (45). Despite the lack of significant effect of the primary end points, patients in two studies had a significant increase in lean body mass measured by DEXA (44) or muscle volume measured by CT (43). The lack of efficacy in these studies may be in part due to the dosages that were used. Albuterol is an FDA-approved drug; however, clinical trials are limited to dosages previously approved to be safe and effective in clinical trials. Although the aforementioned studies used the maximum approved doses, it is possible that higher doses would provide a benefit. Also of note is that the maximum voluntary isometric contraction testing of patients at study start compared with the end of the study (52 weeks) did not decrease (44) or decreased only minimally (43) in both placebo- and albuterol-treated patients, suggesting that the rate of muscle strength decline had leveled off or was too slow to detect spar-
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...ing of additional loss. Treatment for longer periods or earlier in the disease may be required to provide benefit.

Although the lack of evidence to support the use of albuterol in FSHD patients is discouraging, the findings that β2AR agonists can potentially lower the expression of DUX4 in FSHD patients, in conjunction with the anabolic effects of β2AR agonists, make this treatment approach a very attractive one. Whereas current β2AR agonists can potentially lower the expression of DUX4 in FSHD patients, in conjunction with the anabolic effects of β2AR agonists, this treatment approach may prove beneficial for patients with FSHD.

Experimental procedures

Cell culture

FSHD patient primary myoblasts were purchased from the Coriell Cell Repositories (ID nos. GM17940 and GM17869). Skmdc myoblasts were purchased from Cook myosite (SK-1111, SkMDC, adult donor, lot P101061-51M). Myoblasts were cultured in GM consisting of Ham’s F-10 (Cellgro 10-070-CV) supplemented with 20% fetal bovine serum (HyClone, SH30071.03), 5% chicken embryo extract (MP Biomedical, 2850145), 1.2 mM CaCl2 (EMD OmniPur 3000), 1% antibiotic/antimycotic (Corning, 30-004-CI). For assays, myoblasts were seeded on 0.1% gelatin-coated multiwell tissue culture plates in GM (6.25 × 10^4 cells/cm^2), and 24 h after seeding, differentiation into myotubes was started by switching the confluent myoblast monolayer to differentiation medium (DM5) consisting of Dulbecco’s modified Eagle’s medium (Invitrogen, 1111, SkMDC, adult donor, lot P101061-51M). Myoblasts were cultured in GM consisting of Ham’s F-10 (Cellgro 10-070-CV) supplemented with 20% fetal bovine serum (HyClone, SH30071.03), 1% chicken embryo extract (MP Biomedical, 2850145), 1.2 mM CaCl2 (EMD OmniPur 3000), 1% antibiotic/antimycotic (Corning, 30-004-CI). For assays, myoblasts were seeded on 0.1% gelatin-coated multiwell tissue culture plates in GM (6.25 × 10^4 cells/cm^2), and 24 h after seeding, differentiation into myotubes was started by switching the confluent myoblast monolayer to differentiation medium (DM5) consisting of Dulbecco’s modified Eagle’s medium (Invitrogen, 11965-092) and nutrient mixture F-12 (Invitrogen, 11765-054) 1:1 containing 5% fetal bovine serum (HyClone, SH30071.03), 5 μg/ml ITS (BD Biosciences, 354351), 0.1% BSA (Sigma, A1595), 1% antibiotic/antimycotic (Corning, 30-004-CI), and 1% glutamine (Invitrogen, 25030081). Cultures were differentiated up to 7 days.

ZSCAN4 RGA and compound screening

This assay was used to screen for low-molecular weight inhibitors of Dux4-dependent gene expression in FSHD940 myoblast by means of a ZSCAN4 RGA. ZSCAN4 adenovirus transductions of FSHD940 myoblast were performed at onset of differentiation day 0. Briefly, myoblasts were plated in 0.1% gelatin-coated 384-well plates at 6.25 × 10^3 cells/well in growth medium. After 24 h, growth medium was removed and replaced with DM5, and at the same time, cells were transduced with 1 × 10^9 viral particle/ml ZSCAN4-NSucP reporter gene assay adenovirus. Three days after onset of differentiation, compounds were added, and cells were incubated for an additional 48 h. Chemiluminescence was quantified using the Nano-Glo™ luciferase assay system (Promega, N1120) and EnVision Multi-label Plate Reader (PerkinElmer Life Sciences) following the manufacturer’s protocol.

Adenoviruses

The ZSCAN4 RGA was constructed by inserting exon 2 of human ZSCAN4 upstream of the Nano Luciferase P ORF followed by the SV40 poly(A) sequence. The CMV-SEAP transcriptional unit consisted of the CMV promoter followed by the SEAP ORF and the BGH poly(A) sequence. Both transcriptional units were cloned into pAd363, a bipartite adenovirus construct from O.D.260 Inc. PKA constructs were as described previously (25). The PKA ORFs were cloned into the pAd/CMV adenoviral vector using the Gateway technology (Thermo Scientific). Control adenovirus contained no ORF after the CMV promoter. All adenovirus constructs were sent to Welgen Inc. (Worcester, MA) for production and purification.

Immunostaining and antibodies

As counterscreens, the compounds were also tested for their effects on differentiation, toxicity, and morphology by MYHC immunostaining and high-content imaging using the same assay and compound addition protocol as for the ZSCAN4 RGA. Briefly, at day 5, cells were fixed in 4% paraformaldehyde for 15 min at room temperature. Cells were rinsed once in PBS and then incubated in 0.5% Triton in PBS for 5 min at room temperature. For blocking of nonspecific binding, cells were rinsed in PBS and incubated at room temperature in normal goat serum (InVitrogen, 500622) for 1 h. Primary antibodies were diluted 1:1000 in PBS and incubated overnight at 4 °C. After primary antibody incubation, cells were washed twice in PBS, 5 min each. Secondary antibodies and DAPI (2 μg/ml) were diluted in PBS and incubated for 1 h at room temperature and protected from light. Cells were washed three times in PBS for 10 min and stored in PBS. Myotubes were imaged with an INCell Analyzer 2000 (GE Healthcare).

The following antibodies were used: anti-myosin heavy chain, clone A4,1025 (Upstate, 05-716); Dux4 clone E55 (Abcam, ab124699); HA (Sigma-Aldrich, 11867423001); phosphorylated PKA substrates (Cell Signaling Technologies, 9624); phosphorylated CREB (Cell Signaling Technologies, 9198); CREB (Cell Signaling Technologies, 9197); anti-rabbit, rat, and mouse IgG horseradish peroxidase (Rockland, 18-8816-33, 200-5138-0100, and 610-703-002, respectively); goat anti-mouse IgG Alexa Fluor 594 (Molecular Probes, A-11020); and goat anti-rabbit IgG Alexa Fluor 488 (Molecular Probes, A-11008). All primary antibodies were used at 1:1000. Secondary antibodies were used at 1:1000 for immunostaining and 1:10,000 for immunoblotting.

Compounds

The following compounds were used: formoterol fumarate dihydrate (Sigma-Aldrich, F9552-10MG), albuterol (Tocris, 0634), 8-Br-cAMP (R&D Systems, 1140), ICI 118,551 (Tocris, 72795-01-8), CGP20712 (Tocris, 1216905-73-5), K7720 (Tocris, 1288), H-89 dihydrochloride (Tocris, 2910), DAPI (Promo- kine, PK-CA707-40043), and isobutylnethylxanthine (Adipogen, AG-CR1-3512-M500).

RNAi

For RNAi experiments, FSHD940 myoblasts were transfected on day 3 of differentiation with a final concentration
of 10 nM siRNA and 2 μl/ml Dharmafect 1 (Dharmacon, T-2000). Dux4 siRNA and nontargeting control siRNA 1 were from Qiagen (GS22947) and Dharmacon (D-001210-01-05), respectively.

**RNA analysis**

For measuring gene expression levels of all genes except *DUX4*, myoblasts were plated on gelatin-coated, 24-well plates at 1.25 × 10^5 cells/well and switched to DM5 24 h later. Differentiating cells were treated on day 5 postdifferentiation and lysed on day 7. RNA was isolated from cell lysates using the Qiagen RNeasy Mini Plus kit (catalog no. 74106) followed by cDNA synthesis using the High Capacity cDNA Reverse Transcription Kit (Applied Biosystems) and cDNA was synthesized using the Taqman Gene Expression Assays and Master Mix. To detect *DUX4* levels normally undetectable using cDNA from total RNA, mRNA was isolated from 15 μg of total RNA using a Dynabeads mRNA purification kit (Ambion by Life Technologies, catalog no. 61006) followed by cDNA generation from 250 ng of mRNA and qPCR as above. The following Taqman primer probe sets were used: *DUX4* (double homeobox 4, Hs03037970_g1), *TRIM43* (tripartite motif-containing 43, Hs00299174_m1), *ZSCAN4* (zinc finger and SCAN domain-containing 4, Hs00537549_m1), *CKM* (creatine kinase, M type, Hs00176490), *MYOG* (myogenin, Hs01072232), *MEF2A* (myocyte enhancer factor 2A, Hs01054060), *DMD* (dystrophin, Hs00758098_m1), *MBD3L2* (methyl CpG-binding protein 3-like 2, Hs00544743_m1), *LEUTX* (Leucine Twenty Homeobox, Hs00128718_m1). They were normalized to reference genes *TBP* (TATA-box-binding protein, Hs04400364_m1) and *ACTB* (actin β, Hs00357333_g1).

**cAMP detection**

cAMP levels were measured using the cAMP GS Dynamic Kit (Cisbio, 62AM4PEB). FSHD940 primary myoblasts were plated at 6.25 × 10^3 cells/well in growth medium and incubated overnight at 37 °C and 5% CO_2_. The following day, the medium was exchanged with DM5 differentiation medium, and the cells were differentiated for 5 days at 37 °C and 7.5% CO_2_. On day 5 of differentiation, the cells were treated with formoterol and cAMP levels were measured using the cAMP GS Dynamic Kit (Cisbio, 62AM4PEB). FSHD940 primary myoblasts were from Qiagen (GS22947) and Dharmacon (D-001210-01-05). Dux4 siRNA and nontargeting control siRNA 1 were from Qiagen (GS22947) and Dharmacon (D-001210-01-05), respectively.

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**Statistical analysis**

Significance was determined using unpaired *t* test. All analyses were parametric assuming Gaussian distribution using GraphPad prism. Individual samples were compared with the vehicle control groups.

**Quantification of myoblast differentiation by high-content imaging**

Myoblasts and myotubes were fixed and immunostained for MYHC as described above. Cells were imaged and analyzed with the Cellomics Arrayscan VTi (Thermo Scientific). Nine adjacent 10× fields were captured and analyzed per well. DAPI-stained nuclei were identified, and a mask 1 pixel larger than the nuclei was generated and used for MYHC quantification. Positive MYHC staining within the mask was determined by comparison with myoblasts immunostained normally and myotubes immunostained with secondary antibody alone. A threshold value was set where negative control samples exhibited no MYHC-positive nuclei. On average, >9,000 nuclei were analyzed per well.
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