Expression of the Human FSHD-Linked *DUX4* Gene Induces Neurogenesis During Differentiation of Murine Embryonic Stem Cells

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Misexpression of the double homeodomain protein DUX4 in muscle is believed to cause facioscapulohumeral muscular dystrophy (FSHD). Although strategies are being devised to inhibit DUX4 activity in FSHD, there is little known about the normal function of this protein. Expression of DUX4 has been reported in pluripotent cells and testis. To test the idea that DUX4 may be involved in initiating a germ lineage program in pluripotent cells, we interrogated the effect of expressing the human DUX4 gene at different stages during in vitro differentiation of murine embryonic stem (ES) cells. We find that expression of even low levels of DUX4 is incompatible with pluripotency: DUX4-expressing ES cells downregulate pluripotency markers and rapidly differentiate even in the presence of leukemia inhibitory factor (LIF) and bone morphogenetic protein 4 (BMP4). Transcriptional profiling revealed unexpectedly that DUX4 induced a neuroectodermal program. Embryoid bodies exposed to a pulse of DUX4 expression displayed severely inhibited mesodermal differentiation, but acquired neurogenic potential. In a serum-containing medium in which neurogenic differentiation is minimal, DUX4 expression served as a neural-inducing factor, enabling the differentiation of Tuj1+ neurites. These data suggest that besides effects in muscle and germ cells, the involvement of DUX4 in neurogenesis should be considered as anti-DUX4 therapies are developed.

Introduction

DUX4 IS A DOUBLE HOMEODOMAIN protein encoded by a tandem repeat array referred to as D4Z4 [1]. Alterations in the repeat number, specifically contractions from the usual approximately 100 copies down to 10 or fewer, cause the dominant genetic disease facioscapulohumeral muscular dystrophy (FSHD) [2,3] and many lines of evidence suggest that these contractions lead to low-level expression of *DUX4*, which is normally silent in skeletal muscle [4–6]. The homeodomains of DUX4 belong to the paired homeodomain family, and within this family are most similar in sequence to those of PAX3, PAX6, and PAX7. *Pax6* is an important regulator of embryonic and adult neurogenesis [7]. *Pax3* and *Pax7* are key determinants of myogenic development [8–10] and muscle satellite cell physiology [11]. Accordingly, when DUX4 was expressed in myoblasts at low levels, it was found to interfere with myogenic gene expression, block differentiation into multinucleated myotubes, and display competitive interactions with both PAX3 and PAX7 [12]. Interference with PAX3/7-regulated gene expression pathways governing muscle regeneration is thus a likely pathogenic effect of *DUX4* expression, which may explain its involvement in FSHD.

On the other hand, the normal function of *DUX4* remains mysterious. The mouse has a homologue named *Dux*, which is also arrayed in tandem repeats (although fewer in number than those of the human) and expressed in various tissues, but most prominently in neurogenic tissues [13]. In humans, expression has been reported in testis [6] and a role in the expression of germ cell cancer/testis antigens demonstrated [14]. *DUX4* message has also been detected at low levels in human embryonic stem (ES) cells [15].

To investigate the role of *DUX4* in early development, we created a mouse ES (mES) cell line that would inducibly express DUX4. The early stages of embryo development are well recapitulated in mES cell growth and embryoid body (EB) differentiation; thus, these cells allowed the interrogation of the activity of this transcription factor on the development of all of major lineages. Although we had anticipated that DUX4 might drive germ line differentiation, we found only a very modest effect on germ line-specific genes and no evidence of directing pluripotent cells into a germ cell fate. Rather, DUX4 induced a neuroectodermal gene expression program at the expense of other major lineage-specific programs, and was able to drive neurogenesis in vitro even under nonoptimized conditions in which minimal neurogenesis normally occurs.

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Materials and Methods

iDUX4 ES cell generation and culture

The *DUX4* ORF was subcloned into p2Lox, the targeting vector for the inducible cassette exchange (ICE) recombination system [16]. *DUX4*-inducible ES cells were generated by cassette-inducible exchange recombination (ICE) into the doxycycline-inducible locus upstream of HPRT in ZX1 mES cells, an improved version of A2Lox.cre [17]. ZX1 cells were used as the parent control cell line in experiments. *iDUX4* ES cells were cultured on mouse embryonic fibroblasts (MEFs) in Dulbecco's modified Eagle's medium (DMEM) (Invitrogen 10829-018) supplemented with 15% fetal bovine serum (FBS), 0.1 mM nonessential amino acids (Gibco), 2 mM glutamax (Invitrogen), penicillin/streptomycin (Gibco), 0.1 mM β -mercaptoethanol (Sigma), and 1,000 U/mL leukemia inhibitory factor (LIF) (Millipore), at 37°C in 5% CO₂.

Embryoid body differentiation

ES cells were differentiated as EBs by preplating for 30 min to remove MEFs by attachment, followed by suspension culture in hanging drops (100 cells per 10 μ L drop) in the EBD medium: Iscove's modified Dulbecco's medium (IMDM, Invitrogen) supplemented with 15% FBS, 200 μ g/mL iron-saturated transferrin (Sigma), 4.5 mM monothiolglycerol (Sigma), 50 μ g/mL ascorbic acid (Sigma), penicillin/streptomycin (Invitrogen), and 2 mM glutamax (Invitrogen) at 37°C in 5% CO₂, 5% O₂. After 48 h, EBs were harvested from hanging drops by collecting and settling in IMDM. They were then resuspended in 10 mL of EBD and plated in nonadherent 10-cm dishes on a swirling rotator (70 rpm). EBs were fed after 48 h by exchanging 50% of a spent medium for fresh EBD medium. Doxycycline induction was started at day 2 with the concentrations indicated, and EBs were harvested either at day 4 or 6.

Growth curve

ES cells on MEFs were disassociated with trypsin (Invitrogen) and the MEFs were removed by preplating for 30 min. The 1×10^5 cells were plated on 35-mm tissue culture dishes in the knockout serum replacement (KOSR) medium (Gibco) supplemented with 1,000 U/mL LIF and 10 ng/mL bone morphogenetic protein 4 (BMP4) (Peprotech) along with 0, 100, 200, 300, 400, or 500 ng/mL doxycycline. Every 2 days, cells were passaged, cell numbers were counted from triplicate wells of each passage, and 1×10^5 cells were replated in the same conditions.

Fluorescence-activated cell sorting

Live cells were gated based on forward scatter/side scatter profile, and negativity for propidium iodine staining to eliminate debris and dead cells. The following antibodies were used: c-Kit-allophycocyanin (APC) (2B8), CD41-phycoerythrin (PE) (eBioMWREG30), CD31-Cy7-PE (390), CD45-PE (30-F11), Flk1-APC (Avas12a1), Flk1-PE (Avas12a1), platelet-derived growth factor receptor alpha (PDGFR α)-PE (APA5), SSEA1 (eBioMC-480), Tie-2 (TEK4), (all from eBiosciences) and polysialylated neuronal cell adhesion molecule (PSA-NCAM)-APC (130-093-272; Miltenyi

Biotec). Cells were incubated with 1 μ L of antibody per one million cells and analyzed and/or sorted on a fluorescence-activated cell sorting (FACS) Aria II (BD Biosciences).

Cell staining

Live cells or cells fixed first with 4% paraformaldehyde were imaged using phase-contrast microscopy (Evos; Advanced Microscopy Group). For immunostaining, cells were grown as described and fixed with 4% paraformaldehyde in the same plates as they were grown in. Cells were permeabilized, blocked, and immunostained according to the manufacturers' directions for each antibody: *DUX4* (E5-5; Epitomics) or Nestin (AB0968100; Abcam) both used with goat anti-rabbit Alexa Fluor-555 conjugated secondary antibody (Invitrogen A21429), or Tuj1 conjugated with Alexa Fluor 488 (A488-435; Covance). Nuclei were imaged with DNA-staining 4',6-diamidino-2-phenylindole (DAPI) dye. Images were taken with a Zeiss Observer Z1 using an AxioCam MRM camera and software.

Quantitative reverse transcription-polymerase chain reaction

RNA was extracted with Ambion RNAqueous[®] Kit (Life Technologies/Invitrogen); reverse transcription was performed with ThermoScript[™] Reverse Transcriptase (RT) (Invitrogen). Quantitative polymerase chain reaction (qPCR) was performed using real-time PCR (7500 or 7900 Real-Time PCR Systems; Applied Biosystems). In brief, 40 cycles at 95°C for 12 s and 60°C for 30 s using TaqMan Gene Expression Assay primer-probe sets (Applied Biosystems). Quantitative analysis of gene expression was conducted using the comparative cycle threshold (Ct) method and means were normalized first to *GAPDH*, and then to noninduced controls and were compared by the *t*-test. All PCRs were done in triplicate with one control reaction containing cDNA that was reverse transcribed without the RT enzyme.

Microarray experiments

DUX4 was induced with 100 μ g/mL doxycycline from EB day 2 and harvested after 48 h of doxycycline induction. The RNA from three independent replicates of both +doxycycline and control was used to generate the cRNA probe, which was hybridized to MouseWG-6 Bead Chip Arrays (Illumina). Raw data were processed using Beadstudio (Illumina) and analyzed using Genespring GX 7.3.1 (Agilent).

Results

Low-level expression of DUX4 in mES cells downregulates the pluripotent program and promotes neuroectodermal gene expression

Previously, we found that high levels of *DUX4* expression (from a construct that contained the ORF +1.5 kb of 3' UTR) are toxic to myoblast cells and that low levels of *DUX4* inhibited myogenic differentiation, suggesting a mechanism for the involvement of misexpressed FSHD in muscle pathology [12]. We generated a doxycycline-inducible *DUX4* (*iDUX4*) mES line by recombining the *DUX4* ORF into mES cells with an ICE locus upstream of *HPRT* [16]. In these ES

FIG. 1. DUX4 perturbs pluripotency and induces neural gene expression. **(A)** The DUX4 protein was detected by immunofluorescence in iDUX4 embryonic stem (ES) with induction of 100 ng/mL of doxycycline for 24 h and not in uninduced cells. DUX4 is localized to the nucleus (Dux4 images), which was made visible by DAPI staining and merging of the images (Dux4 DAPI images). **(B)** Quantitative reverse transcription polymerase chain reaction (qRT-PCR) analysis showing the dose responsiveness of *DUX4* expression from 50 to 300 ng/mL doxycycline for 24 h. **(C)** Growth curves of iDUX4 ES cells cultured in different concentrations of doxycycline. A dose-dependent inhibition of growth was observed, but this was not severe at 100 ng/mL. **(D)** Phase-contrast photomicrographs of iDUX4 ES colonies in the presence or absence of low-dose doxycycline. **(E)** FACS analysis of SSEA1 and polysialylated neuronal cell adhesion molecule (PSA-NCAM) in iDUX4 ES cells induced with 100 ng/mL doxycycline for 48 h. Controls and iDUX4 no doxycycline is positive for SSEA1 and negative for PSA-NCAM. Cells induced with doxycycline have greatly reduced SSEA1, and demonstrate expression of PSA-NCAM. Shaded area represents unstained cells. **(F)** qRT-PCR for markers of pluripotency, germ line, and endo/meso/ecto-derm in iDUX4 ES cells induced with doxycycline for 48 h at 100 ng/mL. Means were normalized first to *GAPDH*, and then to the noninduced samples and were compared by the *t*-test with the probability of a larger *t* value symbolized as * $P < 0.01$, ** $P < 0.001$, and *** $P < 0.0001$. Scale bars: 100 μ m in **(A)** and **(D)**.

cells, DUX4 protein expression was visible in the nuclei of cells at 24 h postinduction in *iDUX4* cells, but not in uninduced cells (Fig. 1A) and there was a direct relationship between the amount of doxycycline added and the amount of DUX4 RNA (Fig. 1B and Supplementary Fig. S1A; Supplementary Data are available online at www.liebertpub.com/scd). Overexpression of DUX4 at high levels was clearly deleterious and cells could not be expanded (Fig. 1C; 500 ng/mL). At low levels, DUX4 was not overtly toxic, however, these cultures proliferated somewhat more slowly than the controls (Fig. 1C; 100 ng/mL). It should be mentioned that DUX4 sensitizes mouse myoblasts to oxidative stress, and that acute toxicity can be moderated somewhat by culturing cells with antioxidants such as beta mercaptoethanol [12], an additive that is present in the ES culture medium. Colonies were clearly altered in morphology becoming less refractive with flattened separating cells with doxycycline (Fig. 1D).

To determine whether this morphological change indicated a change in pluripotency, we induced DUX4 at low levels for 48 h and evaluated a panel of pluripotent markers and differentiation, both by FACs as well as qRT-PCR (Fig. 1E, F). With the exception of *Sox2*, which was upregulated, all markers of pluripotency, *SSEA1*, *Nanog*, *Klf4*, and *Oct4* were downregulated with low-dose DUX4 induction. Since DUX4 was reported to be expressed in testis and to induce germ cell antigens (among many other changes) when expressed in human myoblasts [6,14], we examined germ cell lineage markers. These results were not consistent with the activation of a germ cell program: the master regulator, *Prdm1*, was significantly downregulated; the germ cell marker, *Dazl*, was upregulated, while *Stella* and *Vasa* showed no change. Since a primordial germ cell lineage was not being induced, we evaluated markers of meso-, endo-, and ectoderm. Early mesodermal cell fate markers showed no change (*Mesp1*) or were downregulated (*Brachyury*), while endoderm markers *Foxa2* and *Sox17* showed no change. Although *Sox2* is expressed in pluripotent cells, it is also a key element of the early neurogenic program [18,19], so its upregulation might be interpreted as the induction of a neurogenic program. Supporting this idea, all the neuroectoderm markers evaluated (PSA-NCAM, *Neurog2*, and *Pax6*) were upregulated. In addition, it should be noted that the epiblast marker *Fgf5* was upregulated, suggestive of a transition through epiblast to neuroectoderm.

DUX4 promotes neuroectodermal differentiation during EB differentiation

Although the monolayer differentiation study above clearly shows that DUX4 interferes with the pluripotent program, monolayer differentiation is not an efficient way to generate the mesoderm or endoderm. To test the effect of DUX4 expression on the generation of the three germ layers, we subjected *iDUX4* ES cells to EB differentiation. During the first 2 days of EB differentiation, cells progress from the ES stage to epiblast. After this time point, the three germ layers are specified, with the earliest germ layer-specific markers appearing shortly afterward. We therefore applied 100 ng/mL of doxycycline at 48 h of differentiation and evaluated EBs 48 h later (after 4 days of differentiation). Under these conditions, doxycycline induction of DUX4 caused EBs to be somewhat more compact (Fig. 2B). We then performed mi-

croarray profiling on total EB RNA. We found no significant differences between induced and uninduced samples for most germline-specific or endodermal markers (Fig. 2C).

For genes associated with early mesodermal lineages, results were mixed with some early markers upregulated, however, this likely reflects lack of appropriate downregulation due to impaired differentiation of early specified mesoderm lineage founders as these markers normally peak at day 3 and are much lower at day 4. By this time point, the first wave of mesodermal differentiation is well underway, and this first wave prominently produces hematopoietic lineage founders, while other mesodermal lineages arise slightly later [20]. Looking at the signature for this first wave, we observed clear downregulation of both hematopoietic transcription factors and globins. Coordinate with this was a decrease in most endothelial markers. However, the most striking signature change was that of the neuroectoderm and neural genes. This lineage is typically quite underrepresented in EBs grown in serum, however, when low levels of DUX4 are induced, almost every marker is upregulated. These results are consistent with DUX4 expression in early development inhibiting various mesodermal lineages particularly the lateral plate and promoting neuroectoderm/neurogenesis.

To support this interpretation, we repeated low-dose DUX4 inductions starting at 48 h, and interrogated various markers specifically, including surface markers that have been associated with specific lineages, and RNA markers by qRT-PCR (Fig. 3A). Day 4 EBs were examined for the early mesoderm markers Flk1 for lateral plate [21], and PDGFR α for paraxial mesoderm [22] by FACS analysis (Fig. 3B). In uninduced cultures, differentiating EBs developed into Flk1⁻PDGFR α ⁺ (10.6%), Flk1⁺PDGFR α ⁺ (34.3%), and Flk1⁺PDGFR α ⁻ (9.6%) populations on day 4 of differentiation. Doxycycline induction increased the PDGFR α single-positive population to 44.4%, and decreased Flk1 expression to negligible levels. While there was a similar proportion of PDGFR α ⁺ cells in treated and untreated samples (45% and 46%), most of these were single-positive with doxycycline treatment. PSA-NCAM is expressed in early neuronal progenitor cells [23–25] and functions in cell migration [26]. Interestingly, this protein was expressed with DUX4 induction, but almost absent in uninduced EBs and also absent in control induced and uninduced cells from the parent cell line of *iDUX4* (Supplementary Fig. S1B). Although PDGFR α marks the paraxial mesoderm, it also marks cells that will become oligodendrocyte progenitors [27]. The presence of double-positive PSA-NCAM/PDGFR α cells suggests DUX4 is promoting a neuronal cell fate (Fig. 3C). To examine the effects of DUX4 on the lateral plate mesoderm, we performed FACs with PSA-NCAM/Flk1 and found that DUX4 inhibits the lateral plate mesoderm, while at the same time promoting neuroectoderm (Fig. 3D). We further explored the induction of neurogenesis by quantitative reverse transcription PCR (qRT-PCR) analyses for expression of selected neuroectodermal genes. *Pax6*, *Neurog2*, *Gbx2*, *Otx2*, *Sox2*, and *Nestin* were all significantly elevated in DUX4-induced EBs (Fig. 3E and Supplementary Fig. S1C). These results support the interpretation that DUX4 promotes early neural progenitors, but inhibits mesoderm, particularly the lateral plate mesoderm.

We performed additional experiments at a later stage of EB development to examine the effects of DUX4 on

FIG. 2. Microarray profiling of day 4 embryoid bodies (EBs) demonstrates neurogenesis. **(A)** Scheme of differentiation indicating times of induction and analysis. **(B)** iDUX4 ES cells were aggregated into EBs for 2 days, and induced with 100 ng/mL doxycycline for 48 h while shaking. Scale bars: 500 μ m. **(C)** Heat map of lineage-specific markers. Total RNA from day 4 EBs was used for Affymetrix microarray gene chip analysis. Expression levels are compared with the median calculated intensity value, as indicated in the scale at the bottom. Each heat map box represents an independent biological replicate. Color images available online at www.liebertpub.com/scd

hematopoietic, endothelial, and cardiac progenitors. DUX4 was induced by applying doxycycline at 100 ng/mL from days 2–6 and cells were assayed by FACS and qRT-PCR (Fig. 4A). In accordance with the previous results, we obtained fewer undifferentiated hematopoietic progenitors (c-Kit/CD41, c-Kit/CD45), and fewer Flk1/Tie2 or Flk1/CD31 endothelial cells (Fig. 4B). To evaluate cardiogenesis, we analyzed two early cardiac markers *Nkx2.5*, *Tbx5* [28] and the terminal marker cardiac troponinT2 (*cTnnt2*) by qRT-PCR and found the expression of all of these markers significantly inhibited by DUX4 induction in comparison to uninduced samples (Fig. 4C). These results demonstrate that DUX4 is incompatible with the differentiation of the major mesodermal lineages assayable in EBs.

DUX4 promotes neurogenesis

We wished to follow up on the observation that DUX4 promotes neuroectoderm, to see whether it could generate differentiated neural cell types under conditions in which, few

such cells are normally formed. EBs were formed by hanging drops for 2 days, and then moved to a swirling suspension with doxycycline being added from day 2 through day 6, when EBs were allowed to attach to a solid surface and grow for an additional 3 days to allow for terminal differentiation (Fig. 5A). In samples not treated with doxycycline, the attached EBs gave rise to minimal neurite-like sprouts, while doxycycline induced a tremendous number of sprouts. To quantify this observation, we counted the number of EBs displaying sprouts. The DUX4-induced EBs had significantly more neurite-like sprouting outgrowths compared to uninduced samples (Fig. 5B, C and Supplementary Fig. S2A). These cultures were fixed and stained for Tuj1, which identifies β -tubulin III, a structural protein of mature neurons. There was minimal Tuj1 staining in control EBs, but in DUX4-induced EBs, Tuj1 was visible throughout the attached colonies, and particularly strongly detected in the sprouts, confirming their neuronal identity (Fig. 5D). This differentiation was in the absence of conventional neuronal-inducing or -supportive factors like retinoic acid, basic fibroblast growth factor, or a

FIG. 3. DUX4 inhibits lateral plate mesoderm and promotes neuroectoderm in day 4 EBs. **(A)** Scheme of differentiation showing doxycycline induction and analysis. **(B)** FACS for Flk1 and PDGFR α ; **(C)** for PSA-NCAM and PDGFR α ; and **(D)** for PSA-NCAM and Flk1 in EBs that were induced with 100 ng/mL doxycycline on days 2–4 and analyzed on day 4. **(E)** qRT-PCR results for day 4 EBs examining neuroectodermal markers, *Pax6*, *Neurog2*, *Nestin*, *Sox2*, *Gbx2*, and *Otx2*. EBs were induced with 100 ng/mL doxycycline on days 2–4 and analyzed on day 4. Means were normalized first to *GAPDH*, and then to noninduced samples and were compared by the *t*-test with the probability of a larger *t* value symbolized as **P* < 0.01, ***P* < 0.001, and ****P* < 0.0001.

medium optimized for neuronal differentiation of ES cells. We also examined cultures in which, EBs had been trypsinized and cells plated directly on plastic for Tuj1 immunofluorescence 2 days later, which also demonstrated Tuj1⁺ cells (Fig. 5E and Supplementary Fig. S2B). Since Tuj1 is a marker associated with later stage development of neurons, we also tested for an early marker of neuronal development, Nestin. We dissociated cells from day 6 EBs and examined them 2 days after plating onto gelatin-coated dishes. We found that DUX4 potently induced Nestin⁺ cells, while there was very little staining in uninduced cultures (Fig. 5F and Supplementary Fig. S2C). Finally, we evaluated several neural markers over a time course from days 6 through 9. Three terminal neural differentiation markers were examined. Tuj1 was dramatically upregulated by DUX4 (Fig. 5G). There was also significant upregulation of expression of two glial genes for terminal differentiation: *GFAP*, a marker for astrocytes and *Olig2*, a marker for oligodendrocytes. These experiments

FIG. 4. DUX4 inhibits cardiac and hemangiogenic progenitors in day 6 EBs. **(A)** Scheme of differentiation showing doxycycline induction and analysis. **(B)** FACS expression profiles of EBs that were induced with 100 ng/mL doxycycline on days 2–6 and analyzed at day 6 for early hematopoietic markers, c-kit and CD41/45 or endothelial markers, Flk1 and Tie2/CD31. **(C)** qRT-PCR analysis of EBs that were induced with 100 ng/mL doxycycline on days 2–6 and analyzed at day 6 for early cardiac markers, *Nkx2.5*, *Tbx5*, and *cTnnt2*. Means were normalized first to *GAPDH*, and then to noninduced samples and were compared by the *t*-test with the probability of a larger *t* value symbolized as **P* < 0.01, ***P* < 0.001, and ****P* < 0.0001.

show that DUX4 not only promotes neuroectodermal fates, it can induce the formation of neuronal progenitors that are capable of terminal differentiation.

Discussion

The recent correlation of FSHD pathogenesis with the chromosome 4 permissive allele that contains a signal sequence allowing polyadenylation of *DUX4* RNA from the last repeat [5], has encouraged researchers to consider using methods to reduce *DUX4* RNA levels as a therapy for patients [29]. However, it is likely that *DUX4* expression has a role in normal development because the open reading frame encoding DUX4 is clearly under positive selection, that is, if it were not functional and required for something essential, it would have acquired mutations that disrupt the ORF [13]. Therefore, it is important to investigate the normal function of DUX4 when therapies reducing the level of DUX4 in cells are considered. For this reason as well as to gain a further understanding of the effects of ectopic expression of *DUX4* in cells, we have determined the effect of expressing the human *DUX4* gene during mES cell in vitro differentiation, a powerful system for determining the function of genes involved in development.

FIG. 5. DUX4 promotes neuronal differentiation. **(A)** Schematic diagram of differentiation protocol and induction with 100 ng/mL doxycycline. **(B)** Percentage of EBs displaying neurite-sprouting outgrowths. **(C)** Phase-contrast image at day 11 of attached and differentiated EBs (*left panel*) and enlarged images (*second panel*) with or without 100 ng/mL doxycycline induction. Scale bars: 500 μ m. **(D)** Immunostaining for Tuj1 (*second panel from left*) and merged with the DAPI-stained images (*third from the left panel*) at day 11 of attached and differentiated EBs. Scale bars: 200 μ m. **(E)** Immunostaining for Tuj1 (*left*) and merged with the DAPI-stained images (*right*) after cells from day 9 EBs were dissociated with trypsin and replated onto gelatin-coated dishes for 2 days. Scale bars: 200 μ m. **(F)** Immunostaining for Nestin merged with the DAPI-stained image in attached EBs. For this image, day 6 EBs were dissociated with trypsin and replated onto gelatin-coated dishes for 2 days before imaging. Scale bars: 100 μ m. **(G)** Three terminal neural differentiation markers (*GFAP*, *Olig2*, and *Tuj1*) were examined by qRT-PCR. Means were normalized first to *GAPDH*, and then to noninduced samples and were compared by the *t*-test with the probability of a larger *t* value symbolized as **P* < 0.01, ***P* < 0.001, and ****P* < 0.0001.

Using a doxycycline-inducible promoter to express DUX4 allowed us to titrate down the level of expression, to a level at which DUX4 was no longer potentially cytotoxic to ES cells growing in a medium with beta-mercaptoethanol. Achieving low levels of expression is important, as the physiological level of DUX4 expression in primary cells, even in FSHD muscle cells, is low [4,6,15,30]. Most previous studies on DUX4 action in cells have used overexpression from strong promoters [4,14,31], and there is clearly a high dose-dependent toxicity of DUX4. However, very low doses that do not cause overt cell death are able to inhibit MyoD expression and block myogenic differentiation of C2C12 cells [12], effects that may be more relevant to FSHD than is cell death.

We found that pluripotency was rapidly compromised by low levels of DUX4. Within 2 days, pluripotent markers were virtually eliminated (*SSEA1*, *Nanog*, *Oct4*, and *Klf4*). This strongly suggests that DUX4 does not play a role in maintaining cells in the pluripotent stage. However, there have been reports of DUX4 RNA expression in WT hES and induced pluripotent stem (iPS) cells [6,15]. We suspect that this may be due not to expression in pluripotent cells, but to expression in some type of early differentiated cells, of

which, there are always a few in healthy karyotypically normal ES cell cultures. Because germ cells are closely related to ES cells, because DUX4 has been demonstrated to be expressed in human testis [6], and because overexpression of DUX4 in human myoblasts induced the expression of a selection of germ cell cancer antigens [14], we presumed initially that such rare DUX4-expressing cells in hES cultures might be early germ lineage progenitors, and therefore, we would find that DUX4 would drive ES cells into the germ lineage. However, we did find strong evidence for this. In ES monolayers, by qRT-PCR, the master regulator of primordial germ cell development, *Prdm1*, was downregulated. One germ cell marker, *Dazl*, was upregulated, while others, *Vasa* and *Stella*, were unchanged. By microarray in day 4 EB cells after a 48-h pulse of DUX4, *Nanos* was upregulated, but *Prdm1* and *Dazl* were unchanged. On the other hand, a clear effect of DUX4 expression in both ES cells and in very early differentiating EBs was the induction of markers indicative of neurogenesis (*PSA-NCAM*, *Sox2*, *Neurog2*, *Pax6*). ES cells differentiated in vitro as EBs in a serum-containing medium give a preponderance of lateral plate mesoderm derivatives [32]. We found that ES cells differentiated under these

conditions, but in the presence of *DUX4* induction showed a severely inhibited lateral plate mesoderm and derivatives (blood and cardiac tissue), but had acquired neurogenic potential, even to the point of producing terminally differentiated neuronal cells. These were obtained when a pulse of low-level *DUX4* expression was applied during EB differentiation, but then removed as EBs were attached to plastic and allowed to differentiate for several more days. Differentiated Tuj1+ neurons were observed by day 11. It should be emphasized that in these experiments, the growth medium was devoid of exogenous retinoic acid, fibroblast growth factor 5 (FGF5), or other growth factors and cytokines that are usually required for neural induction of ES cells. In addition, they were done in the presence of serum, which is inhibitory to neurogenesis in EBs.

We have previously demonstrated a competitive interaction between *DUX4* and Pax3/Pax7 for regulation of MyoD and other targets, and proposed that the similarity of the homeodomains of *DUX4* to those of Pax3 and Pax7 may enable inappropriate interaction with Pax3/7 targets in satellite cells, which would perturb muscle regeneration [12]. It is interesting to note that both Pax3 and Pax7 are expressed in early neural development, and that the *DUX4* homeodomains are equally similar to those of Pax6, an important neurogenic transcription factor. Based on the molecular similarities therefore, a role for *DUX4* in neural differentiation is not unreasonable. We interrogated the neuroectodermal markers used in this study (*Sox2*, *Pax6*, and PSA-NCAM) for *DUX4* consensus DNA-binding elements, and found no conserved sites for *Sox2* or *Pax6*, and only a few nonconserved sites for *NCAM*, so these genes may not be direct targets. Most of the direct *DUX4* targets in myoblast cells appeared to be within primate-specific MaLR repeat elements, possibly suggesting acquired human-specific activities [14]; however, we note that several without primate-specific MaLR sites, have functions in some aspect of neurogenesis or neural function, for example, RFPL1 and 2 [33], and PPP2R2B [34]. Studies of a related gene in mouse, *Dux*, found expression in brain tissues [13]. The particular expression pattern observed suggested that mouse *Dux* might have a role in embryonic development within granule neurons and when young mouse brains were examined, expression was observed throughout the cortex with localized areas of increased expression. Taken together, the experiments presented here suggest the importance of looking beyond muscle and germ line function to understand *DUX4* and that we should consider the possibility that *DUX4* has a role in neurogenesis.

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Author Disclosure Statement

The authors declare no competing financial interests exist.

References

- Gabriels J, MC Beckers, H Ding, A De Vriese, S Plaisance, SM van der Maarel, GW Padberg, RR Frants, JE Hewitt, D Collen and A Belayew. (1999). Nucleotide sequence of the partially deleted D4Z4 locus in a patient with FSHD identifies a putative gene within each 3.3 kb element. *Gene* 236:25–32.
- Wijmenga C, JE Hewitt, LA Sandkuijl, LN Clark, TJ Wright, HG Dauwerse, AM Gruter, MH Hofker, P Moerer, et al. (1992). Chromosome 4q DNA rearrangements associated with facioscapulohumeral muscular dystrophy. *Nat Genet* 2:26–30.
- van Deutekom JC, C Wijmenga, EA van Tienhoven, AM Gruter, JE Hewitt, GW Padberg, GJ van Ommen, MH Hofker and RR Frants. (1993). FSHD associated DNA rearrangements are due to deletions of integral copies of a 3.2 kb tandemly repeated unit. *Hum Mol Genet* 2:2037–2042.
- Dixit M, E Anseau, A Tassin, S Winokur, R Shi, H Qian, S Sauvage, C Matteotti, AM van Acker, et al. (2007). *DUX4*, a candidate gene of facioscapulohumeral muscular dystrophy, encodes a transcriptional activator of PITX1. *Proc Natl Acad Sci U S A* 104:18157–18162.
- Lemmers RJ, PJ van der Vliet, R Klooster, S Sacconi, P Camano, JG Dauwerse, L Snider, KR Straasheijm, GJ van Ommen, et al. (2010). A unifying genetic model for facioscapulohumeral muscular dystrophy. *Science* 329:1650–1653.
- Snider L, LN Geng, RJ Lemmers, M Kyba, CB Ware, AM Nelson, R Tawil, GN Filippova, SM van der Maarel, SJ Tapscott and DG Miller. (2010). Facioscapulohumeral dystrophy: incomplete suppression of a retrotransposed gene. *PLoS Genet* 6:e1001181.
- Osumi N, H Shinohara, K Numayama-Tsuruta and M Maekawa. (2008). Concise review: Pax6 transcription factor contributes to both embryonic and adult neurogenesis as a multifunctional regulator. *Stem Cells* 26:1663–1672.
- Relaix F, D Rocancourt, A Mansouri and M Buckingham. (2004). Divergent functions of murine Pax3 and Pax7 in limb muscle development. *Genes Dev* 18:1088–1105.
- Kassar-Duchossoy L, E Giacone, B Gayraud-Morel, A Jory, D Gomes and S Tajbakhsh. (2005). Pax3/Pax7 mark a novel population of primitive myogenic cells during development. *Genes Dev* 19:1426–1431.
- Relaix F, D Rocancourt, A Mansouri and M Buckingham. (2005). A Pax3/Pax7-dependent population of skeletal muscle progenitor cells. *Nature* 435:948–953.
- Seale P, LA Sabourin, A Girgis-Gabardo, A Mansouri, P Gruss and MA Rudnicki. (2000). Pax7 is required for the specification of myogenic satellite cells. *Cell* 102:777–786.
- Bosnakovski D, Z Xu, EJ Gang, CL Galindo, M Liu, T Simsek, HR Garner, S Agha-Mohammadi, A Tassin, et al. (2008). An isogenetic myoblast expression screen identifies *DUX4*-mediated FSHD-associated molecular pathologies. *EMBO J* 27:2766–2779.
- Clapp J, LM Mitchell, DJ Bolland, J Fantes, AE Corcoran, PJ Scotting, JA Armour and JE Hewitt. (2007). Evolutionary conservation of a coding function for D4Z4, the tandem DNA repeat mutated in facioscapulohumeral muscular dystrophy. *Am J Hum Genet* 81:264–279.
- Geng LN, Z Yao, L Snider, AP Fong, JN Cech, JM Young, SM van der Maarel, WL Ruzzo, RC Gentleman, R Tawil and SJ Tapscott. (2012). *DUX4* activates germline genes, retroelements, and immune mediators: implications for facioscapulohumeral dystrophy. *Dev Cell* 22:38–51.
- Snider L, A Asawachaicharn, AE Tyler, LN Geng, LM Petek, L Maves, DG Miller, RJ Lemmers, ST Winokur, et al. (2009).

- RNA transcripts, miRNA-sized fragments and proteins produced from D4Z4 units: new candidates for the pathophysiology of facioscapulohumeral dystrophy. *Hum Mol Genet* 18:2414–2430.
16. Iacovino M, C Hernandez, Z Xu, G Bajwa, M Prather and M Kyba. (2009). A conserved role for Hox paralog group 4 in regulation of hematopoietic progenitors. *Stem Cells Dev* 18:783–792.
 17. Iacovino M, D Bosnakovski, H Fey, D Rux, G Bajwa, E Mahen, A Mitansoska, Z Xu and M Kyba. (2011). Inducible cassette exchange: a rapid and efficient system enabling conditional gene expression in embryonic stem and primary cells. *Stem Cells* 29:1580–1588.
 18. Li M, L Pevny, R Lovell-Badge and A Smith. (1998). Generation of purified neural precursors from embryonic stem cells by lineage selection. *Curr Biol* 8:971–974.
 19. Kishi M, K Mizuseki, N Sasai, H Yamazaki, K Shiota, S Nakanishi and Y Sasai. (2000). Requirement of Sox2-mediated signaling for differentiation of early *Xenopus* neuroectoderm. *Development* 127:791–800.
 20. Kouskoff V, G Lacaud, S Schwant, HJ Fehling and G Keller. (2005). Sequential development of hematopoietic and cardiac mesoderm during embryonic stem cell differentiation. *Proc Natl Acad Sci U S A* 102:13170–13175.
 21. Yamaguchi TP, DJ Dumont, RA Conlon, ML Breitman and J Rossant. (1993). flk-1, an flt-related receptor tyrosine kinase is an early marker for endothelial cell precursors. *Development* 118:489–498.
 22. Takakura N, H Yoshida, Y Ogura, H Kataoka, S Nishikawa and S Nishikawa. (1997). PDGFR alpha expression during mouse embryogenesis: immunolocalization analyzed by whole-mount immunohistochemistry using the monoclonal anti-mouse PDGFR alpha antibody APA5. *J Histochem Cytochem* 45:883–893.
 23. Blass-Kampmann S, S Reinhardt-Maelicke, A Kindler-Rohrborn, V Cleaves and MF Rajewsky. (1994). *In vitro* differentiation of E-N-CAM expressing rat neural precursor cells isolated by FACS during prenatal development. *J Neurosci Res* 37:359–373.
 24. Chen EW and AY Chiu. (1992). Early stages in the development of spinal motor neurons. *J Comp Neurol* 320:291–303.
 25. Mayer-Proschel M, AJ Kalyani, T Mujtaba and MS Rao. (1997). Isolation of lineage-restricted neuronal precursors from multipotent neuroepithelial stem cells. *Neuron* 19:773–785.
 26. Nguyen L, JM Rigo, B Malgrange, G Moonen and S Belachew. (2003). Untangling the functional potential of PSA-NCAM-expressing cells in CNS development and brain repair strategies. *Curr Med Chem* 10:2185–2196.
 27. Calver AR, AC Hall, WP Yu, FS Walsh, JK Heath, C Betsholtz and WD Richardson. (1998). Oligodendrocyte population dynamics and the role of PDGF *in vivo*. *Neuron* 20:869–882.
 28. Hiroi Y, S Kudoh, K Monzen, Y Ikeda, Y Yazaki, R Nagai and I Komuro. (2001). Tbx5 associates with Nkx2-5 and synergistically promotes cardiomyocyte differentiation. *Nat Genet* 28:276–280.
 29. Wallace LM, SE Garwick, W Mei, A Belayew, F Coppee, KJ Ladner, D Guttridge, J Yang and SQ Harper. (2011). DUX4, a candidate gene for facioscapulohumeral muscular dystrophy, causes p53-dependent myopathy *in vivo*. *Ann Neurol* 69:540–552.
 30. Jones TI, JC Chen, F Rahimov, S Homma, P Arashiro, ML Beermann, OD King, JB Miller, LM Kunkel, et al. (2012). Facioscapulohumeral muscular dystrophy family studies of DUX4 expression: evidence for disease modifiers and a quantitative model of pathogenesis. *Hum Mol Genet* 21:4419–4430.
 31. Kowalijow V, A Marcowycz, E Ansseau, CB Conde, S Sauvage, C Matteotti, C Arias, ED Corona, NG Nunez, et al. (2007). The DUX4 gene at the FSHD1A locus encodes a proapoptotic protein. *Neuromuscul Disord* 17:611–623.
 32. Ismailoglu I, G Yeaman, GQ Daley, RC Perlingeiro and M Kyba. (2008). Mesodermal patterning activity of SCL. *Exp Hematol* 36:1593–1603.
 33. Bonnefont J, SI Nikolaev, AL Perrier, S Guo, L Cartier, S Sorce, T Laforge, L Aubry, P Khaitovich, et al. (2008). Evolutionary forces shape the human RFPL1,2,3 genes toward a role in neocortex development. *Am J Hum Genet* 83:208–218.
 34. Holmes SE, EE O'Hearn, MG McInnis, DA Gorelick-Feldman, JJ Kleiderlein, C Callahan, NG Kwak, RG Ingersoll-Ashworth, M Sherr, et al. (1999). Expansion of a novel CAG trinucleotide repeat in the 5' region of PPP2R2B is associated with SCA12. *Nat Genet* 23:391–392.

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