ARTICLE

A unique library of myogenic cells from facioscapulohumeral muscular dystrophy subjects and unaffected relatives: family, disease and cell function

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To explore possible mechanisms of pathology in facioscapulohumeral muscular dystrophy (FSHD), we generated a novel library of myogenic cells composed of paired cultures derived from FSHD subjects and unaffected first-degree relatives. We prepared cells from biopsies of both biceps and deltoid muscles obtained from each of 10 FSHD and 9 unaffected donors. We used this new collection to determine how family background and disease affected patterns of growth and differentiation, expression of a panel of candidate, and muscle-specific genes, and responses to exogenous stressors. We found that FSHD and unaffected cells had, on average, indistinguishable patterns of differentiation, gene expression, and dose-response curves to staurosporine, paraquat, hydrogen peroxide, and glutathione depletion. Differentiated FSHD and unaffected cultures were both more sensitive to glutathione depletion than proliferating cultures, but showed similar responses to paraquat, staurosporine, and peroxide. For stress responses, the sample size was sufficient to detect a 10% change in effect at the observed variability with a power of >99%. In contrast, for each of these properties, we found significant differences among cells from different cohorts, and these differences were independent of disease status, gender, or muscle biopsied. Thus, though none of the properties we examined could be used to reliably distinguish between FSHD and unaffected cells, family of origin was an important contributor to gene-expression patterns and stressor responses in cultures of both FSHD and unaffected myogenic cells.

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INTRODUCTION

Facioscapulohumeral muscular dystrophy (FSHD) is a progressive, autosomal dominant disease that usually presents with weakness in the face, shoulder girdle and arms, and has variable age of onset, though often in adolescence and young adulthood.¹ In ~95% of FSHD cases, the disease is genetically linked to an abnormally shortened D4Z4 repeat array near the chromosome 4q telomere. Unaffected individuals have unshortened arrays with >10 D4Z4 repeats, whereas contraction to 1–10 repeats is associated with FSHD.^{2,3} A small percentage of FSHD subjects (FSHD2) show the FSHD muscle weakness pattern but do not have a contracted 4q D4Z4 array.⁴ Recent work has suggested that both types of FSHD are characterized by expression and stabilization of mRNA produced from an open reading frame in the most telomeric D4Z4 repeat that encodes a potentially toxic protein: a long isoform of DUX4.^{3,5,6}

Weakness in FSHD appears to be of myogenic, rather than neurogenic, origin.⁷ Many studies have, therefore, used primary

myogenic cell cultures derived from FSHD subject biopsies to examine potential pathogenic mechanisms and therapeutic strategies.^{8–15} Such studies have suggested several possible FSHD-specific phenotypes, including increased sensitivity to oxidative stress^{14,16} and altered gene expression,^{17–20} as well as expression of the long form of DUX4.^{5,6} These findings have, however, sometimes not been consistent among laboratories. Potential confounding variables include (i) muscles used for biopsy, (ii) genetic backgrounds of unaffected and diseased donors, (iii) extent of disease progression in the donor muscles, (iv) methods used to derive and purify myogenic cells, (v) culture conditions, (vi) number of different individual donors available, or (vii) replicative age of the cells.

To extend the usefulness of muscle cell cultures for FSHD studies, we have now generated a unique library of primary myogenic cell cultures derived from genetically related pairs of FSHD and unaffected donors. We generated primary cultures from both biceps and deltoid muscle biopsies obtained from seven ‘cohorts’ of donors, where each

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cohort was composed of at least one affected individual with genetically and clinically verified FSHD, and at least one unaffected first degree relative with unshortened D4Z4 alleles and normal strength. Both biceps and deltoid biopsies were included in the protocol because typically the biceps is involved early in the disease whereas the deltoid is relatively spared. Other variables such as time of day of biopsy and immediate nutritional intake of volunteers were also controlled within cohorts.

We used this unique muscle cell resource to search for possible differences between FSHD and unaffected cells in patterns of growth and differentiation, expression of a panel of FSHD candidate and muscle-specific genes, and responses to exogenous stressors. Our studies showed that cellular properties and gene expression, when averaged across all cohorts, did not consistently differ between cultures of FSHD and unaffected myogenic cells, whereas family of origin significantly influenced myogenic cell properties independent of disease status.

MATERIALS AND METHODS

The study was approved by The Johns Hopkins School of Medicine Institutional Review Board. Open muscle biopsy was performed on both biceps and deltoid muscles of 10 FSHD affected and 9 unaffected subjects (Table 1). Molecular diagnosis was done by the University of Iowa Diagnostic Laboratories and indicated that each donor with a clinical diagnosis of FSHD also had a contracted 4q D4Z4 region as identified by the presence of an EcoRI/BlnI fragment of <35 kb detected with the p13-E11 probe after pulsed-field gel electrophoresis (Table 1). Additional experimental details are provided in Supplementary Methods.

RESULTS

Myogenic cell cultures from FSHD subjects and unaffected relatives

We collected ~1 g of tissue each from both biceps and deltoid muscles of 19 individual donors in seven 'cohorts,' where each cohort consisted of one or more FSHD subjects and unaffected first degree relatives (Table 1). Donors included ten individuals with FSHD, who had shortened 4q D4Z4 repeat arrays (4–8 repeats) and nine of their relatives, who were unaffected by FSHD and had unshortened 4q D4Z4 arrays. Each of the resulting 38 biopsies was subdivided, and a 300–500 mg portion of each was dissociated and used to generate primary cell cultures (Supplementary Methods). Cultures initially consisted of myogenic and non-myogenic cells in varying proportions as in our previous study.¹⁵ Each culture strain was designated with the number of the cohort (eg, 01), whether the donor was FSHD-affected (A for the first affected donor in the cohort, B for the second, etc) or unaffected (U for the first unaffected donor in the cohort, V for the second, etc), and the muscle (bic=biceps or del=deltoid) from which the biopsy was obtained. For example, 01Abic cells were obtained from the biceps of the first FSHD-affected donor in cohort 01.

To prepare cultures with consistently high proportions of myogenic cells for the studies described below, we purified myogenic cells on the basis of CD56 expression. As in our previous study, we used expression of CD56 to identify myogenic cells because (i) purified CD56-positive, but not CD56-negative, cell populations expressed the skeletal muscle marker desmin and fused to form multinucleate myotubes; and (ii) CD56-positive and -negative populations were stable as cells did not switch between CD56-positive and -negative

Table 1 Clinical characteristics of FSHD subjects and unaffected donors

Donor ^a	Familial relations	Gender	Age at enrollment (years)	EcoRI/BlnI fragment sizes ^b (kb)	Deltoid strength ^c	Biceps strength
01A	Proband	M	42	18 , >48	4+/5	4+/5
01U	Brother of 01A	M	46	>48, >48	5/5	5/5
03A	Proband	F	40	20 , 57	5/5	4+/5
03U	Sister of 03A	F	42	80, 157	5/5	5/5
09A	Proband	F	31	25 , >112	5/5	4+/5
09U	Mother of 09A	F	57	47, >112	5/5	5/5
12A	Daughter of 12B	F	22	18 , 63	4+/5	4+/5
12B ^d	Proband	M	49	18 , >112	4+/5	4+/5
12U	Daughter of 12B	F	24	>112, >112	5/5	5/5
12V ^d	Sister of 12B	F	45	>112, >112	5/5	5/5
14A	Proband	M	50	19 , >60	5/5	4/5
14B	Brother of 14A	M	53	19 , >60	4+/5	4+/5
14V	Sister of 14A	F	49	60, 112	5/5	5/5
14W	Brother of 14A	M	47	72 ^e	5/5	5/5
15A	Proband	M	67	28 , >112	5/5	4+/5
15B	Brother of 15A	M	69	28 , >112	5/5	5/5
15V	Sister of 15A	F	60	107, >112	5/5	5/5
16A	Proband	F	56	20 , 97	5-/5	4+/5
16U	Sister of 16A	F	60	59, 93, 97 ^e	5/5	5/5

^aDonors are designated by cohort (family) number (01, 03, etc.) followed by A or B for the FSHD subjects or U, V, or W for the unaffected first degree relative(s).

^bFSHD was confirmed by presence of both clinically apparent muscle weakness and a shortened 4q D4Z4 repeat array identified by an EcoRI/BlnI restriction fragment of <35 kb coupled with a 4qA telomere allele (Supplementary Methods). Shortened repeat arrays are shown in bold.

^cMuscle strength is presented using a modified MRC scale where 5/5 is full strength.

^dSubjects 12B and 12V were originally named 11A and 11U for internal analyses, but were renamed for publication to reflect familial relationship.

^eSee Supplementary Methods for discussion of 14W and 16U genotypes.

phenotypes.¹⁵ All experiments described here were carried out with cultures established with purified CD56-positive cells. As an example, in one series of experiments with cohorts 03, 09, 14, 15, and 16, the average $\pm$ SE percentage of desmin-positive cells in proliferating cultures was 89.3 ± 2.9 ($n=12$) for FSHD cells and 90.2 ± 2.9 ($n=14$) for unaffected cells, indicating both high purity and no difference in purity between FSHD and unaffected cultures. In all cases, cultures were examined at 20–35 population doublings after the initial isolation.

Cultures of FSHD and unaffected cells did not show consistent differences in patterns of proliferation or differentiation, including myotube formation. In one set of experiments, for example, we grew FSHD and unaffected biceps and deltoid cells from five cohorts (01, 03, 09, 15, and 16), and quantified the percentage of nuclei within myotubes (fusion index). After 7 days of differentiation, for example, the average fusion indices $\pm$ SE for FSHD ($59 \pm 3.9\%$, $n=12$) and unaffected ($64 \pm 3.2\%$, $n=12$) cultures were not significantly different ($P=0.33$, *t*-test) (Supplementary figure 1). There was also no difference in the average density of nuclei in the FSHD (435 ± 66 nuclei/mm², $n=10$) and unaffected ($484 \pm$ nuclei/mm², $n=10$) cultures ($P=0.49$, *t*-test). After 2 and 4 days of differentiation, there was also no difference in average fusion between FSHD and unaffected cultures (data not shown). Also, though myotubes showed a wide range of morphologies in different cultures, FSHD and unaffected cultures had similar variability in myotube morphology (data not shown).

All types of myogenic cells within a single cohort (ie, FSHD and unaffected, biceps, and deltoid) tended to have similar fusion indices, but average fusion indices in some cases differed significantly between cohorts (Supplementary Figure 1). For example, the average fusion index $\pm$ SE was $44 \pm 6.0\%$ ($n=4$) for cells from cohort 03, but was $70 \pm 6.5\%$ ($n=4$) for cells from cohort 09 ($P<0.05$). In contrast, nuclear densities for cohort 03 and cohort 09 cultures did not differ significantly. There was also no correlation of fusion percentage with percentage of desmin-positive cells ($R=0.11$). Furthermore, ANOVA showed that there was no significant difference in fusion index based on disease state, muscle biopsied, or an interaction between these ($P>0.3$), whereas there was a significant difference based on cohort ($P=0.009$). These results indicate that differences in the extent of myotube formation were correlated with family of origin, but not with disease status.

Gene expression in cultures of FSHD and unaffected control cells

We next used nanoliter qPCR to analyze mRNAs collected from myogenic cell cultures: (i) after 2 days in growth medium when myoblasts are proliferating at low density (GM2); (ii) at the onset of differentiation when the cultures reached ~ 90 –95% confluence and the growth medium was replaced with differentiation medium (DM0); and (iii) after 2, 4, and 7 days in differentiation medium as myotube formation occurred (DM2, DM4, and DM7). With the qPCR nanoliter platform, we determined a cycle threshold (Ct) value²¹ for each of 75 genes (listed in Supplementary Methods) that were in three groups. The first group included 11 genes that were expected to be expressed at approximately constant levels for use as potential controls (normalization gene set). The second group included 44 genes that were known to be either regulated upon differentiation or to be involved in cell growth and survival (growth and differentiation gene set). The third group included 20 genes that had been identified in previous studies as potentially associated with FSHD (candidate gene set).^{12,22} Because our data sets were obtained before the paper of Lemmers *et al*⁵ was published, we did not include analyses of the DUX4 short- and full-length isoforms in the studies

described here. As described below, we found that all myogenic cell cultures, whether derived from FSHD or unaffected donors, expressed similar patterns and average amounts of the different mRNAs that we examined (Figure 2). Indeed, when we compared the average Ct values for each gene at each stage of differentiation, we found that FSHD and unaffected cell cultures had highly similar average expression patterns with a linear correlation coefficient ($R=0.994$, $n=375$).

All 11 of the normalization genes showed little change in expression, with typical variability of ± 0.5 Ct at different stages of culture (not shown). In addition, several genes, not in our original normalization set, also proved to be expressed at nearly constant levels under all culture conditions. Examples included the transcription factor GLI3, the p53 regulator MDM2, p53 itself (TP53), and beta-2 microglobulin (B2M) (Figure 1). There was no significant difference in the expression of any normalization gene between FSHD and unaffected, or between female and male myogenic cell cultures.

For the growth and differentiation gene set, we also found that FSHD and unaffected cultures had similar average levels of expression and differentiation-induced changes in gene expression (Figures 1a and b). As differentiation progressed, for example, the myoblast-specific transcription factor MYF5 was downregulated in all cultures, whereas myotube-specific genes, including muscle creatine kinase and multiple myosin heavy chain isoforms were upregulated (Figure 1b). Changes in muscle-specific gene expression followed similar kinetics and showed similar quantitative changes in FSHD and unaffected cultures. Thus, for each of these genes, we found no consistent, overall differences in mRNA expression patterns or levels during differentiation, when comparing FSHD *versus* unaffected myogenic cell cultures.

For the candidate gene set, we again found no consistent differences in mRNA expression levels between FSHD and unaffected cell cultures (Figure 1c and not shown). Among these genes were the mitochondrial translocator ANT1, the thyroid hormone binding protein mu-crystallin (CRYM), and two genes located just centromeric to the chromosome 4q D4Z4 array, FRG1 and FRG2_chr4. Transcripts produced from the FRG2 coding sequences on chromosomes 3 and 10 were also expressed at similar levels in FSHD and unaffected cultures.

To further analyze the qPCR data sets, we carried out comparative marker selection and hierarchical clustering analyses to test for differences in gene expression not only for FSHD *versus* unaffected cells, but also male *versus* female and biceps *versus* deltoid. By comparative marker selection,²³ none of the genes that we analyzed had expression patterns that consistently differed between FSHD *versus* unaffected, male *versus* female, biceps *versus* deltoid, or donors of different ages (not shown). Furthermore, hierarchical clustering did not place FSHD *versus* unaffected, male *versus* female, or biopsy *versus* deltoid into two separate clusters, but rather produced mixed clusters (Figure 2a). In contrast, there was evidence of clustering by cohort, for example, all cultures (FSHD, unaffected, biceps, and deltoid) from cohort 03 were placed in a single cluster, as were all cultures from cohort 09 (Figure 2a). Cohorts 01, 12, and 15 showed partial clustering with a majority (3 of 4, or 4 of 6) of each cohort's cultures placed in a single cluster.

Examination of gene expression differences among individual cultures also provided evidence that cohort of origin, but not disease status, was for some genes a factor in determining expression level. Examples of genes that were differentially expressed by cohort irrespective of disease status included: (i) mu-crystallin (CRYM), which, in differentiating cultures, was more highly expressed by cells from cohort 01 than cohort 15, and (ii) NCAM1, which, in both proliferating and differentiating cultures, was more highly expressed

Figure 1 FSHD and unaffected cultures had similar patterns of gene expression as determined by nanoliter qPCR. Expression is reported as Ct value, which is inversely related to expression level (lower Ct=higher expression). Ct values at five stages of culture are shown for each gene; from left to right, two stages of proliferation (2d in growth medium and the day of switch to differentiation medium) and three stages of differentiation (2d, 4d, and 7d). Results for FSHD (red squares, dotted lines) and unaffected (green circles, solid lines) cells were without significant differences. A total of 75 genes were assayed (Supplementary Methods) of which panels **a** and **b** show results for 24 of the 44 growth and differentiation genes and panel **c** shows the results for 12 normalization or candidate genes. Results for the genes not shown here also did not differ between FSHD and unaffected. Error bars=SE.

by cells of cohort 09 than cohort 03 (Figure 2b). On the other hand, MDM2 was an example of a gene that was expressed at approximately the same level by all cultures in all cohorts, including cohorts 01, 03, 09, and 12 (Figure 2b). Because of the different expression levels in different cohorts, we also examined the possibility that FSHD and unaffected cells within an individual cohort might show consistently higher or lower expression of one or more genes. However, as shown by the examples of CRYM and NCAM1 (Figure 2b), as well as analyses of the other genes we examined, calculation of such paired differences did not identify consistent differences between FSHD and unaffected cells, even within individual cohorts.

Figure 2 Gene expression did not differ between FSHD and unaffected cultures, but was correlated with cohort of origin. **(a)** Hierarchical clustering of qPCR data was used to examine relatedness of growth and differentiation gene expression among cell cultures from different individual donors and biopsies. Cells are designated by cohort (01, 03, etc); whether FSHD (A and B) or unaffected (U, V, W), whether biceps (bic) or deltoid (del); and gender (F or M). Four plausible clusters are boxed and designated 1–4. FSHD versus unaffected, male versus female, or biceps versus deltoid were not placed into two distinct clusters. In contrast, there was evidence of clustering by cohort, for example, all cohort 03 cultures were in cluster 1 and all cohort 09 cultures were in cluster 4. **(b)** Expression patterns differed between cohorts. Irrespective of whether cells were FSHD (dotted lines) or unaffected (solid lines), mu-crystallin (CRYM, top panel) was more highly expressed by cohort 01 (blue) than by cohort 12 (gold) cells in differentiating cultures (DM2, DM4, and DM7), and NCAM1 (bottom panel) was more highly expressed by cohort 09 (orange) than by cohort 03 (brown) cells in both proliferating (GM2 and DM0) and differentiating cultures. In contrast, MDM2 (middle panel) was expressed similarly by all cultures of cohorts 01, 03, 09, and 12. Error bars=SD, $n=4$.

Further analyses of the qPCR data sets showed that some genes appeared to be more tightly regulated than others. When the average Ct value $\pm$ SE was calculated for each gene, we found that $\sim 20\%$ of the genes had a low SE of <0.2 Ct unit, whereas another $\sim 20\%$ had a high SE of >1.0 . As expected, SE tended to increase with increasing Ct, but genes with higher SE were found at all levels of expression (not shown). The average SE for all genes was 0.41 ± 0.011 Ct unit ($n=660$) with a range of 0.033–1.57. Genes with high variability included CRYM and NCAM1 (Figure 2b), as well as LAMA1 and PAX7 (Figure 1). Less variable genes included MDM2 (Figure 2b), as well as FRG1, LAMA2, and TP53 (Figure 1a and b). The variability of gene expression in FSHD cultures was similar to that in unaffected cultures, as measured by the size and distribution of SE values for individual genes, cultures, or cohorts (data not shown).

The qPCR analyses were highly reproducible from laboratory to laboratory and over time. In one set of experiments, for example, we examined three sets of 01Abic cultures that were grown several months apart in two different laboratories and analyzed by independent RNA/cDNA preparation and qPCR arrays. We found that the three replicates had very similar quantitative and temporal patterns of

gene expression during proliferation and differentiation (not shown). Across the three independent experiments, the SE for individual genes ranged from 0.04–1.85 Ct units and the average SE for all genes was 0.61 Ct units. These values were similar to the SE range of 0.033–1.57 for individual genes and average SE=0.41 for the experiments with all cohorts described above. Linear correlation coefficients among the three independent replicates were $R=0.94$ – 0.96 . We have found similar reproducibility in additional independent replicates of genes from cultures of multiple cohorts (data not shown).

Stressor responses in cultures of FSHD and unaffected control cells

We next examined whether FSHD myogenic cells were more sensitive than unaffected cells to exogenous stresses, a possibility suggested by previous studies.^{14,16} In particular, we examined the effects on cell viability of four types of stressors: (i) buthionine sulfoximine (BSO), which decreases intracellular glutathione by inhibition of gamma-glutamylcysteine synthetase;²⁴ (ii) staurosporine, a kinase inhibitor that induces apoptotic cell death;²⁵ (iii) paraquat, a superoxide generator; and (iv) hydrogen peroxide. Each stressor was tested at a range of doses and times as used in previous studies^{14,16,24,25} for the effect on cell viability (Supplementary Methods).

FSHD and unaffected cultures had the same average dose-response curves (when averaged across cells from all cohorts) for each of the four stressors (Figure 3). The overall linear correlation coefficient was $R=0.975$ ($n=60$) for comparison at each experimental condition of average FSHD *versus* average unaffected response. Each stressor produced a significant decrease in viability at higher concentrations, but the effect on viability did not differ between FSHD and unaffected cells. In addition, dose-response curves for FSHD and unaffected cells were not significantly different in either proliferating (myoblast) or differentiating (myotube-forming) cultures (Figure 3).

Though FSHD and unaffected cells had similar average responses to glutathione depletion by BSO, we unexpectedly found that differentiated cultures of both FSHD and unaffected myogenic cells were more sensitive than proliferating cultures to BSO (Supplementary Figure 2). After treatment for 24 h (Figure 3) or 48 h (Supplementary Figure 2) with 0.001 mM and higher concentrations of BSO, differentiating cultures had consistently fewer cells remaining than proliferating cultures, with, typically, about two out of three as many differentiating cells surviving. In contrast, for staurosporine, paraquat, and hydrogen peroxide, we found that both proliferating and differentiating cultures

of FSHD and unaffected cells had the same dose-response curves (Figure 3 and data not shown). Thus, the increased sensitivity of differentiating FSHD and unaffected cells was specific to BSO, and did not indicate a general susceptibility of myotubes to all stressors.

As with myotube fusion and gene expression levels, we found evidence that family of origin, though not disease status, had a role in determining responses to stressors. For example, the average responses of differentiating FSHD and unaffected cultures (when averaged across all cultures from all cohorts) to 1 μ M staurosporine for 24 h did not differ (Figure 4). Nonetheless, cultures established with cells from different cohorts did show significantly different responses to staurosporine. For example, all cultures from cohort 09 (ie, FSHD and unaffected from both biceps and deltoid) were significantly less sensitive to staurosporine-induced death

Figure 4 Sensitivity to staurosporine was correlated with cohort of origin, but not with disease status. After 5 days in differentiation medium, cultures were treated with 1 μ M staurosporine and the percentage of cells surviving 24 h of treatment was determined. When averaged across cells from all cohorts, FSHD (red bar) and unaffected (green bar) cultures had the same average sensitivity to staurosporine. In contrast, cells from three different cohorts (03, 09, and 16), including all FSHD (Abic, and Adel) and unaffected (Ubic, and Udel) cells, showed different sensitivities (gray bars). Error bars=SE; $n=14$ for FSHD average, 12 for unaffected average, and 4 for individual cell strains. * $P<0.05$ (for 01Ubic *versus* 09Adel and 09Udel), ** $P<0.01$ for all other comparisons between individual cultures from different cohorts (eg, 03 Abic *versus* 09Abic or 03 Adel *versus* 16Udel).

Figure 3 FSHD and unaffected myogenic cells had similar responses to stressors. Parallel cultures of CD56-positive cells were established, and cultures in growth medium (GM) or differentiation medium (DM) were incubated for 12, 24, or 48 h as noted with increasing doses (indicated by the right triangles) of stressors (see Supplementary Methods for details). Each point is the average for either all FSHD cultures (red squares, dotted lines, $n=14$ – 19) or all unaffected cultures (green circles, solid lines, $n=12$ – 18) that were tested. For each of the four stressors there was no significant difference between the average responses of the FSHD cultures and the corresponding unaffected cultures. Error bars=SE.

and also significantly less sensitive than all cultures from cohorts 03 and 16 (Figure 4).

As an additional method to assess the influence of cohort on stress responses, we generated linear correlation coefficients for the stress responses of all possible intra-cohort (eg, 01Abic *versus* 01Udel) and inter-cohort (eg, 01Abic *versus* 16Udel) pairs. From these data, we calculated the average intra-cohort correlation for each individual cohort, for example, for cohort 09 the average $\pm$ SE correlation for all comparisons within the cohort was $R=0.86 \pm 0.019$ ($n=6$). Similarly, we calculated the average inter-cohort correlation for each pair of different cohorts, for example, the average correlation between cohort 09 and cohort 16 stress responses was 0.57 ± 0.026 ($n=16$). In this case, a comparison of the average intra-cohort 09 correlation to the average inter-cohort 09 *versus* 16 correlation detected a significant difference ($P<0.01$). The results of all such intra- *versus* inter-cohort comparisons are shown in Supplementary Figure 3. In 42 comparisons, we found that the intra-cohort correlation was significantly higher than the inter-cohort correlation 23 times ($P<0.001$ compared with a 5% expectation of significance). In one case, the inter-cohort correlation was higher than the intra-cohort (intra-cohort 14 *versus* cohort 15), and 19 comparisons showed no significant difference. The average intra-cohort correlation ($R=0.64 \pm 0.052$; $n=7$; range 0.43–0.86) was significantly greater than the average inter-cohort correlation ($R=0.52 \pm 0.021$; $n=21$; range 0.32–0.70) ($P<0.02$, *t*-test). In contrast, we did not find significant differences in average correlation coefficients for FSHD *versus* unaffected, male *versus* female, or biceps *versus* deltoid cultures. Thus, for responses to stressors, cells within a cohort were more often similar to each other than to cells from other cohorts.

DISCUSSION

The new library of myogenic cells that we produced and analyzed here is unique in that cells were derived from biopsies of genetically-related pairs of FSHD subjects and their first degree relatives, as well as from two muscles (biceps and deltoid) in which pathology typically progresses at different rates. Our analyses of seven cohorts of these genetically-paired myogenic cell cultures showed that myogenic cells from FSHD and unaffected donors, when averaged across all cohorts, had indistinguishable patterns of myotube formation, gene expression, and responses to stressors. We also found, however, that cellular properties were often significantly correlated with the family of origin, irrespective of disease status, gender, donor age, or biopsied muscle. Our strategy thus allowed us to demonstrate that family background can be an important variable when comparing diseased and unaffected cells.

Recent studies have led to a proposed pathogenic mechanism for FSHD in which expression and stabilization of mRNA produced from an open reading frame in the most telomeric D4Z4 repeat leads to expression of a potentially toxic, long isoform of DUX4.^{3,5,6} Furthermore, expression of this long form of DUX4 is detected in only a small percentage ($\sim 0.1\%$) of nuclei in cultures of FSHD subject-derived myogenic cells.⁶ Data acquisition for our study was completed before publication of this disease model, so we did not include analyses of the long form of DUX4 here. In our study, we found that expression of a panel of genes, myotube formation, and stressor responses were, on average, indistinguishable between FSHD and unaffected cell cultures. Our results are not inconsistent, however, with the possibility that the cultures contained a small fraction of cells with DUX4-induced pathology, as we would not have expected pathology in only 0.1% of the cells to produce detectable, significant changes in the overall properties of the FSHD cultures compared with the unaffected cultures. It remains to be determined how the cells that express DUX4

from the endogenous promoter in culture might differ from the large majority of DUX4-negative cells.

We did not find significant differences between the FSHD and unaffected cells, when averaged across all cohorts. With the variability and number of cultures that we examined, it was possible to detect significant differences of $\sim 10\%$ in average stress responses (*cf.* Supplementary Figure 2). For gene expression, the difference required to reach significance depended on how variable expression was for the specific gene, as some genes showed low variability ($SE< \pm 0.2$ Ct unit), whereas others were more variable ($SE> 1.0$ Ct unit). Furthermore, none of the specific cell properties or gene expression patterns that we examined proved to be a biomarker that could reliably distinguish FSHD from unaffected myogenic cells, even within a single cohort. For every property examined, FSHD and unaffected cultures had overlapping ranges, so that there was no property for which all FSHD cultures differed from all unaffected cultures.

Myogenic cells derived from FSHD subject biopsies have been analyzed in multiple studies.^{6,8–15} Some of these studies have suggested possible FSHD-specific phenotypes, including increased sensitivity to oxidative stress,^{14,16} altered myotube morphology,¹⁴ and altered expression of FRG1¹⁸ or FRG2.¹² We found that proliferating and differentiating cultures of both FSHD and unaffected cells expressed similar patterns of FRG2 mRNA, and a recent study also found FRG2 mRNA in unaffected myoblasts and myotubes.²⁷ In contrast, previous studies have reported that FRG2 mRNA in differentiated cultures of FSHD, but not unaffected cells, and not at all in proliferating cultures.^{12,26} The reasons behind these discrepancies remain to be determined, but the very low level of FRG2 expression and different qPCR assays might contribute. Also unlike previous studies,^{14,16} we found no difference between FSHD and unaffected cells in responses to paraquat or hydrogen peroxide. Our results, however, are consistent with previous work¹² which found no significant differences between FSHD and unaffected myogenic cells in expression of FRG1 or other 4q genes such as PDLIM3. Our strategy also allowed us to show that family of origin was a significant contributor to determining cellular properties. The differences among families that we found were often of similar magnitudes to differences that have been reported between FSHD and unaffected cells. In studies of cells from a small number of individuals with unknown genetic relationships, it is possible that differences detected between FSHD and unaffected cells could inadvertently arise because of different family backgrounds of the diseased and unaffected cells, rather than to disease.

As with all studies of myogenic cell cultures, our study has limitations. In culture, myogenic cells have limited contractility and lack the interactions with other cell types – the neurons, connective tissues, and blood vessels – that are found in the skeletal muscle. If a disease mechanism were activated only upon extensive contraction or by interactions with other cell types, then it would not be activated in myogenic cell cultures. Myogenic cell cultures also cannot uncover any disease mechanism that may occur in a non-muscle cell type. Also, differences between FSHD and unaffected cells might only appear under particular culture conditions that are different from those we used here. Whether family of origin, but not disease, influences gene expression in the muscle tissue as in cultured cells remains to be determined. Finally, the FSHD subjects in this study had 4–8 D4Z4 repeats and generally mild clinical impairment of muscle function, and it remains to be determined if cells from subjects with 1–3 D4Z4 repeats or more severe muscle weakness show different properties.

Despite these limitations, we can conclude from our results that, within narrow effect detection ranges, none of the properties we examined can be reliably used to distinguish between FSHD and unaffected myogenic cell cultures under our experimental conditions. Moreover, our results also demonstrate that family of origin can be an important contributor to gene expression patterns and stressor responses in cultures of both FSHD and unaffected myogenic cells. Our finding of the significance of family of origin may prove to be important when designing studies of FSHD, as well as other diseases, in which cultures of diseased and unaffected cells are to be compared.

CONFLICT OF INTEREST

The authors declare no conflict of interest.

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