DUX4 and DUX4 downstream target genes are expressed in fetal FSHD muscles

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Facioscapulohumeral muscular dystrophy (FSHD) is one of the most prevalent adult muscular dystrophies. The common clinical signs usually appear during the second decade of life but when the first molecular dysregulations occur is still unknown. Our aim was to determine whether molecular dysregulations can be identified during FSHD fetal muscle development. We compared muscle biopsies derived from FSHD1 fetuses and the cells derived from some of these biopsies with biopsies and cells derived from control fetuses. We mainly focus on DUX4 isoform expression because the expression of DUX4 has been confirmed in both FSHD cells and biopsies by several laboratories. We measured DUX4 isoform expression by using qRT-PCR in fetal FSHD1 myotubes treated or not with an shRNA directed against DUX4 mRNA. We also analyzed DUX4 downstream target gene expression in myotubes and fetal or adult FSHD1 and control quadriceps biopsies. We show that both DUX4-FL isoforms are already expressed in FSHD1 myotubes. Interestingly, DUX4-FL expression level is much lower in trapezius than in quadriceps myotubes, which is confirmed by the level of expression of DUX4 downstream genes. We observed that *TRIM43* and *MBD3L2* are already overexpressed in FSHD1 fetal quadriceps biopsies, at similar levels to those observed in adult FSHD1 quadriceps biopsies. These results indicate that molecular markers of the disease are already expressed during fetal life, thus opening a new field of investigation for mechanisms leading to FSHD.

INTRODUCTION

Facioscapulohumeral muscular dystrophy (FSHD) is one of the most frequent muscular dystrophies with an incidence of 4/100 000 (www.orpha.net, June 2013). The pathology often

begins during late adolescence with an asymmetric atrophy of muscles located in the face, the shoulder and in the arms. A high inter-individual variability is observed with very variable phenotypes being reported for the same genotype (1,2). In general, the pathology progresses slowly. The genetic cause of

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the disease was proposed 20 years ago (3): FSHD is classically associated with the contraction of a macrosatellite repeat array in the sub-telomeric region of chromosome 4q35 (for review see (4)). This region normally consists of 11–100 tandem repeats of a 3.3 kb D4Z4 unit, and deletions leaving only 1 to 10 D4Z4 repeats have been associated with FSHD1 patients. In FSHD2 patients, the contraction of the D4Z4 array is not observed although the clinical features are identical to those observed in FSHD1 patients (5).

D4Z4 repeats are not restricted to chromosome 4, but FSHD1 has only been associated with D4Z4 contractions occurring on chromosome 4. Moreover, a complex genomic context (for review see (6)) is required for FSHD onset. Two allelic variants of chromosome 4q (4qA and 4qB) exist in the region distal to D4Z4 and FSHD seems to be almost always associated with 4qA (7–11), which contains a polyadenylation signal for transcripts encoded from the D4Z4 unit (12). Each D4Z4 region contains one open reading frame encoding the double homobox gene *DUX4* (13), but owing to the presence of the polyadenylation signal located immediately after the D4Z4 repeats, only the last D4Z4 unit is able to generate a stable mRNA (12,14). In most of the cases, full-length DUX4 is expressed in FSHD patients whereas it is not expressed in control individuals (15–17). The expression of DUX4, always associated with the 4qA variant, has introduced the notion of a permissive chromosome for FSHD (12). Combined with the fact that at least one D4Z4 unit is required to develop FSHD (18), it has been proposed that DUX4 may play a role in the development of the pathology, although its role is still unclear. The pre-messenger RNA can generate at least three different mRNAs varying in size (15). The shortest one (DUX4-S) can be found in both control and FSHD biopsies as well as in cell culture, whereas the largest one (DUX4-FL) is mainly found in FSHD individuals (16,17). The two DUX4-FL isoforms differ by the retention or not of the first intron located in the 3′UTR. Overexpression of DUX4-FL protein appears to be highly toxic (19–22), and its expression has been proposed to generate a disorganization of the nuclear envelope (23), atrophic myotubes (20), an activation of the PITX1 gene (14), but also of germline genes, retroelements and immune mediators (24), and may cause a p53-dependent myopathy *in vivo* (25). Interestingly, DUX4 expression has also been described in FSHD2 patients (16) and these patients also carry the 4qA haplotype (12).

In FSHD1, D4Z4 contraction has been associated with more relax chromatin structure leading to the expression of DUX4. FSHD2 patients do not have D4Z4 contraction but do also reveal the more relax chromatin structure and subsequent DUX4 expression. Recently, Lemmers *et al.* identified the causal determinant for most FSHD2 patients and have clearly demonstrated that mutations in the epigenetic modifier SCHMD1 gene are a causal determinant for most FSHD2 patients by derepressing DUX4 transcription (26). DUX4 mRNA expression is so far the only molecular determinant in common between FSHD1 and FSHD2 patients, which strongly suggests its direct implication in the FSHD phenotype.

Despite the fact that DUX4-FL is always expressed in FSHD myotubes, the consequence of this expression on muscle function is not yet clearly understood and the link between the DUX4 expression and the development of FSHD is not well established.

We had access to quadriceps and trapezius muscle biopsies isolated from a 14-week FSHD1 fetus from which we established primary muscle cultures and investigated whether some molecular hallmarks of the pathology were already present in fetal biopsies and muscle cultures derived from these biopsies. We observed that DUX4-FL is expressed in the myogenic cells derived from the two FSHD1 biopsies and interestingly, myotubes derived from FSHD1 quadriceps express four times more DUX4-FL than myotubes derived from FSHD1 trapezius. Moreover, some genes downstream of DUX4-FL were specifically activated in FSHD1 biopsies and not in controls, thus showing that a molecular signature potentially leading to FSHD1 phenotype is already present in the developing fetal muscle. Finally, we observed that TRIM43 and MBD3L2, which are already overexpressed in FSHD1 fetal quadriceps muscle biopsies, are also expressed in adult quadriceps biopsies at similar levels to those observed in FSHD1 fetal quadriceps muscle biopsies, thus opening a new field of investigation concerning the molecular mechanisms leading to FSHD.

RESULTS

DUX4-FL is expressed in cells isolated from fetal FSHD1 muscles

As DUX4 has been described to be expressed in differentiated cells, we used myotubes at day 5 of differentiation and compared DUX4 mRNA expression in myoblasts derived from either fetal FSHD1 trapezius (FTrap) or quadriceps (FQuad) and quadriceps control cells (MCQ). Three primer sets were used (Fig. 1A): DUX4-all primers are able to amplify all the DUX4 mRNA isoforms described previously (16), DUX4-FL primers can only amplify the DUX4 long isoform where intron 1 is retained (Fig. 1B) and DUX4-UTR primers are flanking the two introns of DUX4 and can amplify both the DUX4-FL isoforms. When DUX4-all primers were used, we observed that the total expression of the DUX4 isoform mRNAs was 5-fold lower in FTrap as compared with FQuad myotubes (Fig. 1C). In the control myotubes (MCQ), DUX4 was also observed but its expression was 10-fold less than that in FQuad. When specific primers to DUX4-FL2 were used, we observed that the expression of DUX4-FL2 was 3-fold lower in the FTrap than in the FQuad (Fig. 1C) and DUX4-FL2 was never detected in control myotubes (Fig. 1C and data not shown, performed on three other control samples). Finally no amplification was observed in the control cells with the DUX4-UTR primers whereas the two DUX4-FL isoforms were observed in the FSHD fetal cells (Fig. 1D).

These results show that both the DUX4-FL isoforms are already expressed in muscle cells isolated from FSHD1 fetal biopsies. This result was confirmed at the protein level: an immunostaining against DUX4 was carried out on FQuad and revealed that 8.89% of the nuclei within myotubes were DUX4 positive (Fig. 1E).

FSHD fetal quadriceps and trapezius muscles have different differentiation kinetics during development

In order to determine whether this differential DUX4 mRNA expression could be owing to a difference in myotube formation,
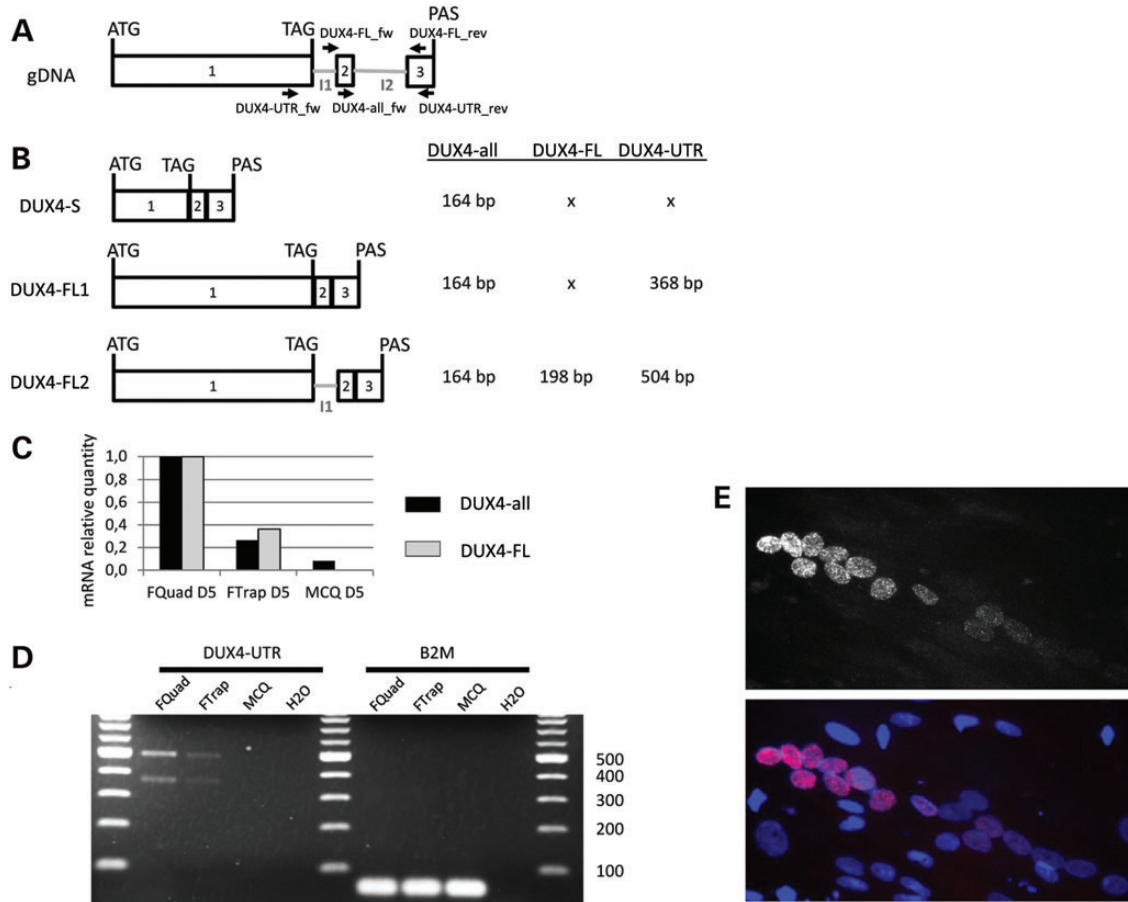
Figure 1. The expression of DUX4-FL mRNA in FSHD and control fetal myotubes. (**A**) Schematic representation of the DUX4 gene and the resulting DUX4 mRNA expression in muscle. The two primer locations that have been used are represented. (**B**) Representation of the DUX4 mRNA isoforms previously described (16). The primers for DUX4-all are able to amplify all of the DUX4 isoforms whereas the primers for DUX4-FL cannot amplify DUX4-s or DUX4-FL1. The PCR product sizes are indicated. (**C**) DUX4 relative expression in fetal-derived FSHD1 (FQuad and FTrap) and control (MCQ) myotubes after 5 days of differentiation. A RT-qPCR (taqman assay) was carried out on 1 μg of total RNA using either DUX4-all (black bars) or DUX4-FL (gray bars) primer sets. The results obtained with FQuad were used as a reference (and arbitrarily put at 1) and were normalized using B2M. (**D**) The amplification products using the DUX4-UTR or B2M primers were separated on a 2% agarose gel. The expected sizes of the PCR product were 368 bp when both introns 1 and 2 are spliced and 504 bp when intron 1 is retained. The molecular weight is a 100 bp ladder. The exons of DUX4 are indicated as gray boxes numbered 1 to 3. I: Intron; gDNA: genomic DNA. (**E**) Immunostaining against DUX4 was realized on FQuad 5 days after the induction of differentiation. On the upper panel, DUX4 was labeled with the E5.5 antibody (dilution 1/10). In the lower panel, nuclei have been counterstained with Dapi. The percentage of DUX4-positive nuclei represents 8.89% of the total number of nuclei within myotubes.

differentiation kinetics were analyzed. Cells were harvested at different time points after differentiation and DUX4-all or DUX4-FL2 mRNA were assessed by qPCR. For FQuad, DUX4-all and DUX4-FL2 mRNA expression increased during differentiation until day 4 and then decreased (Fig. 2A). For FTrap, the expression pattern of DUX4-all mRNA was delayed, with an increase until day 5–7 when a plateau was observed (Fig. 2B). As previously observed, the expression levels of DUX4-all and DUX4-FL were much higher (4–5-fold) in FQuad as compared with FTrap independently of the differentiation time point.

As we observed a delay in the expression of DUX4 mRNA in FTrap as compared with FQuad, we investigated whether the kinetics of differentiation/fusion were similar in both the cultures. The expression of Myogenin (MyoG), a transcriptional activator of the myogenic program that permits the transition from proliferating myoblasts to differentiating myotubes (for review see (27)), was compared between FQuad and FTrap

differentiated cultures. Interestingly, differences were observed: in FQuad cultures, the peak of expression of MyoG mRNA was observed at D1 of differentiation and then its expression decreased (Fig. 3A). In FTrap cultures, the MyoG peak was observed at D4, suggesting a delay in the activation of the genes required for differentiation and thus of the whole differentiation program. This delay in differentiation was confirmed using the inclusion of BIN1 exon11 in BIN1 mRNA. Inclusion of the exon 11 is specific of muscle differentiation, and this generates an isoform of BIN1 implicated in tubular invaginations of the membrane (28). Exon 11 inclusion was clearly observed at D1 for FQuad, whereas it was included only from D4 in FTrap (Fig. 3B and C). These results strongly suggest that there is a delay in the activation of the myogenic program in FTrap as compared with FQuad.

We next investigated whether the differences we observed between FQuad and FTrap are related to the pathology or to an intrinsic difference between the different muscle groups
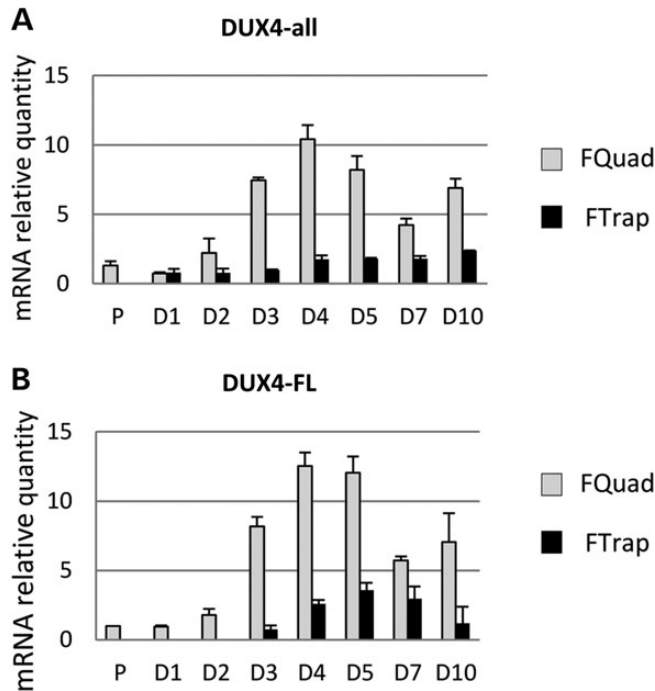
Figure 2. The expression of DUX4 mRNA in FTrap and FQuad during myotubes formation. Differentiation kinetics was performed on FQuad and FTrap cultures. Cells were harvested at different time points. Total RNA was extracted using trizol, and RT-qPCRs using either Dux4-all (**A**) or DUX4-FL (**B**) primers were performed. The results obtained with FQuad in proliferation were used as a reference and were normalized to B2M. The experiments were repeated in duplicate. P: proliferation; D: day after the induction of differentiation. Error bars represent SD of PCR triplicate.

(quadriceps versus trapezius). The same experiments were performed with cells isolated from fetal control biopsies (i.e. without any neuromuscular disease). We searched for DUX4 mRNA expression during the kinetics of differentiation by qPCR but never observed a consistent expression of DUX4-FL mRNA although sporadically amplification could be detected, thus confirming previous reports (17,29). As for the FSHD cultures, we also observed in four control cultures a delay in MyoG mRNA expression (Supplementary Material, Fig. S1), thus confirming that the delay we had initially observed in the FSHD1 fetal trapezius muscle is owing to an intrinsic difference in muscle maturation between trapezius and quadriceps rather than being directly linked to FSHD.

Genes downstream of DUX4-FL are activated in myotubes derived from FTrap and FQuad

DUX4-FL overexpression *in vitro* potentially induces the expression of several genes (14,24). As DUX4-FL mRNA expression levels are different in FTrap and FQuad myotubes, we investigated the expression of these DUX4-FL target genes in cultures derived from FTrap and FQuad. The expression of MBD3L2, ZSCAN4, TRIM43, DEFB103 and ZNF217 mRNAs was analyzed by qPCR. All of these genes, except ZNF217, were activated during differentiation (Fig. 4A). It should be noted that the kinetics of gene expression were different during differentiation of cultures derived from FTrap and
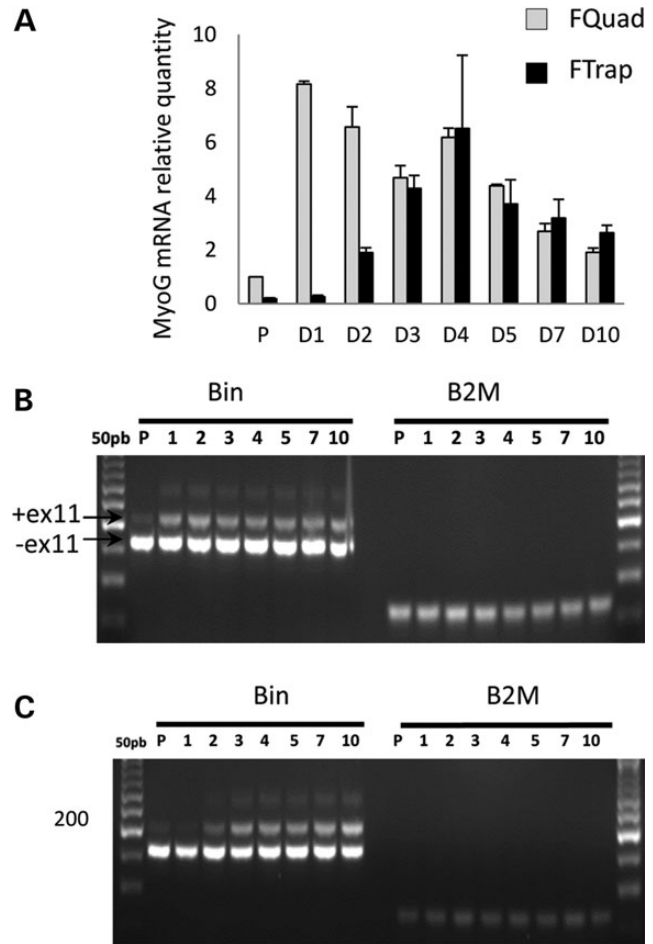

Figure 3. Characterization of the differentiation kinetics in FQuad and FTrap. To analyze differentiation kinetics on FQuad and FTrap, total RNA was extracted at different time points after the induction of differentiation by serum starvation. RT-qPCR was performed for the detection of MyoG (myogenin) (**A**) and BIN1 exon 11 (**B** FQuad; **C** FTrap). B2M was used as the normalizer.

FQuad: whereas the expression of target genes was maximal at day 4 of differentiation in FQuad cultures, this peak was delayed to day 5 to 7 in FTrap cultures, as observed for DUX4 mRNA expression. The relative expression levels of MBD3L2 and ZSCAN4 mRNA were next compared between FQuad and FTrap cultures (Fig. 4B). We observed that the levels of MBD3L2 and ZSCAN4 expression were 3–5-fold lower in FTrap than in FQuad cultures, as was observed for DUX4 mRNA expression.

FQuad and FTrap myoblasts were next transduced using a lentiviral vector containing an shRNA against all DUX4 mRNA isoforms. In cells transduced with the DUX4 shRNA (Fig. 5A), a down-regulation of DUX4-FL mRNA was observed (21 and 44% of residual mRNA in FQuad and FTrap myotubes, respectively, at day 5 of differentiation), demonstrating the efficacy of the shRNA. The depletion of DUX4 was also demonstrated at the protein level using a DUX4-specific antibody (Supplementary Material, Fig. S2). We next investigated the expression level of MBD3L2, ZSCAN4, DEFB103, TRIM43 and ZNF217 in the transduced cells (Fig. 5B). In the cells transduced with the empty shRNA, the mRNA expression level of the target genes
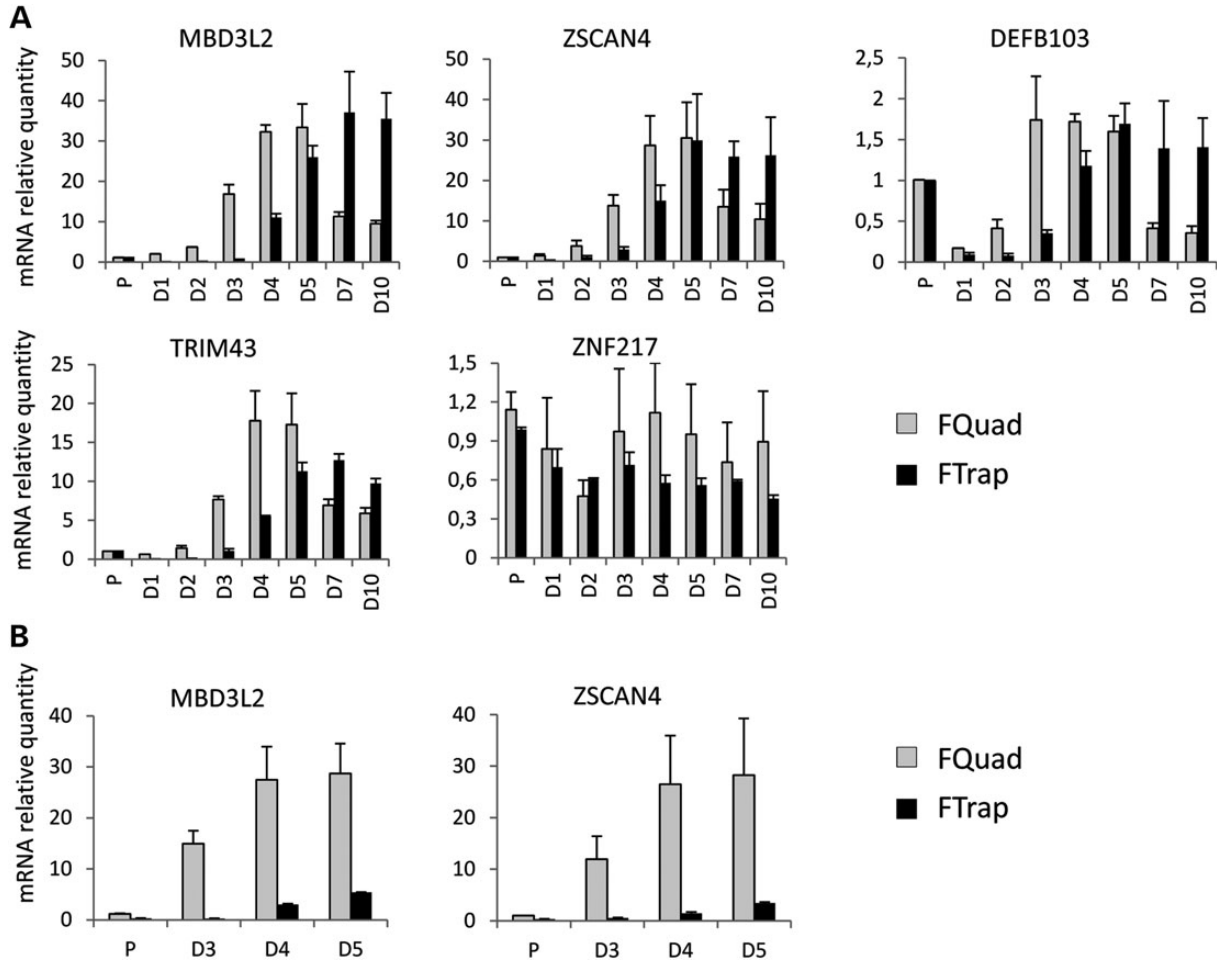
Figure 4. The expression level of genes downstream of DUX4 in FQuad and FTrap. Real-time RT-PCRs were carried out at different time points after the induction of differentiation. Several genes described as being activated by DUX4 were tested. The experiment was made on FQuad (black bars) and FTrap (gray bars). (**A**) Values measured in each cell type were normalized to its level in proliferation condition. (**B**) The results are normalized to the level of expression in proliferating FQuad. FTrap expression level is thus relative to FQuad, which allows a better comparison between both differentiated cultures. Error bars represent SD of PCR triplicates carried out on two independent kinetic experiments.

(except for DEFB013 in FQuad) increases during differentiation as expected. As previously observed in Figure 4A, the differentiation process did not modulate the expression level of ZNF217, thus suggesting that ZNF217 expression is not regulated by DUX4. This was confirmed in the cells transduced with the shDUX4, where the mRNA expression level of ZNF217 was not affected by the presence of the shRNA (Fig. 5B), whereas for the other genes, the presence of the DUX4 shRNA inhibited their overexpression during differentiation, thus confirming the down-regulation of the *DUX4* expression that turns down its target genes. The levels of expression of DUX4-FL downstream targets further confirm the different levels of DUX4 expression observed in the differentiated cultures.

As many genes have previously been described to be modulated by the overexpression of DUX4-FL, we investigated the impact of the shDUX4 expression in FQuad and FTrap on the expression of TP53, THOC4, DBR1, TFiP11, SRSF8, PRAMEF1 and MURF1 (Supplementary Material, Fig. S3). None of these genes were modulated except *PRAMEF1*, which was

dramatically down-regulated in both FQuad and FTrap when the shDUX4 is expressed and MURF1 mRNA, which was up-regulated.

Genes downstream of DUX4-FL are also specifically activated in FSHD1 fetal biopsies

Broucquault *et al.* (29) have shown previously that DUX-FL is expressed in fetal FSHD muscle biopsies and because we have used the same FSHD biopsies as them we decided to investigate the mRNA expression level of genes downstream of DUX4-FL. Interestingly, we found that MBD3L2 and TRIM43 mRNA expression levels were systematically higher in the fetal FSHD biopsies (18–36-fold and 10–26-fold for MBD3L2 and TRIM43, respectively), indicating that these DUX4-FL downstream genes are specifically activated in FSHD samples (Fig. 6). However, DEFB103, ZSCAN4, PRAMEF1 and ZNF217 mRNA expression levels were not different in FSHD biopsies as compared with control biopsies. These results
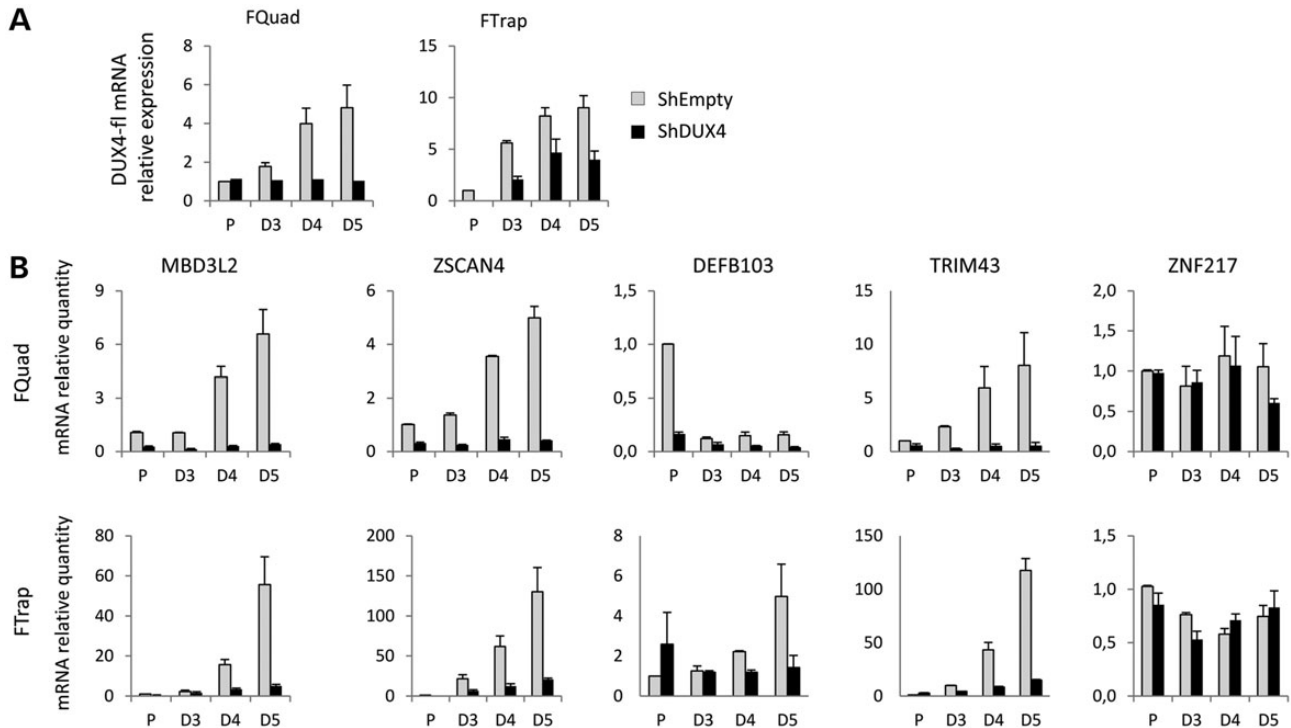
Figure 5. DUX4 inhibition using shDUX4 in FQuad and FTrap and activation of genes downstream of DUX4. A lentivirus (pLL3.7) carrying both an shRNA directed against DUX4 and a CMV-GFP cassette was used to transduce FQuad (left panel) or FTrap (right panel) cultures. After sorting of the CD56+ (a marker for myogenic cells) and GFP+ double positive cells, a differentiation kinetic was carried out. (**A**) DUX4 mRNA expression was analyzed by real-time PCR using the DUX4-all primers in the Fquad (left panel) and FTrap (right panel) transduced cells. At day 5 of differentiation, a student's t-test reveals that $P = 0.04$ and 0.03 for FQuad and FTrap, respectively. (**B**) The mRNA expression level of the downstream target genes was assessed by real-time PCR on FQuad (upper panel) and FTrap (lower panel) cultures transduced with either the shDUX4 (black bar) or the shEmpty (gray bar). A T-test realized at day 5 of differentiation reveals that $P < 0.05$ for MBD3L2, ZSCAN4, TRIM43 and DEFB103. Error bars represent SD of PCR triplicate realized on two independent experiments.

demonstrate that a modification in the expression of some but not all of the genes downstream of DUX4-FL is already present in fetal FSHD1 biopsies, strongly suggesting that DUX4-FL is also expressed during fetal development.

Expression of genes downstream of DUX4-FL is similar in fetal and adult samples

We next investigated the mRNA expression levels of MBD3L2, ZSCAN4, ZNF217, PRAMEF1, DEFB103 and TRIM43 in adult FSHD and control biopsies (Fig. 7A). We did not see any difference for ZNF217 and DEFB103. The overexpression of ZSCAN4 and PRAMEF1 was not confirmed in all adult samples. Finally, we did observe a clear modification in MBD3L2 and TRIM43 mRNA expression levels because the means of mRNA overexpression were 63 and 163, respectively. It should be noted that the FSHD1 samples are heterogeneous with sample #FSH1 always showing an important activation of the genes studied and sample #FSH3 always showing a very low activation of these genes. As FSHD1 has not been described to be a fetal pathology, we investigated whether the mRNA expression levels of TRIM43 and MBD3L2 were similar in adult and fetal biopsies. As shown in Figure 7B, we observed that the expression levels of both genes were comparable in fetal and adult FSHD biopsies.

DISCUSSION

Recent studies have suggested that DUX4 is a central player in FSHD pathophysiology. Indeed, the isoform DUX4-FL is expressed in myotubes isolated not only from FSHD1 but also from FSHD2 patients and has been shown to be toxic for myonuclei (14,16,20,26,30). Nevertheless, despite the fact that DUX4 might play an important role in the pathology, the mechanisms by which DUX4 is involved in FSHD still needs to be deciphered. One key to understanding its action is to clearly define when it first begins to be expressed in the skeletal muscle. Clinical features of FSHD usually appear in the second decade of life. However, a minority of patients carrying the most extended contraction develop the disease during late childhood. Nevertheless, nothing is known about the possible effect the D4Z4 contraction could have during fetal muscle growth and development.

In this article, we investigated the DUX4 expression during fetal development and show that DUX4-FL mRNA is already expressed in primary myogenic cells derived from two muscle biopsies derived from a single FSHD1 fetus (owing to the difficulty in obtaining FSHD fetal cells, our *in vitro* study was restricted to 1 fetus). Interestingly, the percentage of nuclei expressing the protein DUX4 does not seem to be different in fetal and in adult myotubes: DUX4 is present in almost 9% of the nuclei in fetal cells, and it was previously published that between 0.5 and 10% of the nuclei are DUX4 positive in adult
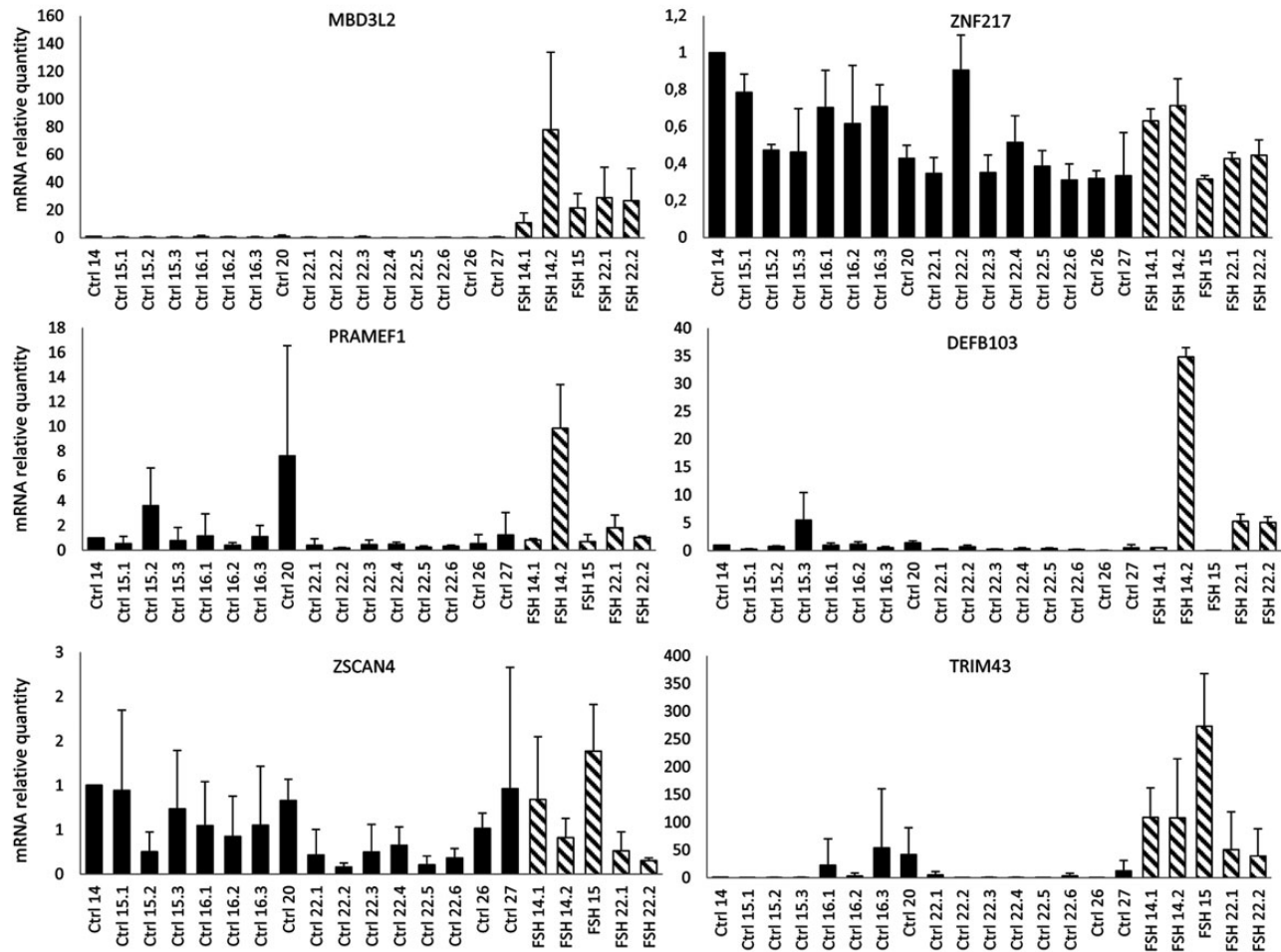
Figure 6. The expression level of genes downstream of DUX4 in quadriceps fetal biopsies. Quadriceps biopsies from five FSHD1 fetuses (hatched bars) and 16 quadriceps control biopsies obtained at different stages of development (back bars) were analyzed. A RT-q PCR was made on each mRNA preparation to determine their relative quantity. B2M was used as the normalizer gene. The result obtained with the Ctrl14 was used as the reference to generate arbitrary units in order to compare results between samples. Statistical analyses (T-test) revealed that the mean expression level for MBD3L2 and TRIM43 was different in control and FSHD1 samples ($P = 0.02$ and 0.03, respectively).

cells (30,31). However, owing to the limited number of control cells available for this study, we cannot speculate that DUX4-FL is not expressed in fetal control cells as it was previously demonstrated with adult cells (17). Actually, Broucqsault and colleagues (29) have observed a DUX4-FL mRNA expression in 8 of 27 fetal control biopsies. Several groups have previously shown that DUX4-FL mRNA is expressed in muscle cells derived from adult FSHD1 muscle biopsies (12,14,16, 17,26,32), but this is the first time that the DUX4 expression has been demonstrated in fetal FSHD1 cells. The two DUX4-FL isoforms, in which the first intron is spliced or not (DUX4-FL1 and DUX4-FL2, respectively), have been described to be expressed in both adult FSHD1 and FSHD2 myotubes, and we have confirmed this result on fetal FSHD1 myotubes. Interestingly, independent of the DUX4 primer set that was used, the DUX4 mRNA expression level was consistently lower in the trapezius than that in the quadriceps. This last result was surprising because the trapezius muscle is described to be one of the first affected muscles and so we were expecting to see a higher level of DUX4 mRNA in this muscle compared

with the quadriceps. Several possibilities could explain this result: (i) as FSHD is a disease affecting muscles asymmetrically by an unknown mechanism, it is possible that the biopsies were realized on a future poorly affected trapezius and a highly affected quadriceps and (ii) DUX4 expression is not the only determinant that explains the pattern of the affected and non-affected muscles in FSHD patients. This is in agreement with the fact that DUX4-FL has also been described to be expressed in control myotubes and biopsies (17,29). A third possibility is that the DUX4 expression is more toxic in Trapezius than that in Quadriceps owing to intrinsic difference between these two muscles. When a myonucleus starts to express DUX4, this will trigger apoptosis and cell death, thus decreasing the number of DUX4-positive nuclei and global DUX4 mRNA expression in FTrap, whereas FQuad myonuclei would not trigger apoptosis in response to the DUX4 expression.

Recently, numerous target genes downstream of DUX4 have been identified after transduction of control primary myoblasts by a lentiviral vector expressing DUX4-FL (24). Similarly, of the five genes we tested (MBD3L2, ZSCAN4, DEFB103,
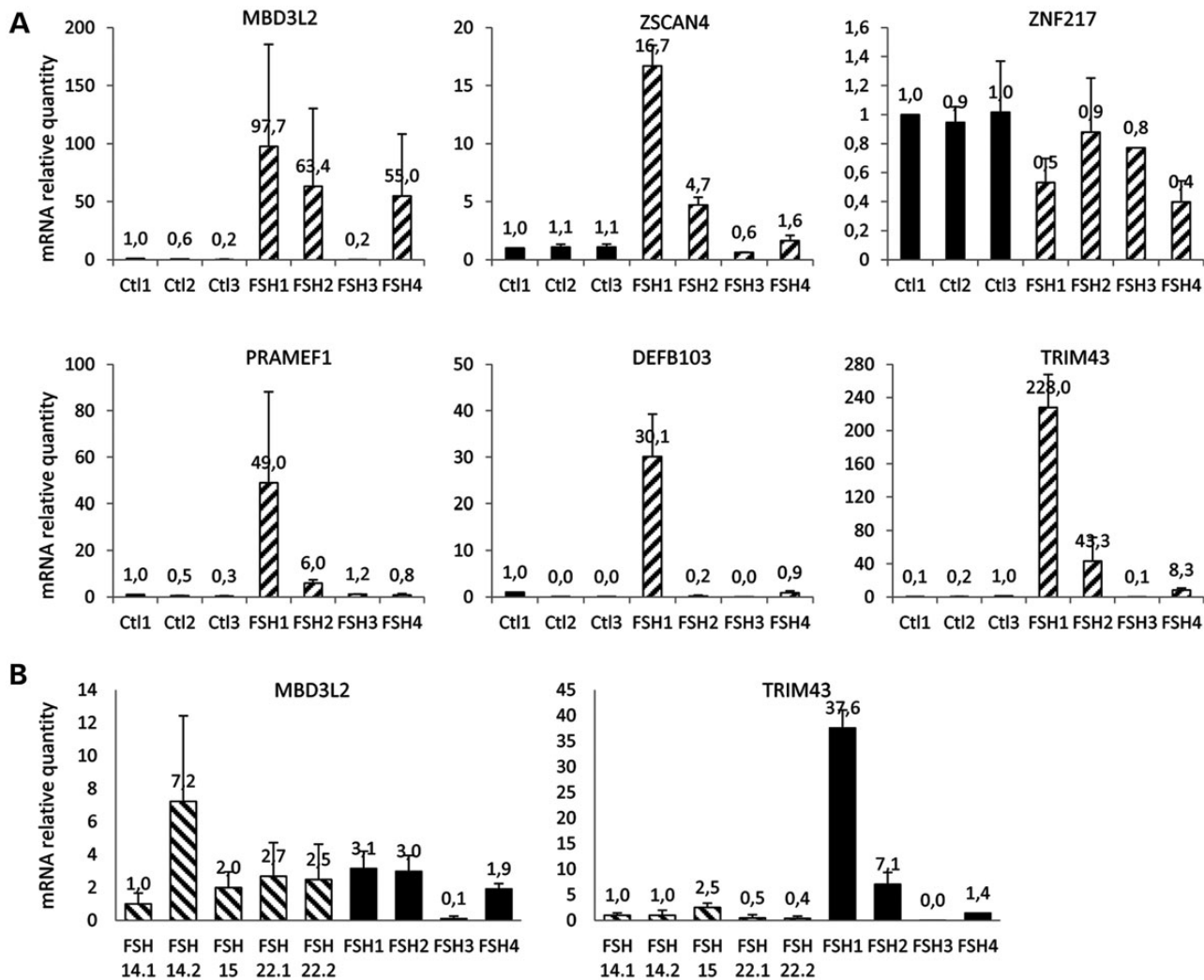
Figure 7. The expression level of genes downstream of DUX4 in quadriceps adult biopsies. (**A**) Four FSHD1 (hatched bars) and three healthy control (black bars) adult quadriceps biopsies were analyzed. For each gene, the result obtained with the Ctrl1 was used as the reference for arbitrary quantification. B2M gene was used as normalizer. Statistical analysis (Student's T-test) reveals that $P < 0.05$ only for MBD3L2 and TRIM43. (**B**) MBD3L2 and TRIM43 expression levels were compared in five fetal (hatched bars) and four adult (black bars) FSHD1 biopsies. Statistical analysis (t-test) does not show any difference in adult and fetal samples ($P = 0.22$ for MBD3L2 and $P = 0.16$ for TRIM43).

TRIM43 and ZNF217), all of them except ZNF217 were up-regulated in the fetal cultures FQuad and FTrap during differentiation. However, in fetal FSHD1 biopsies, we observed that only MBD3L2 and TRIM43 were specifically up-regulated. Considering that FSHD induces a muscle atrophy, which is often asymmetric, it is possible that the modification of the expression of the other genes (ZSCAN4, DEFB103 and PRAMF1) in the FSHD fetal biopsies is influenced by this asymmetry. Moreover, in adult FSHD1 biopsies, we also confirmed that MBD3L2 and TRIM43 are up-regulated. These two genes can therefore be considered as robust markers for the DUX4 expression in both fetal and adult samples. It should also be noted that TRIM43 and MBD3L2 expression levels were similar in fetal and adult FSHD1 biopsies. The molecular events leading to the FSHD phenotype are thus already initiated during fetal development. Because it is difficult to imagine that all five FSHD fetuses would have been infantile cases, which represent 5–10% of all the FSHD cases, our data open a new field of

investigation for mechanisms leading to FSHD. Indeed, FSHD is described as an adult onset myopathy with clinical signs of the disease only appearing during the second decade of life. One hypothesis is that muscle fibers can only tolerate a given amount of DUX4 during a given amount of time, beyond which the delicate balance of muscle maintenance and repair will be lost. In this case, the amount of DUX4 expressed in each fiber is the same throughout life but results in fiber atrophy only after several years once the muscle is no longer able to compensate for the toxic effect of DUX4. FSHD could thus be at least partly attributed to the accumulation of DUX4 and genes downstream of DUX4 throughout life. An alternative hypothesis would result from DUX4 acting already by some epigenetic mechanism during fetal skeletal muscle development without having any major consequences on myogenesis and fiber growth, until a secondary event potentiates its toxic effect.

In conclusion, our results demonstrate that DUX4-FL is already expressed during fetal muscle development, as

evidenced in cultures derived from fetal biopsies, but more importantly by the activation of DUX4 target genes in these biopsies. Whether it is a priming event that requires additional signals or the accumulation of this signal over time that triggers the FSHD phenotype we do not know, and more research will be needed before we understand the complexity of FSHD pathophysiology.

MATERIALS AND METHODS

Ethic statement

This study and the collection of fetal and FSHD1 muscle biopsies was approved by the 'Agence Française de la Biomedecine' of the Ministry of Health to have legal access to the biological material in full accordance with the law (research protocol number PFS12–007). Therapeutic abortions are following a very precise protocol: after discussions with the medical team, the decision can be made to terminate the pregnancy. The parents can then choose to sign an informed consent for a fetal autopsy that will be carried out by pathologists. If the parents indicate that they accept that biological samples are taken for scientific investigations within the frame of scientific research on normal and pathological prenatal development, muscle biopsies will be taken. They will be used either to generate primary cultures of myoblasts or to be frozen for further molecular analyses.

FSHD patient reports

The FSHD1 fetuses were aborted for medical reasons. The fetal muscle biopsies of FSH14.1 fetus were performed by fetopathologists at AP-HP (Assistance Publique—Hôpitaux de Paris). The father was diagnosed for FSHD1 at age 23 and displayed a typical clinical phenotype including facial and scapula fixator muscle weakness. He carried four repeated units on 4qA chromosome 4. He was 32 when the fetus, carrying the same contraction, was aborted and at present (2 years later), the father can still walk but uses a cane. The genetic anomaly was inherited from his father who carried the 4QA D4Z4 contraction. The FSH14.2 fetus was aborted at 14 weeks of development and carried 1.5 D4Z4 repeats. The FSH15 was aborted at 15 weeks of development and carried four D4Z4 repeats. The fetuses FSH22.1 and 22.2 were aborted at 24 weeks of development and they both carried seven D4Z4 repeats.

Adult quadriceps muscle biopsies were obtained from typical FSHD1 patients and healthy control individuals using standardized muscle biopsy protocol. FSH1 and FSH2 were, respectively, 59- and 60-year-old men carrying seven D4Z4 repeats with a clinically non-affected quadriceps. FSH3 was a 62-year-old man carrying seven D4Z4 repeats with clinically affected quadriceps muscles. FSH4 was a 64-year-old woman carrying six D4Z4 repeats with mildly affected quadriceps. Healthy controls were selected in the same age and sex range as the FSHD patients.

Muscle biopsies and cells

Primary cells from FSH14.1 quadriceps (FQuad) and trapezius (FTrap) muscles were derived from the different muscle biopsies coming from the same fetus. Briefly, the biopsies were carefully minced, and the explants were plated in culture dishes previously coated with FBS. Once the cells had migrated from the explants

to colonize the culture dish, they were trypsinized and the percentage of CD56-positive cells was determined by FACS analysis. Briefly, 50 000 cells were incubated with 1 μl of CD56 antibody (CD56-APC, Becton Dickinson, Le Pont de Claix, France) in PBS FBS 2%. A FACSCalibur (Becton Dickinson, Le Pont de Claix, France) was used to determine the myogenicity, i.e. percentage of CD56-positive cells. If this percentage was lower than 80%, the myogenic cells were enriched in CD56-positive cells using MACS columns according to the manufacturer instructions (Miltenyi Biotec, Paris, France).

Cell culture

Fetal FSHD1 and control primary cells were cultivated in proliferation medium (4 vol of DMEM, 1 vol of 199 medium, FBS 20%, Gentamycin at 50 μg/ml, Life technologies, Saint Aubin, France). The differentiation was induced by replacing the proliferation medium by DMEM supplemented with insulin (10 μg/ml). The myogenicity, defined as the percentage of CD56-positive cells, was checked before any experiments performed with the cells, and the cells were always enriched in CD56+ cells using MACS columns immediately before plating if this percentage was <80%.

Cell culture

After 5 days of differentiation, immunostaining was performed using the E5.5 antibody, which recognizes the C-terminus part of DUX4 (33). Cells were fixed with 4% paraformaldehyde for 10 min and permeabilized with 1% triton X-100 (Sigma) in PBS for 10 min at room temperature. Blocking was realized with PBS 2% FBS for 30 min at room temperature. DUX4 antibody (rabbit) was diluted 1/10 in PBS 2% FBS, and MF20 (Mouse IgG2B) was diluted 1/100. Cells were incubated overnight with DUX4 alone or both antibodies and washed three times with PBS 0.5% triton. Secondary antibodies were Ac GAM IgG2B-488 for anti-MF20 and GAR IgG H + L-555 (1/100) for DUX4. Incubation was carried out for 1 h in the dark. Finally, nuclei were counterstained with Hoescht (1/1000, Dako fluorescent mounting).

RNA extraction, PCR and real-time PCR

Total RNAs from either cells or human biopsies of muscles were extracted using trizol according to the manufacturer's protocol (Life technologies, Saint Aubin, France). The quantity of RNA was determined using a nanodrop ND-1000 spectrophotometer (Thermo Scientific, Wilmington, DE, USA). The reverse transcription (RT) was carried out on 1 μg of total RNA using the Roche Transcriptor First Strand cDNA Synthesis Kit (Roche, Meylan, France) at 60°C for 50 min. In a final volume of 13 μl, 1 μl of oligo dT and 2 μl random hexameres were used as RT primers. All of the primers used in this study are listed in Table 1. With DUX4-UTR primers, the PCR was performed in a final volume of 25 μl with 1 μl of RT product, 1 μl of each forward and reverse primers at 20 pmol/μl. Thermal cycling conditions were 94°C for 5 min and then 36 cycles at 94°C for 30 s and 62°C for 45 s. The quantitative PCR was performed in triplicate with the probe mastermix (Roche, Meylan, France) on a LightCycler® 480 Real-Time PCR System (Roche, Meylan, France). DUX4 qPCR was performed in triplicate. Each well contained 0.25 μl of RT product, 4.5 μl of the probe mastermix 2X, 0.18 μl of each primers (20 pmol/μl) and

Table 1. Sequences of the primers

Targeted gene	Primer	Sequence	Size (bp)	Accession number
DUX4-all	DUX4-all_fw	CCCAGGTACCAGCAGACC	164	HQ266761
	DUX4-all_rev	TCCAGGAGATGTAACTCTAATCCA		
DUX4-FL	DUX4-FL_fw	CCTGGGATTCCTGCCTTCTA	198	HQ266761
	DUX4-FL_rev	AGCCAGAATTTCACGGAAGA		
DUX4-UTR	DUX4-UTR_fw	AGGCGCAACCTCTCCTAGAAAC	368 and 504	HQ266761
	DUX4-UTR_rev	TCCAGGAGATGTAACTCTAATCCA		
Myogenin	820U19	GAGTTCAGCGCCAACCCAG	165	NM_002479.5
	965L20	TGCCCGGCTTGGAAGACAAT		
BIN1	BIN F	AGAACCTCAATGATGTGCTGG	162 and 207	NM_139351.2
	BIN R	CGTGGTTGACTCTGATCTCGG		
MBD3L2	MBD3L2_fw	CGTTCACCTCTTTTCCAAGC	142	NM_144614.3
	MBD3L2_rev	AGTCTCATGGGGAGAGCAGA		
ZSCAN4	ZSCAN4_fw	GTGGCCACTGCAATGACAA	143	NM_152677.2
	ZSCAN4_rev	AGCTTCCTGTCCCTGCATGT		
DEFB103	DEFB103_fw	GAGGATCCATTATCTTCTGTTTGC	135	NM_018661.3
	DEFB103_rev	AGGCAGCTGAGCACAGCA		
TRIM 43	TRIM43_fw	ACCCATCACTGGACTGGTGT	100	NM_138800.1
	TRIM43_rev	CACATCCTCAAAGAGCCTGA		
ZNF217	ZNF217_fw	AAGCCCTATGGTGGCTCC	99	NM_006526.2
	ZNF217_rev	TTGATATGACACAGGCCTTTTC		
PRAMEF1	PRAMEF1_fw	CTCCAAGGACGGTTAGTTGC	142	NM_023013.2
	PRAMEF1_rev	AGTTCTCCAAGGGGTTCTGG		
TP53	TP53_fw	GTTCCGAGAGCTGAATGAGG	159	NM_001126118.1
	TP53_rev	TCTGAGTCAGGCCCTTCTGT		
MURF1	MURF1_fw	CTTGACTGCCAAGCAACTCA	80	NM_032588.3
	MURF1_rev	CAAAGCCCTGCTCTGTCTTC		
THOC4	THOC4_fw	CAGCAGACGTGCACTTTGAG	107	NM_005782.3
	THOC4_rev	TGACGTGACAAGCTGAATGTT		
DBR1	DBR1_fw	AAACCATGAAGCCTCAAATCA	92	NM_016216.3
	DBR1_rev	TTACCACACCAGCCAAACCT		
SRSF8	SRSF8_fw	TTAGCTGGGCGCTGATTAGG	71	NM_032102.2
	SRSF8_rev	AATGAAGCCAGGTCCCGAAG		
TFIP11	TFIP11_fw	GCAGATGCCTTTCACAGGTT	95	NM_012143.2
	TFIP11_rev	ACCATCGGGTCACAGTTCC		

0.18 µl of the DUX4 probe (10 pmol/µl) in a final volume of 9 µl. qPCR cycling conditions were as follows: 95°C for 5 min, followed by 53 cycles at 95°C for 30 s and 62°C for 45 s. For the other genes, the qPCR was performed in a final volume of 9 µl with 0.4 µl of RT product, 0.18 µl of each forward and reverse primers 20 pmol/µl, and 4.5 µl of sybergreen mastermix 2X (Roche, Meylan, France). The qPCR cycling conditions were 94°C for 5 min, followed by 50 cycles at 95°C for 30 s and 60°C for 15 s and 72°C for 15 s.

To amplify BIN1, PCR was performed using 2X ReddyMix PCR master mix (Thermo scientific, Rockford, IL, USA) with 1 µl of RT product, 0.5 µl of 20pmol/µl forward and reverse primers, in a final volume of 25 µl. The cycling conditions were as follows: 95°C for 5 min, followed by 32 cycles at 95°C for 20 s, 60°C for 20 s and 72°C for 20 s, then 72°C for 7 min, on a thermocycler GeneAmp PCR System 2700 (Applied Biosystems, Saint Aubin, France).

DUX4 shRNA construct and transduction
The sequence of DUX4 was analyzed for siRNA target using the Clontech software (http://bioinfo.clontech.com/rnaidesigner/sirnaSequenceDesign.do). One siRNA was selected and synthesized to be directly cloned into pLL3.7 (addgene, Plasmid 11795) using the hpaI and XhoI restriction enzyme sites. The oligonucleotides were TGGCAAACCTGGATTAGAGTTTCAA GAGAACTCTAATCCAGGTTTGCCTTTTTC for the sense strand and TCGAGAAAAAGGCAAACCTGGATTAGAGTT CTCTTGAAACTCTAATCCAGGTTTGCCA for the antisense strand. The pLL3.7 shDUX4 vector was produced in human embryonic kidney 293 cells by quadri-transfection of plasmids encoding gag-pol proteins, Rev protein, envelop proteins (VSVg) and the transgene using PEI. 48 and 72 h later; the viral vector is filtered (0.22 µm) before being directly used to transduce myoblasts.

SUPPLEMENTARY MATERIAL

Supplementary Material is available at *HMG* online.

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