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THE JOURNAL OF BIOLOGICAL CHEMISTRY VOL. 288, NO. 49, pp. 34989–35002, December 6, 2013

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Received for publication, July 23, 2013, and in revised form, October 17, 2013 Published, JBC Papers in Press, October 20, 2013, DOI 10.1074/jbc.M113.504522

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Background: FSHD is characterized by the overexpression of double homeobox genes *DUX4* and *DUX4c*.
Results: We found 29 miRNAs differentially expressed between FSHD and normal myoblasts. Twelve of these miRNAs were up-regulated in myoblasts ectopically expressing DUX4c.
Conclusion: *DUX4c* is linked to the abnormal miRNA expression profile observed in FSHD.
Significance: We observe a defective gene regulation by miRNAs in FSHD.

Facioscapulohumeral muscular dystrophy (FSHD) is an autosomal dominant hereditary neuromuscular disorder linked to the deletion of an integral number of 3.3-kb-long macrosatellite repeats (D4Z4) within the subtelomeric region of chromosome 4q. Most genes identified in this region are overexpressed in FSHD myoblasts, including the double homeobox genes *DUX4* and *DUX4c*. We have carried out a simultaneous miRNome/transcriptome analysis of FSHD and control primary myoblasts. Of 365 microRNAs (miRNAs) analyzed in this study, 29 were found to be differentially expressed between FSHD and normal myoblasts. Twenty-one microRNAs (miR-1, miR-7, miR-15a, miR-22, miR-30e, miR-32, miR-107, miR-133a, miR-133b, miR-139, miR-152, miR-206, miR-223, miR-302b, miR-331, miR-362, miR-365, miR-382, miR-496, miR-532, miR-654, and miR-660) were up-regulated, and eight were down-regulated (miR-15b, miR-20b, miR-21, miR-25, miR-100, miR-155, miR-345, and miR-594). Twelve of the miRNAs up-regulated in FHSD were also up-regulated in the cells ectopically expressing DUX4c, suggesting that this gene could regulate miRNA gene transcription. The myogenic miRNAs miR-1, miR-133a, miR-133b, and miR-206 were highly expressed in FSHD myoblasts, which nonetheless did not prematurely enter myogenic differentiation. This could be accounted for by the fact that in FSHD myoblasts, functionally important target genes, including cell cycle, DNA damage, and ubiquitination-related genes, escape myogenic microRNA-induced repression.

Facioscapulohumeral muscular dystrophy (FSHD),[5] a dominant neuromuscular disease, is one of the most frequent muscular dystrophies in the Western world with a prevalence of 1 in 20,000. This dystrophy is characterized by weakness and atrophy of specific groups of muscles of the face, shoulder girdle, and lower extremities (1). The FSHD locus has been mapped to the subtelomeric region on chromosome 4q35, which contains an array of 3.3-kb-long macrosatellite repeats (D4Z4) (2). The length of this array varies from 35 to 300 kb in healthy subjects but is consistently smaller than 35 kb in FSHD patients (3).

Each D4Z4 repeat contains a functional promoter, an open reading frame for DUX4 (double homeobox protein 4) (4, 5), and a number of regulatory elements (reviewed in Ref. 6). In healthy subjects, *DUX4* is expressed during embryogenesis and down-regulated in the course of development (7, 8). In general, its expression level is higher in the skeletal muscles of FSHD patients than in the muscles of healthy controls; however, it is not unusual to find FSHD samples with a normal level of *DUX4* expression (7–9). Therefore, although *DUX4* is generally considered as an FSHD inducer gene, other genes might contribute to the pathological phenotype: *FRG1*, *FRG2* (FSHD region genes 1 and 2), *ANT1* (adenine nucleotide translocator), and *DUX4c* (double homeobox 4, centromeric). Most of these genes have also been shown to be up-regulated in skeletal muscle tissue of some FSHD patients and primary myoblasts derived from them (for a review, see Ref. 6).

Transcriptional profiling performed in FSHD cells has demonstrated a defect in the myogenic differentiation program (9–13), deregulation of genes related to oxidative stress (9, 14, 15), and deregulation of vascular smooth muscle and endothelial cell-specific genes (16) as well as cell cycle-related genes (17).

Ectopic overexpression of several 4q35 genes in mouse tissues or immortalized myoblasts cultured *in vitro* recapitulated

* This work was supported by grants from the Association Française contre les Myopathies (to Y. S. V. and D. L.).
[S] This article contains supplemental Tables S1–S3.
[1] Supported by the Association de Recherche Contre le Cancer, the Association Amis FSH, and the University of Montpellier I.
[2] Recipient of a Coordenaçào de Aperfeiçoamento do Pessoal de Nível Superior - Comité Français d'Evaluation de la Coopération Universitaire et Scientifique avec le Brésil (CAPES-COFECUB) felllowship.
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[5] The abbreviations used are: FSHD, facioscapulohumeral muscular dystrophy; qPCR, quantitative PCR; qRT-PCR, quantitative RT-PCR; miRNA or miR, microRNA; FR-miR, FSHD-related microRNA; pri-miR, primary microRNA precursor; H3K9me3, histone H3 Lys-9 trimethylation; chr., chromosome; TSS, transcription start site.

TABLE 1

Myoblast and myotube samples used in the study

BIO, muscle biopsies; MB, myoblasts; MT, myotubes; ND, non-determined number of D4Z4 repeats.

Sample	Type	Patient	Tissue	Age	Sex	D4Z4 repeats	Source
N1	MB, MT	NO42	Quadriceps	24	Female	ND	This study
N2	MB, MT	NO44	Quadriceps	29	Male	ND	This study
N3	MB, MT	NO46	Quadriceps	31	Male	ND	This study
N4	MB, MT	NO47	Quadriceps	43	Male	ND	This study
F1	MB, MT	MO44	Pyramidal	54	Female	5/7	This study
F2	MB, MT	MO47	Quadriceps	38	Female	7	This study
F3	MB, MT	MO54	Quadriceps	25	Male	4	This study
F4	MB, MT	FSHD10	Trapezius	31	Male	5	This study

TABLE 2

Oligonucleotides used in the study

The coordinates are given relative to the TSSs of the corresponding primary microRNAs (see Fig. 8).

ChiP qPCR primers

code	Human chr.	pri-miRNA	coord. rel. to TSS	Forward	Reverse	Probe
PG1 M37	18	1-2/133a-1	+2358	GTCATTCACGTAGAAAGAAGCAAGAG	CATACAATGCTATGGAATGTAAAGA AGTATGT	6FAM-CCCTTGGTGGTTTATTG
PG2 M38	18	1-2/133a-1	+6050	AGTTTTCTTTAATTTAAATATGCACAC ATCCATGT	CACCTAAAACCCACCTGAACACATA	6FAM-CACACTGTACAATCCC
PG3 M39	18	1-2/133a-1	+5296	GTCTTTGTTTTCAACATCTTTGCTTCTT TTT	TGGTTTTGAAATAGCTTATTGTCTAC ATGTAAGA	6FAM-CAGCCGAAGTCTTTG
PG4 M40	18	1-2/133a-1	+7122	CCTGATGTGATATATGTTGTTTTTAGG TTGGT	TGTGTCTTTGTGGGAATTAGTAAGC AA	6FAM-AACGCCTGTGAAATTA
PG5 M41	18	1-2/133a-1	-94	ACAGCTTTATAGGCAGCTAAGCATT	GGGAAGAGAGAGGCACAAGAGA	6FAM-ACTCCCAGGCAAACAG
PG6 M31	20	1-1/133a-2	+3495	GACGCAAAACCGAGCTACTG	GACAGTGAGCCCCAGAAAGT	6FAM-TCGAGCGCCTGTCCCT
PG7 M32	20	1-1/133a-2	+2563	ACATATCGATACAATTAAGTATTCCA AAGTGCT	GTGCTCACCAGCTCCTAATGA	6FAM-CCACTCGCTAAGTTTAC
PG8 M33	20	1-1/133a-2	+14130	GTGAGTGTCAGTTCCAGAGCAT	CCACCCTCTCGCTCTGC	6FAM-CCCCGACCCTCCCC
PG9 M34	20	1-1/133a-2	+9441	GCACCAAATGGCTTTTCCTTATCC	GAGACCCGAGGTCAAGAGAGA	6FAM-ACGTGGCCGTTCATTT
PG10 M35	20	1-1/133a-2	+11203	CACGTACTGTATCTTCATGGCATCT	GGATACTAGTTGCAGGCAATCTCT	6FAM-CCGGCAGCCCTGTTAA
PG11 M36	20	1-1/133a-2	-201	GGGACCCGCCGTCAAT	GCGCTCCCTGACCATATTTAGTC	6FAM-TCCCACCCTGTTCCCG
PG12 M28	6	206/133b	-11069	ACATCTAGTGTTCTCTGCCCAAGA	TCCCCCCAAATACAAAAGCCTTT	6FAM-CATGCTGAAACCCC
PG13 M29	6	206/133b	-10835	ACCATTCTCCTGACGTCATGTG	ATCGATACAGATCACCAGTCTTCCT	6FAM-CCCTCCCAATTCTCC
PG14 M30	6	206/133b	+7169	GAGTAGGTAGGAGTCAAGGTGCT	AGAGGTAACTTTTTATAAGTATGTTT TCTATAACAGAATCATTAC	6FAM-CACATACGCAAAACAC

pri-miRNA qRT-PCR primers

PM1 PP1	18	1-2	+1988	AGACATTCCTAAGCATTTGACCAG	TCACTCTGAAGACTGAAGAAACAC
PM2 PP2	18	133a-1	+5734	ATTTGAAATCCTTAAGTCATCCATAC ATTT	CATTTGTGAATATCAATTACTTGCCAAC
PM3 PP3	20	1-1	+3556	CTGTCCACTTCTGCCTTTCTG	AACTTCTTCCCTGGAGTCTACTG
PM4 PP4	20	133a-2	+14652	AGTGAAGCCCTGAGTGCC	GAACAAGCAGGAGCACCC
PM5 PP5	6	206	+7996	AAAGGACTGGATAGACTGTAGCTG	GCCACTTGTAGCATCTTTCCTC
PM6 PP6	6	133b	+12958	AACAGAAGAGATTCAACTGCAACT	TAGGGTTGGCACAAACTCCAT

some features of FSHD, suggesting that these genes could indeed contribute to the FSHD phenotype. Specifically, DUX4c and DUX4 have been shown to inhibit myogenic differentiation; DUX4 induced oxidative stress (18, 19) and atrophy of myoblasts cultured *in vitro* (20); FRG1 overexpression in mouse muscles induced muscle atrophy (21).

The mechanism of the overexpression of these functionally important 4q35 genes in FSHD may be attributed, at least partially, to a modification of the nuclear matrix attachment proximally to the D4Z4 array (22) and subsequent perturbation of the three-dimensional structure of the FHSD locus (23, 24). Furthermore, we have previously shown that the D4Z4 repeats contain a potent transcriptional enhancer (25, 26), which interacts with the Krüppel-like transcription factor KLF15 and activates the expression of *DUX4c* and *FRG2* genes (27).

The complexity of gene regulation in FSHD has been further enhanced by the recent finding that non-coding RNAs are implicated in epigenetic regulation of FSHD-related genes (28)

(for a review, see Ref. 29). D4Z4 was found to be transcribed in both directions, and the resulting non-coding RNAs were shown to modulate *DUX4* transcription (30). A high throughput analysis of microRNA expression in FSHD cells revealed several miRNAs differentially expressed in FSHD cells (17, 31).

Here, we have analyzed miRNA expression in primary myoblasts from healthy subjects and FSHD patients and found 29 miRNAs differentially expressed in FSHD samples. Twelve of these miRNAs, including myogenic microRNAs miR-1, miR-206, miR-133a, and miR-133b were induced by DUX4c overexpression, suggesting that *DUX4c* is a novel regulator of miRNA expression. Despite overexpression of several myogenic microRNAs, we did not observe a premature myogenic differentiation of FSHD myoblasts. The expression analysis of target genes of these miRNAs revealed that some of them are not repressed by myogenic miRNAs in FSHD cells.

EXPERIMENTAL PROCEDURES

Cell Culture Conditions and siRNA Transfection—The rhabdomyosarcoma cell line TE-671 (a kind gift of Dr. S. Leibowitz) was grown as described (26). Primary human myoblasts were isolated from skeletal muscles of healthy subjects as described (32) (for details, see Table 1), purified with an immunomagnetic sorting system (MiltenyiBiotec) using an anti-CD56/NCAM antibody according to the manufacturer's specifications. CD56-positive myoblasts were seeded in collagen-coated Petri dishes (P1) and cultured in DMEM, 10% FCS, 1% Ultroser G at 37 °C with 5% CO_2. All experiments were carried out between P1 and P5 to avoid cell senescence. Myoblast purity was determined by staining for desmin. Proliferating primary human myoblasts were transfected as described (20), and RNA was prepared 24 h after transfection. The growth conditions of human immortalized myoblasts (a kind gift of Dr. V. Mouly) have been described previously (33). siRNA transfection has been performed using siPORTNeoFX reagent (reverse transfection protocol) as described (20). pcDNA3-MYOD plasmid was a kind gift of Anna Polesskaya.

Biopsies—The biopsies have been obtained in accordance with French national regulations. The origins of biopsies are listed in Table 1.

Reporter Gene Assays—5×10^3 TE-671 cells were plated onto 24-well plates and after 24 h were cotransfected with 0.5 μg of pGL3-based miR-1/206 or miR-133a/b reporter plasmids containing the luciferase gene fused to the 3′-UTR of the *Hand2* (34) or *Whsc2* gene (35), respectively, and 0.5 μg of pCIneo-DUX4c, pCIneo-DUX4, or a GFP-coding plasmid (Stratagene) using JetPEI (Polyplus). Luciferase activity was determined 48 h after transfection using the Luciferase Assay System (Promega) and normalized to protein concentration (determined by BCA assay; Sigma) or the Dual-Luciferase Assay System and normalized to the activity of the reporter phRL-TK (Promega).

qRT-PCR and RT-PCR—400 ng of total RNA purified via TRIzol (Invitrogen) was converted into cDNA using eight independent pools of primers (catalog no. 4384791, Applied Biosystems) and the TaqMan microRNA reverse transcription kit (catalog no. 4366596, Applied Biosystems). cDNA was quantified via qPCR using TaqMan 2× Universal PCR Master Mix, No AmpErase UNG (catalog no. 4324018, Applied Biosystems), and human microRNA panel version 1.0 TLDA (TaqMan Low Density Array, Applied Biosystems), and data were acquired on an AB7900HT real-time PCR machine. The following probes were used for the miRNAs in this study: miR-1, catalog no. 4373161; miR-133a, catalog no. 4373142; miR-133b, catalog no. 4373172; miR-107, catalog no. 4373154; miR-139, catalog no. 4373176; miR-152, catalog no. 4373126; and miR-331, catalog no. 4373046. The expression analysis of DUX4 and DUX4c genes has been described previously (36, 37).

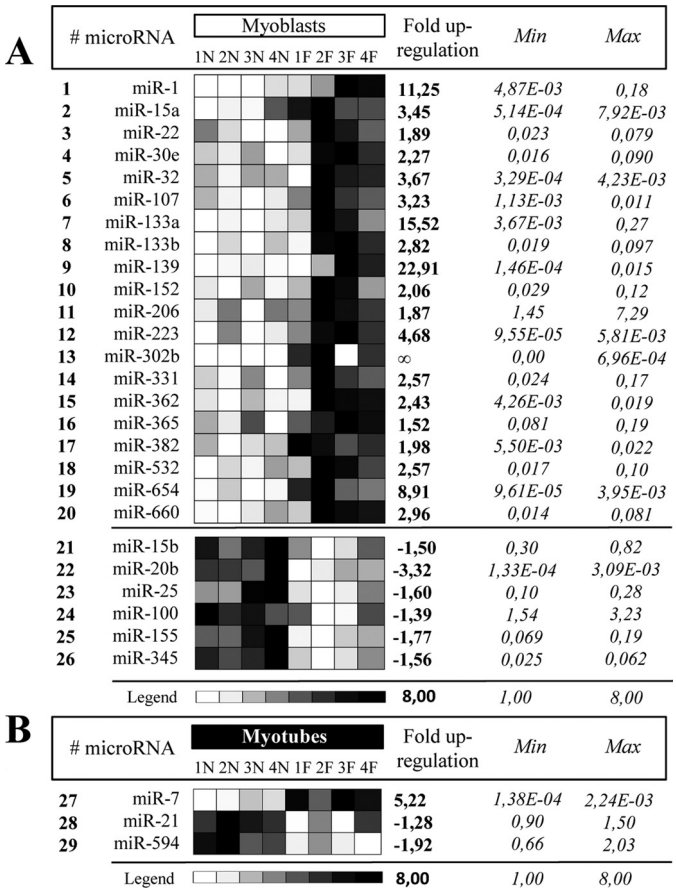

FIGURE 1. **miRNA expression profile of FSHD myoblasts and myotubes.** The expression of miRNA was measured using qRT-PCR in primary myoblasts (*A*) and myotubes (*B*) from four healthy subjects (*1N–4N*) and four FSHD patients (*1F–4F*) (see Table 1 for sample description). *Square intensity* indicates the miRNA expression level normalized to the control (*RNU44*). *Gray gradations* were scaled from minimal and maximal values independently for each miRNA.

Chromatin Immunoprecipitation—Chromatin was prepared from 10×10^6 primary myoblasts and myotubes from FSHD patients and normal controls and sonication-sheared, and 5 μg was used for immunoprecipitation with 1 μg of H3K9me3 antibodies (catalog no. ab8898, Abcam) or preimmune rabbit or mouse IgGs (Abcam) using the ChiP-IT Express Kit (Active Motif). Immunoprecipitated DNA was then amplified using specific primers (see Table 2) and TaqMan 2× Gene Expression Master Mix (catalog no. 4369016, Applied Biosystems) on a custom TLDA array (Applied Biosystems). PCR amplification and data acquisition were performed using AB7900HT real-time PCR machine (Applied Biosystems). To study *DUX4c* binding to myomiR promoters, TE-671 cells were transfected with *DUX4c*-coding plasmid, and chromatin was immunoprecipitated using *DUX4c* antibodies (36).

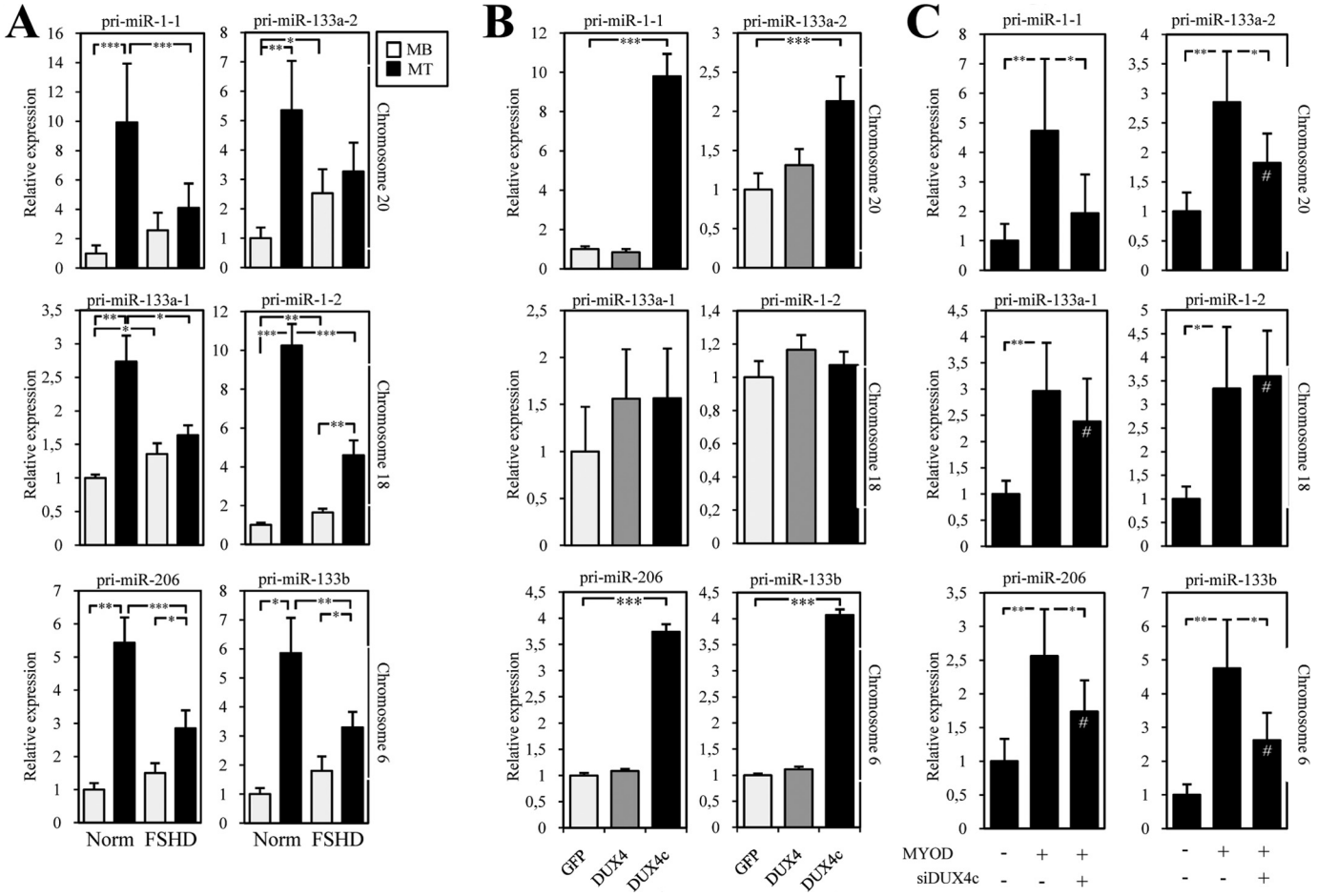

FIGURE 2. **The expression level of pri-miR precursors of myogenic microRNAs measured in primary myoblasts (*MB*) and myotubes (*MT*) from four normal subjects and four FSHD patients (*A*); TE-671 cells transfected with DUX4, DUX4, or control plasmids (*B*); and TE-671 cells co-transfected with MYOD-overexpressing or control plasmids and siRNA against DUX4c or control siRNA (*C*).** *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$ (Student's *t* test); #, $p < 0.05$ relative to control plasmid/control siRNA transfection. *Error bars*, S.E.

Transcriptome Profiling—Human primary myoblasts were sacrificed directly on plates at 30% confluence using TRIzol. RNA was prepared using organic extraction and ethanol precipitation as described (38), followed by silica column cleanup on silica columns (Nucleospin RNA extraction kit, Macherey Nagel). RNA extracted from individual myoblast lines was Cy3-labeled, mixed with a pool of RNA samples labeled with Cy5, and hybridized to Gene Expression microarrays (4 × 44,000, catalog no. G4112F, Agilent) and scanned as instructed by the manufacturer. Scanned images were then analyzed using the Feature Extraction software (Agilent), and the treatment of the gene expression data was performed using R and Bioconductor. Spots with intensity lower than 50 or lower than background in more than 50% of biological replicates were removed from further analysis. The background correction and intensity normalization procedures were applied for the remaining ~30,000 probes using the Bioconductor package vsn (39). A background offset and a scaling factor for each array and dye channel were calculated using the least squares regression procedure, and then the generalized log transformation was applied. The ordinary least squares regression approach is based on the assumption that "most genes are not differentially expressed." However, in the case of myogenic differentiation, where many genes are differentially expressed, this assumption does not hold. Therefore, to apply the above mentioned approach to myogenic differentiation, the vsn least squares model was first applied to a subset of features and then extended for the whole set of features. To select the subset of features, a pool of samples prepared from five different proliferating myoblast lines before and after myogenic differentiation was hybridized to two additional microarrays. Then 14,358 features that did not exceed the cut-off value of 1.23-fold change between the pools were selected. To determine the differentially expressed genes, a *t* test analysis was conducted using the limma package from Bioconductor (39). Using this package, a linear model was fitted to the expression data for each gene. An empirical Bayes moderation of the S.E. values was performed. This method borrows information across genes in order to arrive at more stable estimates of each individual gene's variance, even for experiments with a small number of arrays.

RESULTS

Identification of miRNA Differentially Expressed in FSHD—miRNA expression profiles in total RNA extracted from primary myoblast populations originating from four FSHD patients and four normal individuals were compared using a high throughput TaqMan qRT-PCR approach. To avoid contamination with connective tissue and inflammatory cells, we

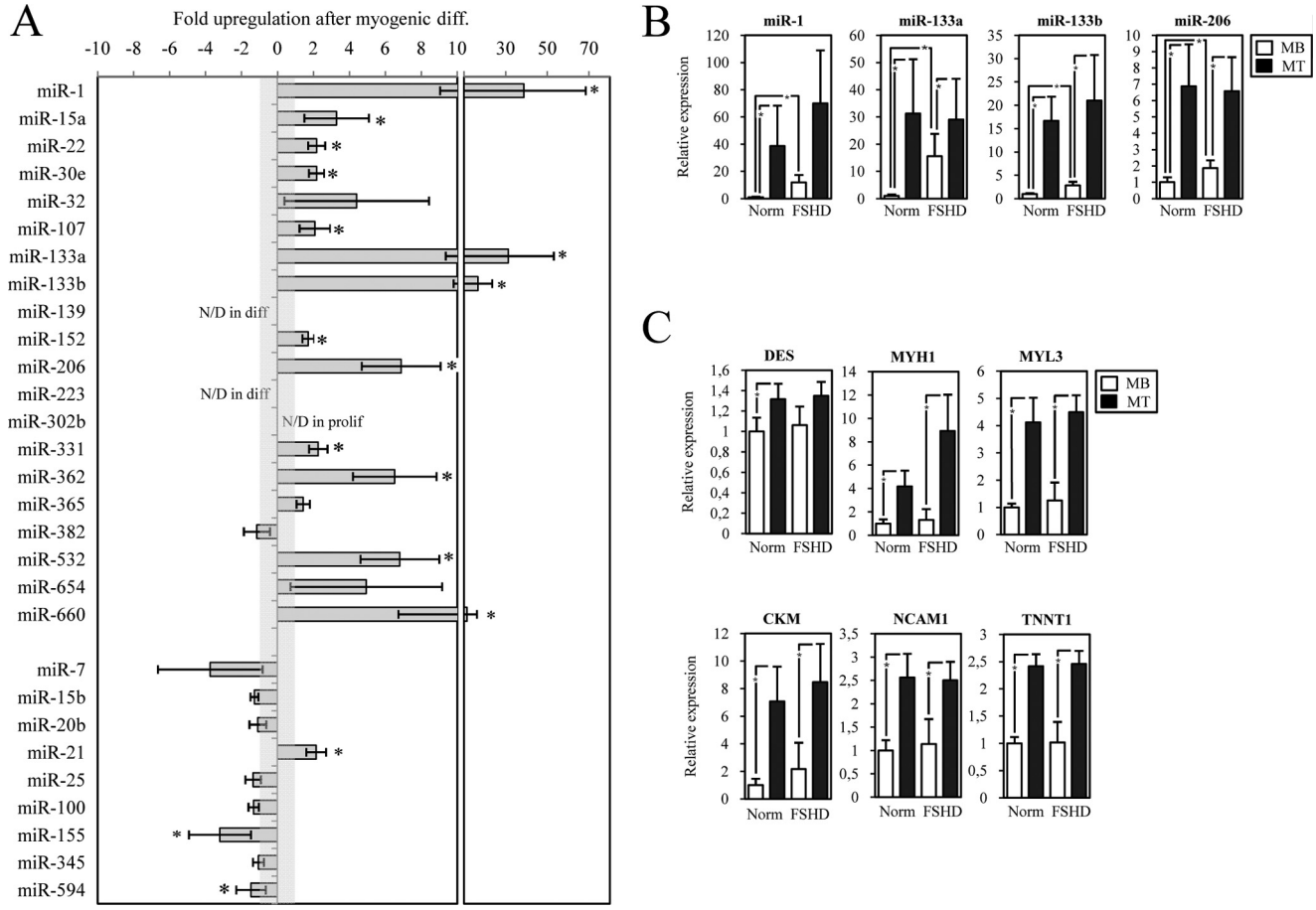

FIGURE 3. **FR-miRs up-regulated during myogenic differentiation.** *A*, the expression of FR-miRs was measured using qRT-PCR in primary myotubes from healthy subjects, 3 days after induction of myogenic differentiation, and normalized to the expression level in primary myoblasts from the same subjects prior to the induction of myogenic differentiation. *B*, the expression of myogenic microRNAs was tested using qRT-PCR in primary myoblasts isolated from healthy subjects and FSHD patients before (*MB*) and after (*MT*) induction of myogenic differentiation. *C*, expression of myogenic markers in FSHD myoblasts. *DES*, desmin; *MYH1*, myosin, heavy chain 1; *MYL3*, myosin, light chain 3; *CKM*, creatin kinase, muscle; *NCAM1*, neural cell adhesion molecule 1; *TNNT1*, troponin T, type 1. The expression of myogenic markers was tested using qRT-PCR in primary myoblasts isolated from healthy subjects and FSHD patients, before (*MB*) and after (*MT*) induction of myogenic differentiation. Mean values are shown, and *error bars* represent S.E. of four independent experiments. *, $p < 0.05$ (Student's *t* test).

used an affinity purification procedure to isolate $CD56^+$ myoblasts. Of 365 microRNAs tested, 29 (hereafter called FSHD-related microRNAs (FR-miRs)) were differentially expressed in FSHD myoblasts as compared with the controls (Fig. 1, *A* and *B*, and supplemental Table S1). These include 20 miRNAs up-regulated and 6 down-regulated in FSHD myoblasts (Fig. 1*A*). Differential expression of some of these FR-miRs, including miR-133a, miR-206, miR-21, and miR-100, was also discovered in FSHD cells in a pilot analysis of miRNA transcriptome using an Ambion microarray in FSHD myoblasts performed prior to this study (data not shown).

miRNA expression has been also profiled in the same myoblasts following the induction of myogenic differentiation. Three microRNAs, miR-7, miR-21, and miR-594, were found differentially expressed in the resulting FSHD myotubes (Fig. 1*B*). In total, of 365 miRNAs tested, the expression of 186 miRNAs, including miR-517*, which was shown previously to be differentially expressed in FSHD muscles (31), could not be detected in our qRT-PCR assay.

Previously, miR-186, miR-15a, miR-23b, and miR-411 have been found to be differentially expressed in FSHD myoblasts (17, 40). Of these, we could only confirm up-regulation of miR-15a (supplemental Table S1). Other miRNAs, including miR-30e and miR-331, shown previously to be overexpressed in Duchenne muscular dystrophy (31), were not found to be differentially expressed in the present study (supplemental Table S1). Therefore, all but one FSHD-related miRNAs identified in our study were not previously associated with FSHD.

The increase in the amounts of mature miRNAs in FSHD myoblasts could be explained by an increased transcription of their genes, resulting in elevated expression of both pri-miRNA precursors and mature miRNAs; alternatively, an elevated rate of miRNA precursor processing could result in the increase in mature forms with or without a decrease in the expression of pri-miRNAs. Classical myogenic microRNAs miR-1, -133a/b, and -206 (41) were among the miRNAs most strongly overexpressed in proliferating FSHD myoblasts (Figs. 1*A* and 3*B* and supplemental Table S1). We have studied the expression of their precursors in the FSHD and control cells. Human myogenic miRNA precursors pri-miR-133a-2 (chr. 20) (see Fig. 8), pri-133a-1, and pri-miR-1–2 (chr. 18) were significantly up-regulated in FSHD myoblasts, whereas pri-miR-1-1 (chr. 20), pri-miR-206, and pri-miR-133b (chr. 6) demonstrated a trend for up-regulation in FSHD cells as compared with the normal

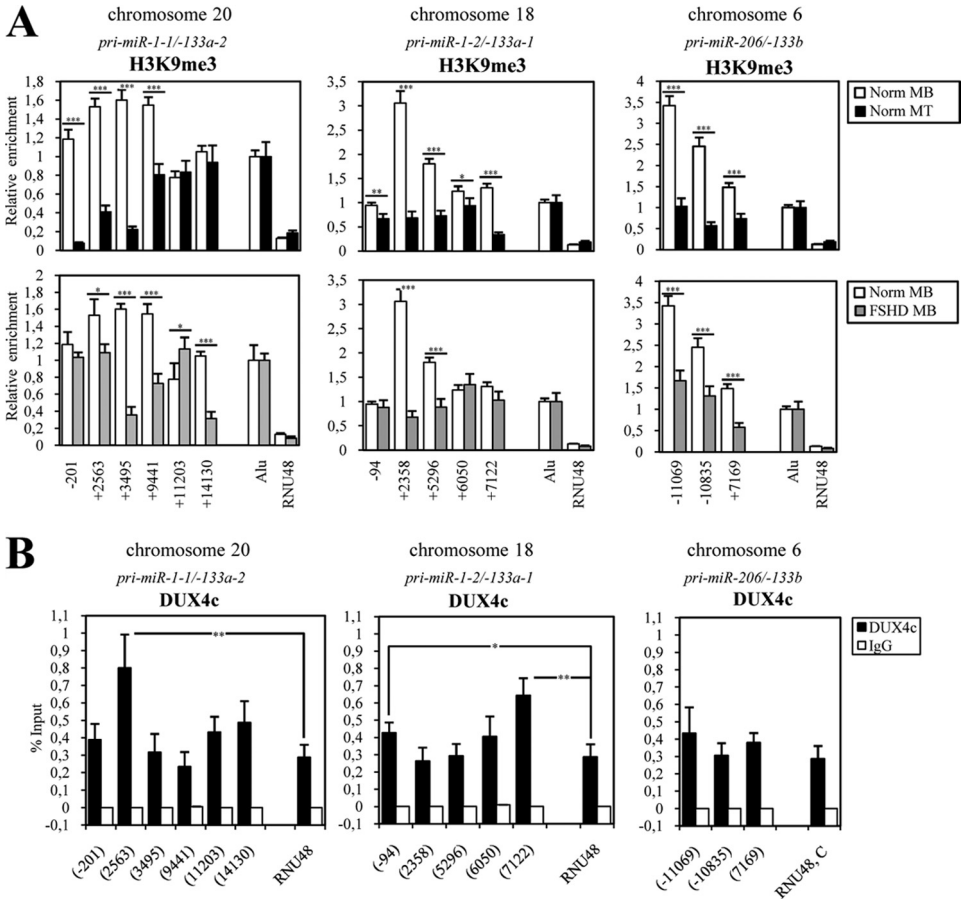

FIGURE 4. *A*, the promoter regions of myogenic microRNAs adopt an open chromatin structure during myogenic differentiation and in FSHD. The genomic DNA associated with H3K9me3 was immunoprecipitated from normal and FSHD myoblasts (*MB*) and myotubes (*MT*) via specific antibodies and quantified using qPCR (primers indicated in Fig. 2 and Table 2; their positions relative to the transcription start site of the corresponding pri-miRNA are indicated *below*). Relative enrichment was calculated as target DNA enrichment compared with input (percentage of input) normalized to percentage of input of the Alu repeat. *B*, DUX4c is bound to the promoter region of miR-1-1/miR-133a-2 pri-miRNA on chromosome 20 and miR-1-2/miR-133a-1 pri-miRNA on chromosome 18. Chromatin was immunoprecipitated using antibodies against DUX4c or preimmunization IgGs and quantified using qPCR. The RNU48 promoter region was used as a control, and data are expressed as percentage of input; *, $p < 0.5$; **, $p < 0.01$ (Student's *t* test). *Error bars*, S.E.

controls (Fig. 2*A*). As expected, the expression of human myogenic miRNA precursors and their mature forms was strongly up-regulated following myogenic differentiation *in vitro* of normal and FSHD myoblasts (Figs. 2*A* and 3*B*). In FSHD myotubes, we have observed a decrease in the expression level of all myogenic miRNA precursors (Fig. 2*A*) but not in the expression level of mature forms of these miRNAs as compared with controls (Fig. 3*B*).

Next, we have used ChIP followed by qPCR to examine the chromatin structure of promoters of miR-1, miR-133a/b, and miR-206 before and after myogenic differentiation and in FSHD. As expected, we have observed a significant reduction of the heterochromatin-specific histone 3 modification H3K9me3 on these promoters. A similar reduction of H3K9me3 was observed in proliferating FSHD myoblasts as compared with normal controls, supporting our observation that the expression of myogenic miRNA genes is higher in FSHD cells (Fig. 4). We conclude that the up-regulation of the mature forms of myogenic microRNAs can be explained by the up-regulation of their precursors in myoblasts and myotubes from the FSHD patients.

FR-miRs Are Differentially Expressed during Myogenic Differentiation—A defect of myogenic differentiation in FSHD has been documented previously by several groups (9, 13, 32). Here, we have found that classical myogenic microRNAs, miR-1, -133a, -133b, and miR-206 were up-regulated in FSHD myoblasts. This prompted us to test whether the expression of other FR-miRs is also myogenesis-dependent. Intriguingly, nine FR-miRs up-regulated in FSHD myoblasts were also up-regulated during normal myogenic differentiation (Fig. 3*A*).

The up-regulation of myogenesis-dependent miRNAs in proliferating FSHD myoblasts could be explained by a premature myogenic differentiation of FSHD cells. We thus measured the expression of myogenic markers, including desmin, myosin heavy chain 1, and others, and did not find evidence of their up-regulation in FSHD myoblasts (Fig. 3*C*). Therefore, the elevated expression of myogenesis-related FR-miRs in FSHD myoblasts was not due to their premature myogenic differentiation.

DUX4c Activates FR-miRs—Recent studies have indicated that the DUX4 and DUX4c transcription factors are overexpressed in FSHD and could play an important role in the onset of this disease (18–20). To test whether *DUX4* and *DUX4c* could be responsible for the observed up-regulation of FR-miRs in FSHD, we overexpressed these transcription factors in rhabdomyosarcoma TE-671 cells. Twelve of FR-miRs up-regulated in FSHD were found to be also up-regulated by DUX4c overexpression (Fig. 5*A*). Interestingly, six of these FR-miRs, including

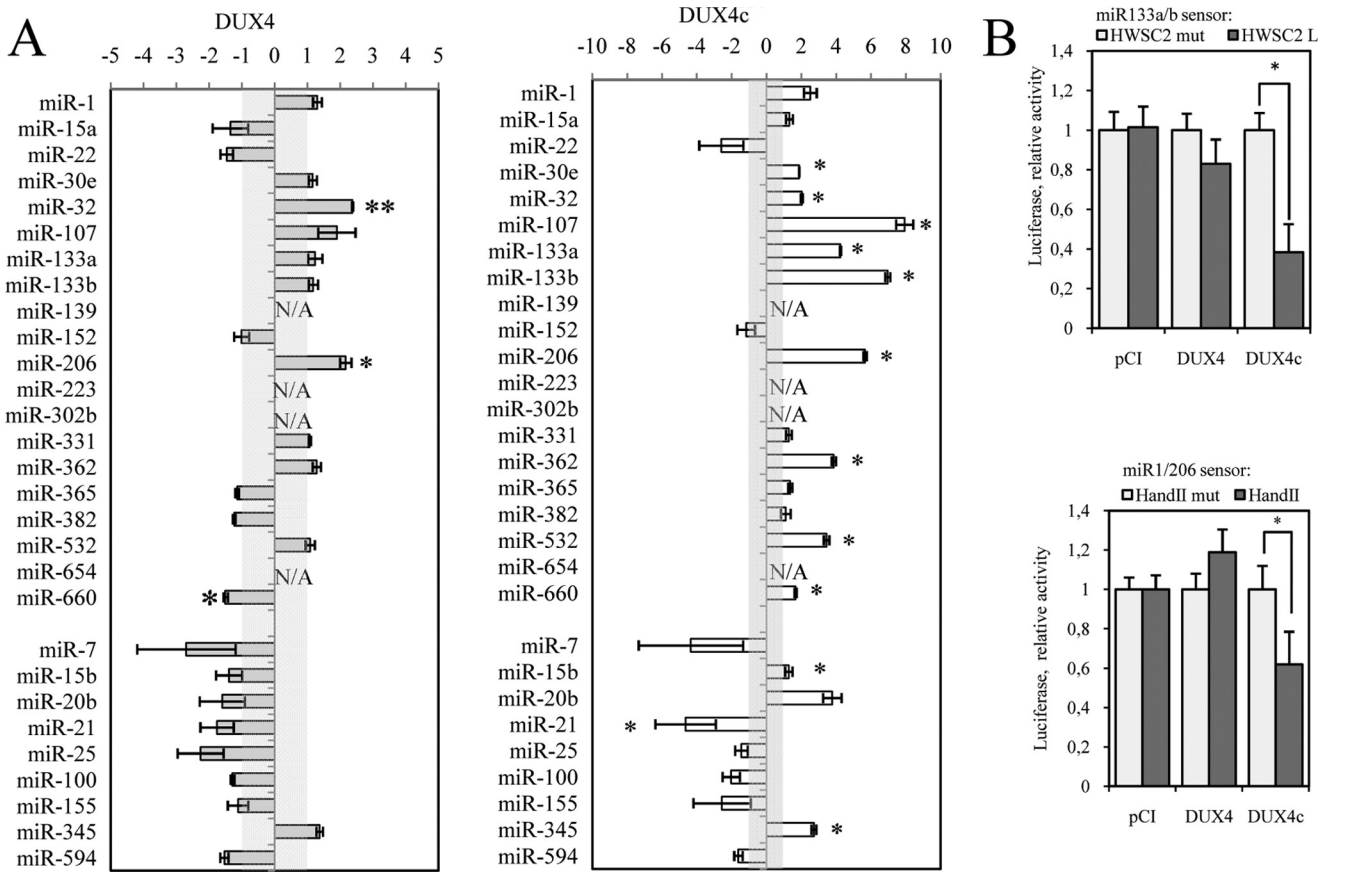

FIGURE 5. *A*, microRNAs activated by DUX4c and DUX4. The rhabdomyosarcoma cell line TE-671 was transiently transfected either with DUX4- or DUX4c-expressing plasmids. miRNA expression level was quantified by qRT-PCR normalized to RNU44. *N/A*, not applicable. *B*, effect of DUX4 and DUX4con expression of miR-1/133 and miR-206 sensors. Immortalized human myoblasts were transiently co-transfected with a combination of DUX4- or DUX4c-expressing plasmids and corresponding luciferase microRNA sensor plasmids. Luciferase activity was assayed 24 h post-transfection normalized to *Renilla* luciferase activity. Mean values are shown; *error bars* represent S.E. of four independent experiments. *, $p < 0.05$ (Student's *t* test).

TABLE 3

MicroRNAs differentially expressed in FSHD myoblasts (MB) and myotubes (MT) and during myogenic differentiation induced in normal human myoblasts

*, this study. Arrows pointing upward indicate up-regulation, and those pointing downward indicate down-regulation. —, no change. ND, microRNA is undetectable.

	MicroRNA	FSHD MB (this study)	FSHD MT (this study)	Normal myogenesis	TE-671 overexpressing: DUX4c (this study)	TE-671 overexpressing: DUX4 (this study)	Other muscular dystrophies
1	miR-1	↑	—	↑ (*) ↑ (64)	—	—	—
2	miR-7	—	↑	↓ (65)	—	—	—
3	miR-15a	↑ (*) ↑ (17)	—	↑ (*)	—	—	—
4	miR-15b	↓	—	↓ (65)	↑	—	—
5	miR-20b	↓	—	↓ (64)	—	—	—
6	miR-21	—	↓	↑ (*) (64)	↓	—	—
7	miR-22	↑	—	↑ (*)	—	—	—
8	miR-25	↓	—	—	—	—	—
9	miR-30e	↑	—	↑ (*)	↑	—	↑ (31)
10	miR-32	↑	—	—	↑	↑	—
11	miR-100	↓	—	—	—	—	—
12	miR-107	↑	—	↑ (*)	↑	—	—
13	miR-133a	↑	—	↑ (*) ↑ (64)	↑	—	—
14	miR-133b	↑	—	↑ (*) ↑ (64)	↑	—	—
15	miR-139	↑	—	↑ (65)	ND	ND	—
16	miR-152	↑	—	↑ (*)	—	—	—
17	miR-155	↓	—	↓ (*)	—	—	—
18	miR-206	↑	—	↑ (*) ↑ (66)	↑	↑	—
19	miR-223	↑	—	(ND in MT) (*)	ND	ND	—
20	miR-302b	↑	—	(ND in MB) (*)	ND	ND	—
21	miR-331	↑	—	↑ (*)	—	—	↑ (31)
22	miR-345	↓	—	—	↑	—	—
23	miR-362	↑	—	↑ (*)	↑	—	—
24	miR-365	↑	—	—	—	—	—
25	miR-382	↑	—	—	—	—	—
26	miR-532	↑	—	↑ (*)	↑	—	—
27	miR-594	—	↓	↓ (*)	—	—	—
28	miR-654	↑	—	↑ (65)	ND	ND	—
29	miR-660	↑	—	↑ (*)	↑	↓	—

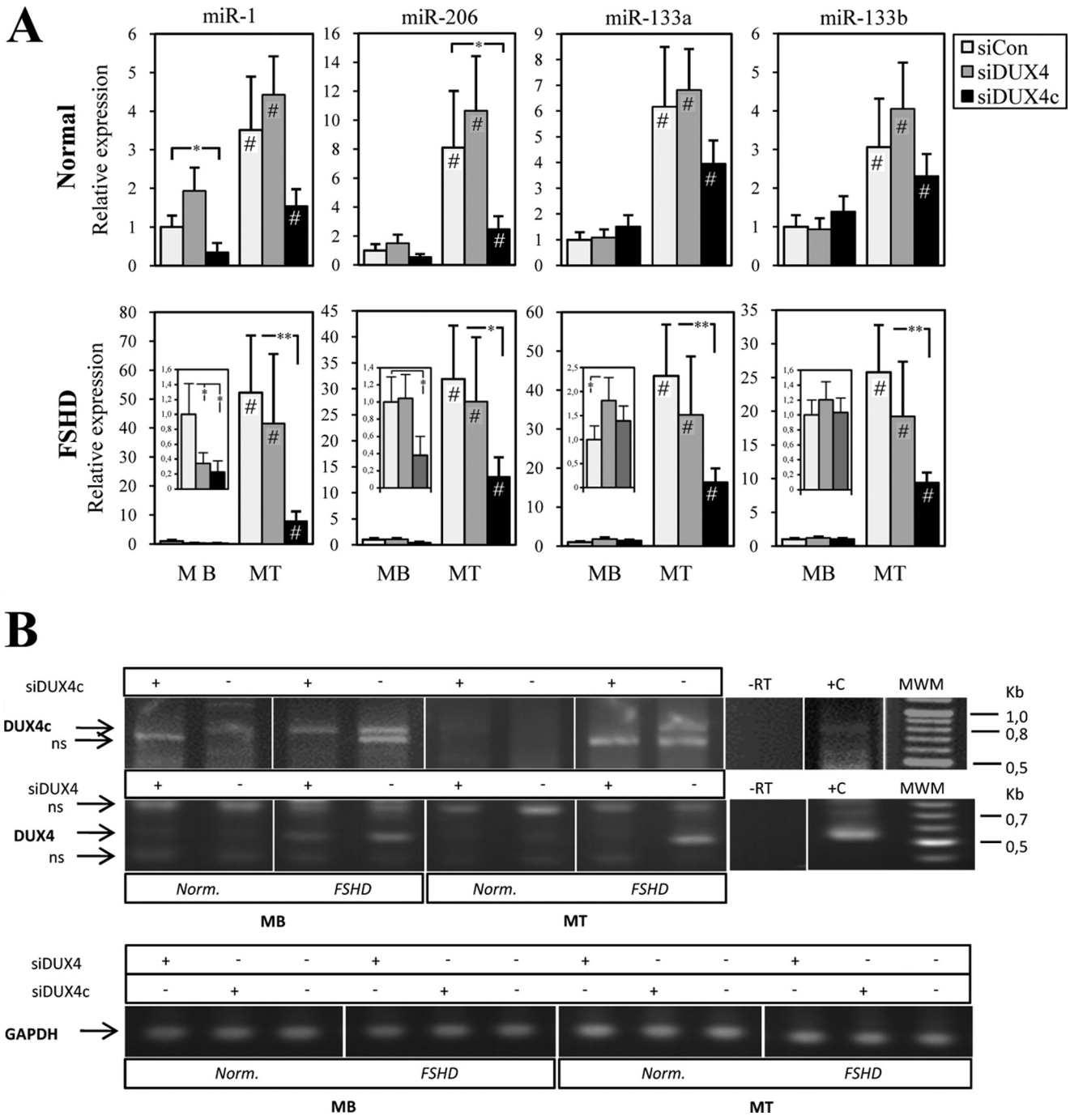

FIGURE 6. ***DUX4c* knockdown prevents myogenic microRNA from up-regulation during myogenic differentiation of both normal and FSHD myoblasts.** Primary myoblasts from one normal subject and one FSHD patient were transfected via siRNA against *DUX4c*, *DUX4*, or control siRNA. *A*, the expression level of miRNAs was quantified via qRT-PCR using the $\Delta\Delta Ct$ method using *RNU44* as control gene and control transfected myoblasts as a reference sample (expression level was arbitrarily set to 1). A representative experiment is shown. *B*, the expression level of *DUX4* and *DUX4c* or *GAPDH* genes was measured via RT-PCR. *Insets* show *close-ups* of miRNA expression data in proliferating myoblasts. *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$; #, statistically significant difference ($p < 0.05$) as compared with the myoblasts transfected with the same siRNAs. *MB*, myoblasts; *MT*, myotubes. *Error bars*, S.E.

the classical myogenic microRNAs miR-1, miR-133a, miR-133b, and miR-206, were also up-regulated during normal myogenic differentiation (Table 3). DUX4 overexpression mostly inhibited the miRNA expression with the exception of two microRNAs, miR-32 and miR-206, that were up-regulated in FSHD and induced by DUX4 overexpression (Fig. 5*A*).

We have next tested the effect of DUX4 and DUX4c overexpression in TE-671 cells on the expression of myogenic microRNA precursors (pri-miRs). We have found that DUX4c overexpression led to the up-regulation of pri-miR-133b and pri-miR-206 precursors from chromosome 6 and pri-miR-1 and pri-miR-133a precursors from chromosome 20 but not from chromosome 18 (Fig. 2*B*). DUX4 overexpression did not significantly alter the expression of myogenic microRNA precursors (Fig. 2*B*). Forced expression of MYOD can induce myogenic differentiation of rhabdomyosarcoma cells, although inefficiently

TABLE 4

Functional classification of genes targeted by miR-1, -133a, -133b and -206 in normal and FSHD myoblasts

The number of target genes down-regulated during myogenic differentiation of normal or FSHD myoblasts is indicated. *, less than 50% of target genes of a given functional class down-regulated during normal myogenesis are down-regulated during myogenic differentiation of FSHD myoblasts. The complete list of target genes of each microRNA analyzed can be found in supplemental Table S2.

	miR-1 target genes	downregulated in Norm	FSHD
Functional classes	transcription, negative reg. transcription, negative reg,	8	5
	cell cycle regulation	7	4
	apoptosis	6	5
	* DNA damage response	5	1
	* ubiquitination/proteolysis	4	2
	* chromatin organization/modification	4	1
	cytoskeleton organization	4	3
	cell motility	3	3
	* protein modification	3	1
	other functional classes	3	2
Target genes classified, total		22	14

	miR-133a target genes	downregulated in Norm	FSHD
Functional classes	transport/localization	9	8
	* DNA damage response	3	1
	phosphorylation	3	2
	RNA splicing	3	3
	* ubiquitination/proteolysis	3	1
	* cell cycle regulation	2	1
	transcription, negative reg.	2	2
	apoptosis	2	2
	other functional classes	2	2
Target genes classified, total		19	15

	miR-133b target genes	downregulated in Norm	FSHD
Functional classes	transport/localization	6	6
	* cell cycle regulation	3	1
	* DNA damage response	3	0
	cytoskeleton organization	3	2
	* phosphorylation	2	0
	RNA splicing	2	2
	* ubiquitination/proteolysis	2	0
	transcription, negative reg.	2	2
	other functional classes	2	2
Target genes classified, total		16	10

	miR-206 target genes	downregulated in Norm	FSHD
Functional classes	transcription, negative reg.	19	10
	* apoptosis	18	8
	* DNA damage response	17	5
	* cell cycle regulation	16	6
	* transport/localization	14	4
	reg. of kinase activity	12	7
	* cytoskeleton organization	11	5
	* nucleotide biosynthesis	10	4
	* phosphorylation	10	3
	cell motility	9	5
	* ubiquitination/proteolysis	9	2
	* chromatin organization/modification	9	2
	* other functional classes	9	3
Target genes classified, total		90	33

(42, 43). We overexpressed MYOD in TE-671 rhabdomyosarcoma cells and observed moderate up-regulation of pri-miRNA precursors of myogenic microRNAs. Interestingly, DUX4c knockdown prevented MYOD-dependent up-regulation of pri-miR-1 and -133a on chromosome 20 and pri-miR-133b and pri-miR-206 on chromosome 6 (Fig. 2*C*). Furthermore, we have found that DUX4c was bound to the promoter regions of pri-miR-1 and -133a on chromosomes 18 and 20 (Fig. 4*B*).

To confirm that the myogenic miRNAs induced by DUX4 and DUX4c, were functional, we next transfected immortalized myoblasts with DUX4- and DUX4c-expressing plasmids along with miR sensor plasmids containing a luciferase reporter gene fused to the 3′-UTRs of a gene containing either miR-133a/b (35) or miR-1/206 (34) recognition sites. DUX4c overexpression resulted in a reduced expression of both sensors but not of their mutant versions, indicating that DUX4c-induced myogenic miRNAs were functionally active, whereas the effect of the DUX4 overexpression on the miR reporter activity was not statistically significant (Fig. 5*B*).

Furthermore, to test whether *DUX4c* is required for the up-regulation of the expression of myogenic microRNAs during myogenic differentiation, we have knocked down *DUX4* and *DUX4c* expression in myoblasts and myotubes originating from one healthy donor and one FSHD patient and tested the expression of mature myogenic microRNAs in these cells. We have observed that *DUX4c* knockdown resulted in a significant decrease of the expression of all four myogenic microRNAs (miR-1, miR-133a, miR-133b, and miR-206) in normal and FSHD myoblasts (Fig. 6, *A* and *B*). We conclude that *DUX4c* might be required for the up-regulation of the myogenic microRNAs during myogenic differentiation in both normal and FSHD cells. Taken together, these results indicate that *DUX4c* contributes to the up-regulation of myogenic microRNAs during myogenic differentiation of both normal and FSHD cells.

Target Genes Escape the Repression by Myogenic miRNAs in FSHD—Our observation that myogenic microRNAs are up-regulated in FSHD myoblasts could be explained by a premature myogenic differentiation of these cells. However, myogenic differentiation markers were not up-regulated in FSHD (Fig. 3*C*). We therefore hypothesized that the overexpression of myogenic microRNAs observed in FSHD cells could be insufficient for the induction of the myogenic differentiation; alternatively, the functional activity of these miRNAs could be perturbed.

miRNAs regulate gene expression either at the level of transcript stability by inducing deadenylation and degradation of mRNA or by inhibiting the translation of their target genes (44). If a gene is controlled at the level of transcript stability by a given miRNA, its expression level will be low when the expression level of the corresponding miRNA is high; in other words, the expression levels of an miRNA and its target gene should be inversely correlated. To test whether the differential expression of miRNAs between FSHD and normal myoblasts had a functional significance, we thus decided to investigate the expres-

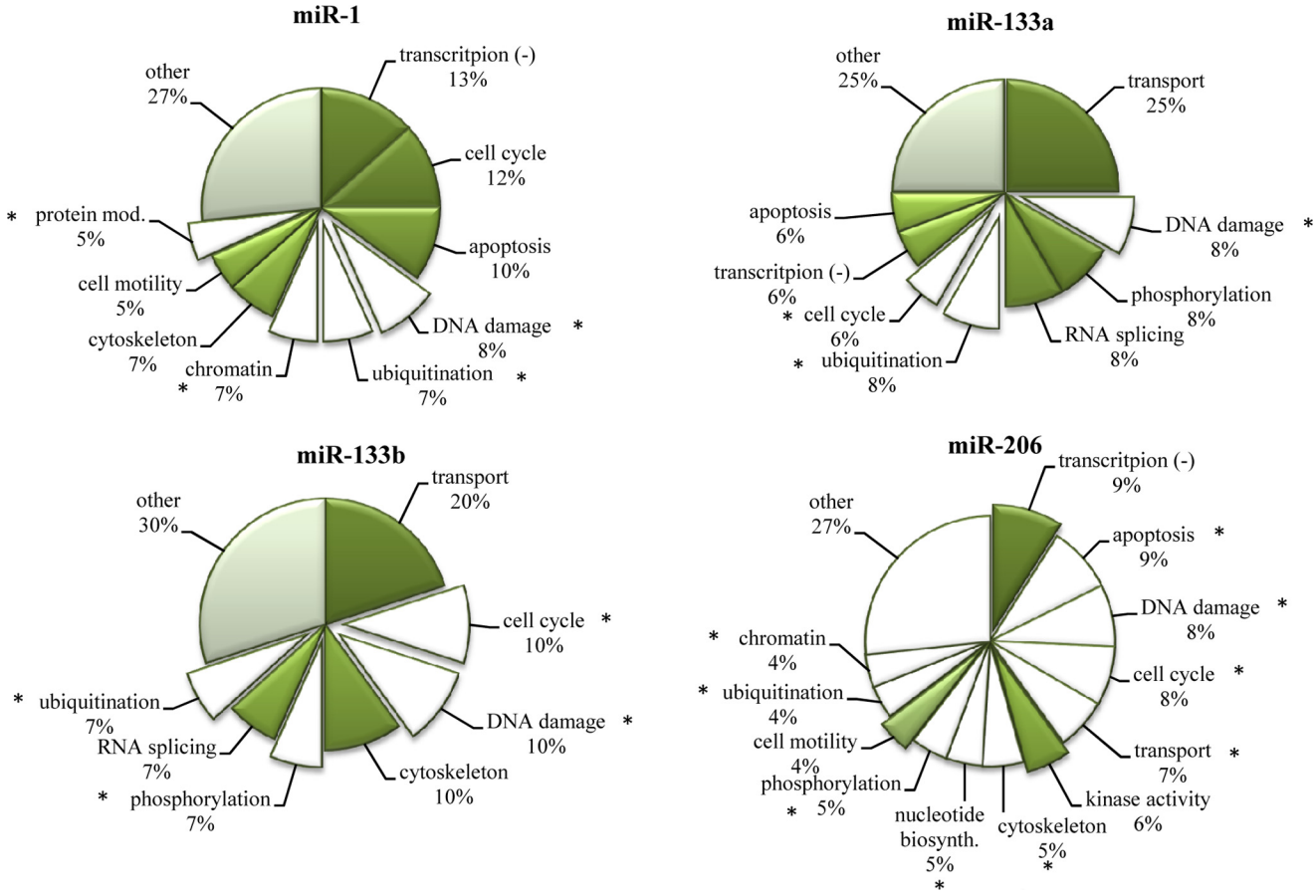

FIGURE 7. **Functional classification of FR-miR target genes.** Functional classes of target genes inversely correlated with FR-miR during normal myogenic differentiation are shown. *Pie chart sections* were left *blank* and *labeled* with an *asterisk* when the expression of 50% or less target genes was inversely correlated with that of FR-miR in FSHD cells.

sion level of their predicted target genes. The majority of validated microRNA target genes that are currently available from the public databases miRTar Base and MiRWalk are regulated at the level of translation, but these are outnumbered by genes regulated by miRNAs at the level of transcription (45, 46).

We thus chose to focus on target genes that are potentially regulated by myogenic miRNAs at the level of transcript stability. For this purpose, the following strategy was adopted. First, the RNA22 algorithm was used to predict target genes for each FR-miR (47). Then the expression level of RNA22-predicted target genes was tested in samples with high and low expression levels of FR-miRs (normal myotubes and myoblasts, respectively). Only those target genes with expression levels inversely correlated with FR-miR expression levels were considered as "supported by microarray data" (supplemental Table S2). We then analyzed the expression of supported myogenic miRNA target genes in FSHD as compared with normal myoblasts. In FSHD myoblasts with myogenic microRNAs up-regulated, a significant number of microarray-supported target genes were not down-regulated (Table 4). In the case of genes of miR-1 and miR-133b, only 57.6 and 68.4% of microarray-supported target genes were found down-regulated in FSHD myoblasts, respectively, indicating that myogenic microRNAs fail to down-regulate the totality of their microarray-supported target genes in FSHD myoblasts. Functional classification of the microarray-supported targets of the myogenic microRNAs has shown that in agreement with previous reports, they might be involved in the control cell cycle and myogenesis-related gene expression (41). In addition, these microRNAs could be implicated in the regulation of kinase activity, DNA damage response, ubiquitination, and others (Fig. 7, Table 4, and supplemental Table S3).

Interestingly, although some functions of myogenic microRNAs were well preserved in FSHD myoblasts, others seemed to be severely compromised with less than 50% of genes still down-regulated. These include cell cycle regulation, DNA damage response, ubiquitination/proteolysis, chromatin organization and modification, and cytoskeleton organization (Fig. 7, *green*). Taken together, our data provide evidence of an abnormal expression of myogenic miRNA target genes in FSHD.

DISCUSSION

Significant progress in the understanding of the pathogenesis of FSHD has been made possible by a combination of genomic (8, 48, 49), transcriptomic (9–11, 13, 17), and proteomic approaches (11, 14). Recently, non-coding RNAs have emerged as major players in FSHD (for a review, see Ref. 29). miRNA profiles of FSHD cells have been reported in several studies (17, 31), but so far, no model explaining the miRNA deregulation observed in FSHD has been put forward.

Here we have identified a specific miRNA profile of FSHD myoblasts and myotubes (Fig. 1). In total, we have found 29

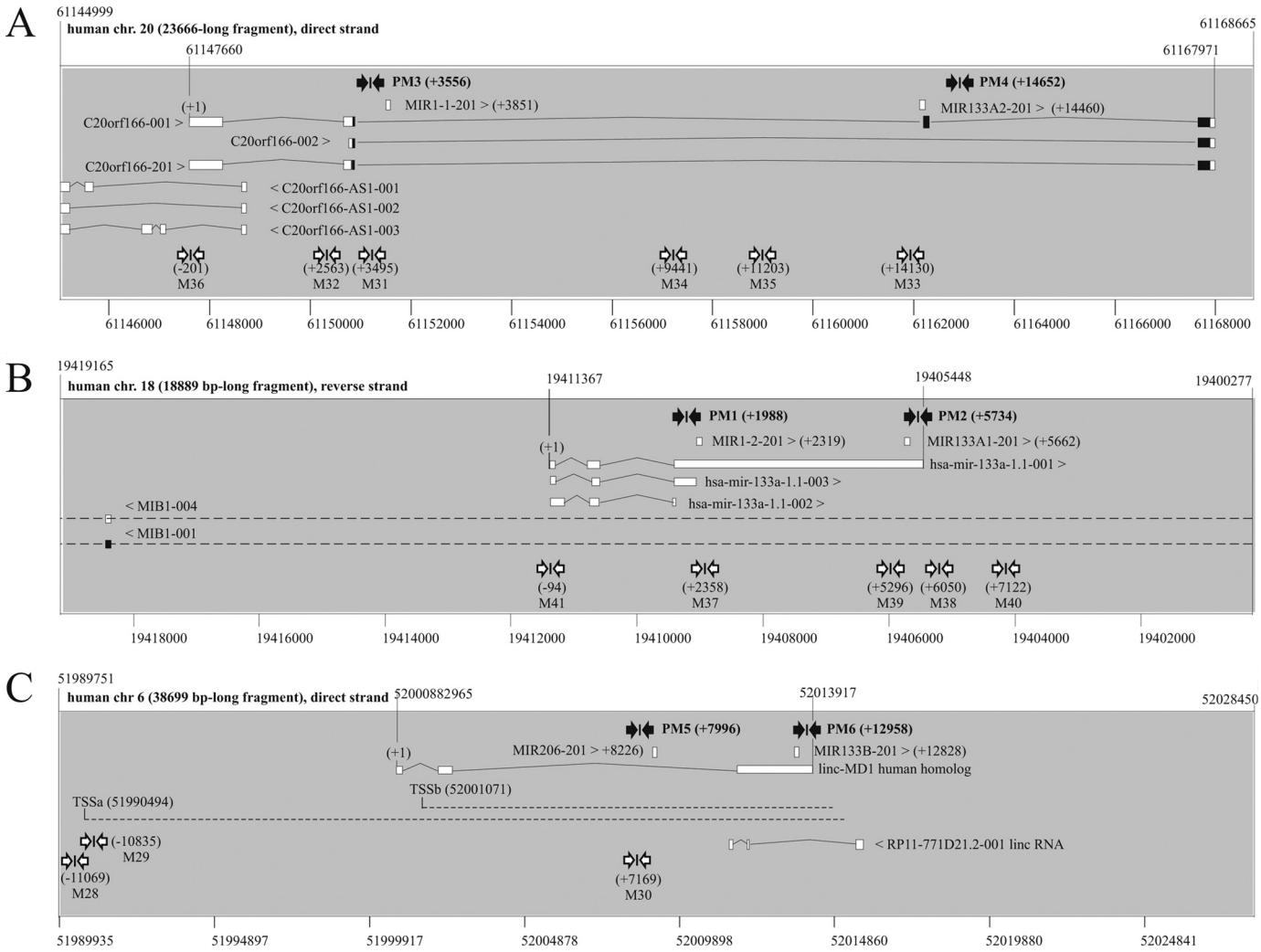

FIGURE 8. **Genes coding for human myogenic microRNAs miR-1, miR-133a, miR-133b, and miR-206.** miRNA genes are transcribed into 5′-capped and 3′-polyadenylated primary microRNA precursors (pri-miRs) that are processed by Drosha/Pasha endonucleases into pre-miRs, which are exported to cytoplasm, where the final Dicer-dependent processing step and mature miRNA production take place (for a review, see Ref. 44). Positions of PCR primers used for pri-miR expression analysis via qRT-PCR and chromatin structure analysis via ChIP are indicated with *black* and *white arrows*, respectively. Human miR-1 and miR-133a are encoded within two common bicistronic genes on chromosomes 20 and 18. *A*, intron-exon structure and genome context (Ensembl) of the putative pri-miR precursor of miR-1-1 and miR-133a-2 on chromosome 20. The transcription start site (TSS) of this precursor is defined by the c20orf166 TSS according to CAGE tags, TSS seq, and H3K4me3 ChIP data (60). *B*, intron-exon structure and genome context (Ensembl) of the putative pri-microRNA precursor of miR-1-2 and miR-133a-1 on chromosome 18. The TSS of this precursor is given according to Ref. 60. The structure of the human miR-206 and -133b genes is currently unknown; however, it has been suggested that it might be similar to that of the corresponding mouse genes (61). In mice, miR-133b is expressed as a common precursor containing both miR-206 and miR-133b; miR-206, however, is expressed from its own internal promoter (61). *C*, putative intron-exon structure and genome context (Ensembl) of primary microRNA precursor of miR-206 and miR-133b on chromosome 6 deduced from its mouse homolog linc-MD1 (61); genomic coordinates are given according to genome assembly hg19; TSSa is according to Ref. 62; TSSb is according to Ref. 60. Genomic coordinates are given according to human genome assembly hg19. Pre-miRNA coordinates were extracted from Ensembl. Genomic coordinates of pri-miRNA precursors, including their putative TSSs, were extracted from the Refs. 60, 62, and 63. Conversion of genomic coordinates, if needed, was performed via the UCSC Genome Browser LiftOver utility.

microRNAs, named here FR-miRs (FSHD-related microRNAs) differentially expressed in FSHD.

Of these, only three microRNAs were found to be differentially expressed in FSHD myotubes *versus* controls. Intriguingly, miRNAs differentially expressed in myoblasts and myotubes are not the same. A loss of differential expression of the majority of FR-miRs in differentiated myotubes may be explained by a higher variability of miRNA expression that we observed in myotubes.

Some FR-miRs described in this study were previously found to be overexpressed in other muscular dystrophies (*e.g.* miR-1 and miR-133 in Duchenne muscular dystrophy and miR-107 in limb-girdle muscular dystrophy) (31, 50). We have not been able to confirm the expression of some miRNAs found to be differentially expressed in FSHD by others (miR-186, -23, -411, and -517*) (17, 31, 40). The reason for this discrepancy might be the difference in cell culture procedures (*e.g.* using $CD56^+$ myoblasts *versus* non-purified myoblasts).

A significant proportion of the FR-miRs identified in this study are also up-regulated during normal myogenesis, among them the classical myogenic miRNAs miR-1, miR-133a, miR-133b, and miR-206 (Fig. 8) that are under control of the myogenic differentiation factors *MyoD*, *Mef2*, and *SRF* (51). However, the up-regulation of myogenesis-dependent miRNA in FSHD cannot be explained by a premature onset of myogenic differentiation in FSHD myoblasts, because myogenic markers were not up-regulated in FSHD cells. This suggests that one or

more factors distinct from classical myogenic differentiation regulators drive the overexpression of these miRNAs in FSHD myoblasts.

One of these factors could be DUX4c, an inducer of the *MYF5* gene, involved in myogenic determination (36). Previously, we had shown that *DUX4c* is overexpressed in FSHD myoblasts and myotubes (27, 36). *DUX4c* is overexpressed in most FSHD patients tested, although in some patients, *DUX4c* is missing from the chromosome 4q with the shortened D4Z4 repeat array (52, 53), which argues against a causal role of this gene in the FSHD etiology. Unfortunately, in these patients, neither *DUX4c* expression levels nor miRNA profiles have been determined; therefore, we cannot exclude the possibility that DUX4c originating from a homologous chromosome contributes to the pathological phenotype in these patients.

Here, we have obtained multiple lines of evidence that *DUX4c* is an activator of the expression of the myogenic microRNAs. We have demonstrated that DUX4c overexpression induced expression of myogenic microRNAs and their precursors in TE-671 rhabdomyosarcoma cells. Conversely, *DUX4c* knockdown resulted in down-regulation of microRNAs in primary myotubes from normal subjects and FSHD patients. Interestingly, the effect of *DUX4c* knockdown on the expression of myogenic microRNAs was stronger in FSHD as compared with control myotubes. At the moment, we cannot conclude whether the stronger impact of *DUX4c* knockdown on the expression of myogenic miRNAs in FSHD cells is a general phenomenon, because only one cell line from a normal control and an FSHD patient was used in the study; however, our results clearly indicate that *DUX4c* might be a novel regulator of the expression of myogenic microRNAs.

Ectopic overexpression of any of the four classical myogenic microRNAs is sufficient for induction of the myogenic differentiation in skeletal myoblasts (54). However, premature differentiation is not observed in FSHD myoblasts expressing all four myogenic miRNAs. Failure to induce myogenic differentiation can be explained by a low level of overexpression of myogenic miRNAs in FSHD myoblasts; alternatively, one can suggest that the function of these miRNAs is impaired in FSHD myoblasts.

Indeed, we observed that several target genes of the myogenic miRNAs were not down-regulated in FSHD myoblasts, as would be expected if they were still under control of these miRNAs (Fig. 4). A similar effect has been observed previously in a mouse model with simultaneous up-regulation of a microRNA, namely miR-206, and its target gene urotropin (55). Functional classification of myogenic miRNA target genes indicated that whereas most functions remained virtually unchanged, genes specifically related to cell cycle control, DNA damage response, and ubiquitination escaped miRNA-dependent repression in FSHD myoblasts.

There are several possible explanations for this phenomenon. First, the miRNA-dependent repression pathway could be impaired in FSHD. Second, some other factors could override the miRNA-dependent regulation of these genes in FSHD. In favor of the former model, genes encoding the components of RNAi machinery, including all four Argonaute genes and the Dicer 1 gene have been reported to be down-regulated in FSHD myotubes (9), suggesting that miRNA-dependent transcript destabilization and pre-miRNA processing might be perturbed in FSHD cells. In addition, altered pre-miR processing might lead to generation of miRNA isoforms with an altered spectrum of target genes (for a review, see Ref. 56). In favor of the latter model, it has been demonstrated that several potent transcription factors, including *DUX4* (7–9) and *KLF15* (27), are up-regulated in FSHD. DUX4 up-regulates the expression of the cyclin A gene (57, 58), which could, at least in part, explain why cell cycle-related genes escape the control of FR-miRs. Furthermore, up-regulation of one miRNA target gene may lead to saturation of RNAi machinery and a consequent reduction in the miRNA-dependent repression of other target genes (59).

The specific escape of cell cycle and DNA damage response-related genes from FR-miR control, whatever the mechanism is, indicates that FSHD myoblasts simultaneously express two competing biological programs: (i) the myogenic differentiation program as indicated by the overexpression of myogenesis-related microRNAs orchestrated by DUX4c and the concomitant repression of the majority of their target genes and (ii) the proliferation program as indicated by the overexpression of cell cycle and DNA damage-related genes. Because the successful completion of myogenic differentiation requires cell cycle arrest, the simultaneous expression of two incompatible programs may explain why FSHD myoblasts demonstrate a defect in myogenic differentiation, as observed previously (10, 11, 13, 14), rather than prematurely engage into the myogenic differentiation program.

Acknowledgments—We thank V. Mouly for donating human immortalized myoblasts, Gianluigi Condorelli and Deepak Srivastava for miR-133a/b and miR-1/206 reporter plasmids, and Cecile Cassan for critical review of the manuscript.

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J. Biol. Chem. 2013, 288:34989-35002.
doi: 10.1074/jbc.M113.504522 originally published online October 20, 2013
