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Three-dimensional imaging in myotonic dystrophy type 1

Linking molecular alterations with disease phenotype

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Abstract

Objective

We aimed to determine whether 3D imaging reconstruction allows identifying molecular: clinical associations in myotonic dystrophy type 1 (DM1).

Methods

We obtained myoblasts from 6 patients with DM1 and 6 controls. We measured cytosine-thymine-guanine (CTG) expansion and detected RNA foci and muscleblind like 1 (MBNL1) through 3D reconstruction. We studied dystrophia myotonica protein kinase (DMPK) expression and splicing alterations of MBNL1, insulin receptor, and sarcoplasmic reticulum Ca(2+)-ATPase 1.

Results

Three-dimensional analysis showed that RNA foci (nuclear and/or cytoplasmic) were present in 45%–100% of DM1-derived myoblasts we studied (range: 0–6 foci per cell). RNA foci represented <0.6% of the total myoblast nuclear volume. CTG expansion size was associated with the number of RNA foci per myoblast (r = 0.876 [95% confidence interval 0.222–0.986]) as well as with the number of cytoplasmic RNA foci (r = 0.943 [0.559–0.994]). Although MBNL1 colocalized with RNA foci in all DM1 myoblast cell lines, colocalization only accounted for 1% of total MBNL1 expression, with the absence of DM1 alternative splicing patterns. The number of RNA foci was associated with DMPK expression (r = 0.967 [0.079–0.999]). On the other hand, the number of cytoplasmic RNA foci was correlated with the age at disease onset (r = −0.818 [−0.979 to 0.019]).

Conclusions

CTG expansion size modulates RNA foci number in myoblasts derived from patients with DM1. MBNL1 sequestration plays only a minor role in the pathobiology of the disease in these cells. Higher number of cytoplasmic RNA foci is related to an early onset of the disease, a finding that should be corroborated in future studies.

MORE ONLINE

Video

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Glossary

CI = confidence interval; **DM1** = myotonic dystrophy type 1; **DMPK** = dystrophia myotonica protein kinase; **IR** = insulin receptor; **MBNL1** = muscleblind like 1; **mRS** = modified Rankin Scale; **qPCR** = quantitative PCR; **RT-PCR** = reverse transcription PCR.

Myotonic dystrophy type I (DM1) is a multisystemic disorder with autosomal dominant inheritance caused by a cytosine-thymine-guanine (CTG) repeat expansion in the 3′UTR of the dystrophia myotonica protein (*DMPK*) gene.[1] The main pathogenic process underlying DM1 is a toxic RNA gain-of-function effect of expanded DMPK transcripts forming hairpin-structured aggregates, called "RNA foci."[2,3] These aggregates are able to sequester splicing factors, thereby affecting the alternative splicing of some pre-mRNAs such as insulin receptor (IR), sarcoplasmic/endoplasmic reticulum Ca(2+)-ATPase 1 (ATP2A1),[4,5] or muscleblind like 1 (MBNL1).[6]

Most studies on DM1 have relied on 2D imaging at a single cell level. Yet, this classic approach does not allow detection of all RNA foci aggregates nor identification of their location inside cells. In addition, with this technique, it is not possible to identify the distribution and sequestration of MBNL1 protein. These limitations have hindered recognition of the full cascade of molecular events associated with the phenotype manifestation of DM1.

Here, we used 3D imaging in whole myoblasts derived from patients with DM1 with different degrees of affectation in an attempt to gain insight into the link between DM1 molecular players and disease phenotype. Thus, we assessed the relationship between CTG repeat expansion size, RNA foci, MBNL1 colocalization, and splicing defects in DM1 patient-derived myoblasts, on the one hand, and the clinical characteristics of the patients, on the other hand. We have obtained new pathophysiology data related to how DM1 might affect patient-derived myoblasts.

Methods

Standard protocol approvals, registrations, and patient consents

This study was approved (reference # PI-15-009) by the Ethic Committee of the University Hospital Germans Trias i Pujol (Badalona, Spain) and was performed in agreement with the Declaration of Helsinki for Human Research. All participants signed a written informed consent to participate in the study.

Participants

Six patients were recruited for this study, with DM1 diagnosis confirmed in our laboratory by bidirectional triplet PCR, as previously described.[7] Patient muscle biopsies were obtained from the left biceps brachialis (n = 5) and vastus lateralis muscle (n = 1). The control biopsies were obtained from the hand muscle abductor pollicis longus of healthy individuals (see supplemental data, links.lww.com/NXG/A291, for more information on the participants and clinical data collection).

Myoblast cultures

We extracted muscle cells from the biopsy explants and placed them on culture plates between 2015 and 2016 in the cell culture room of our institution (see supplemental data, links.lww.com/NXG/A291, for more information) and analyzed data from these myoblasts during 2018 and 2019 as described below.

Expansion repeat sizing in myoblasts

We determined CTG expansion size using long PCR-southern blotting (see supplemental data, links.lww.com/NXG/A291, for a detailed description of the protocol).

Three-dimensional imaging of RNA foci and MBNL1

To detect RNA foci and MBNL1, we performed fluorescence in situ hybridization and immunostaining. The protocol we used and the details of 3D imaging can be found in supplemental data, links.lww.com/NXG/A291.

Alternative splicing analysis and *DMPK* expression

We analyzed alternative splicing and *DMPK* expression with reverse transcription (RT) PCR and quantitative PCR (qPCR), respectively (see supplemental data, links.lww.com/NXG/A291, for more information on the protocol and primers).

Statistical analysis

Details of the statistical analysis can be found in supplemental data, links.lww.com/NXG/A291.

Data availability

The data that support the findings of this study are available from the corresponding author on reasonable request.

Results

Patients' phenotype

We studied 6 patients with DM1—4 women and 2 men—with age at disease onset ranging from 15 to 48 years (table 1). Patients were unrelated except P3 and P4 (who were sisters). The majority of patients had cardiac problems, with 3 showing mild ECG alterations, one (P2) a structural cardiopathy (valvulopathy), and another one (P5) using a pacemaker. Half of the patients needed nocturnal mechanical ventilation, and the other 3 had mild changes in respiratory functional tests without actual clinical impairment. The muscular impairment status according to the Muscular Impairment Rating Scale was

Table 1 Clinical data of patients with DM1

Patient with DM1	Sex	Age at onset (y)	Biceps MRC	Myotonia (s)	Cataracts	Cardiopathy	Spirometry	6MWD (m)	MIRS	mRS
P1	F	15[a]	4	0.52	No	LAFB	Altered PFT	348	4	2
P2	M	48	5	0.67	Yes	Valvulopathy	NMV	251	3	2
P3	F	36	5	0.73	Yes	None	NMV	368	2	1
P4	F	42	5	0.98	Yes	1st-degree AV block	NMV	338	3	1
P5	F	27	4	NP	Yes	Pacemaker	Altered PFT	NP	4	4
P6	M	36	5	0.96	No	LAFB	Altered PFT	519	3	2

Abbreviations: 6MWD = 6-minute walking distance; AV = atrioventricular; NP = not performed; LAFB = left anterior fascicular block; mRS = modified Rankin Scale; MIRS = Muscular Impairment Rating Scale; MRC = Medical Research Council Grade; NMV = nocturnal mechanical ventilation; PFT = pulmonary function tests.
[a] It was not possible to determine the actual age at onset of patient P1, but since at the first visit (age 36 years), she had obvious signs that commonly appear in the early patient's life—including oval pallor and temporal atrophy—we considered that the disease onset occurred during adolescence.

variable, with P3 showing minimal signs of muscular impairment on exploration, P2, P4, and P6 presenting with distal weakness, and P1 and P5 with mild-to-moderate proximal weakness. Most patients were independent in activities of daily living (modified Rankin Scale [mRS] score 0–2), yet one (P5) had severely limitations (mRS score 4). P5 did not to do the 6-minute walking distance test or the myotonic evaluation because she died unexpectedly due to respiratory insufficiency at age 40 years.

CTG expansion correlates with RNA foci

We determine the CTG expansion size and the RNA foci and MBNL1 staining analyses in the same cells by dividing the cell lines in different pools. The CTG expansion size in DM1 myoblast cell lines ranged from 195 to 1568 CTG repeats (table 2).

For the staining of RNA foci and MBNL1, we studied 71 and 84 myoblasts from patients and controls, respectively. There was a high heterogeneity among DM1 myoblasts with regard to number of RNA foci. Thus, there was an average of 3 RNA foci (cytoplasmic and/or nuclear) per cell (range 0–6). On the other hand, there were no RNA foci in the control myoblasts (supplemental figure 1, links.lww.com/NXG/A298).

Heterogeneity was also found for the percentage of cells carrying RNA foci. Thus, 4 patients had RNA foci in 100% of their myoblasts, whereas P2 and P6 had RNA foci in 80% and 45% of myoblasts, respectively. Of interest, the cell lines from P2 and P6 were those carrying the lowest CTG repeat number (195 and 230, respectively). We found a strong positive correlation between CTG repeat number and (1) the average number of total (cytoplasmic + nuclear) RNA foci per myoblast (figure 1A), as well as with (2) the average number of nuclear RNA foci per myoblast (r = 0.830; 95% confidence interval [CI] 0.056–0.981).

RNA foci in DM1 myoblasts are mostly, but not only, intranuclear

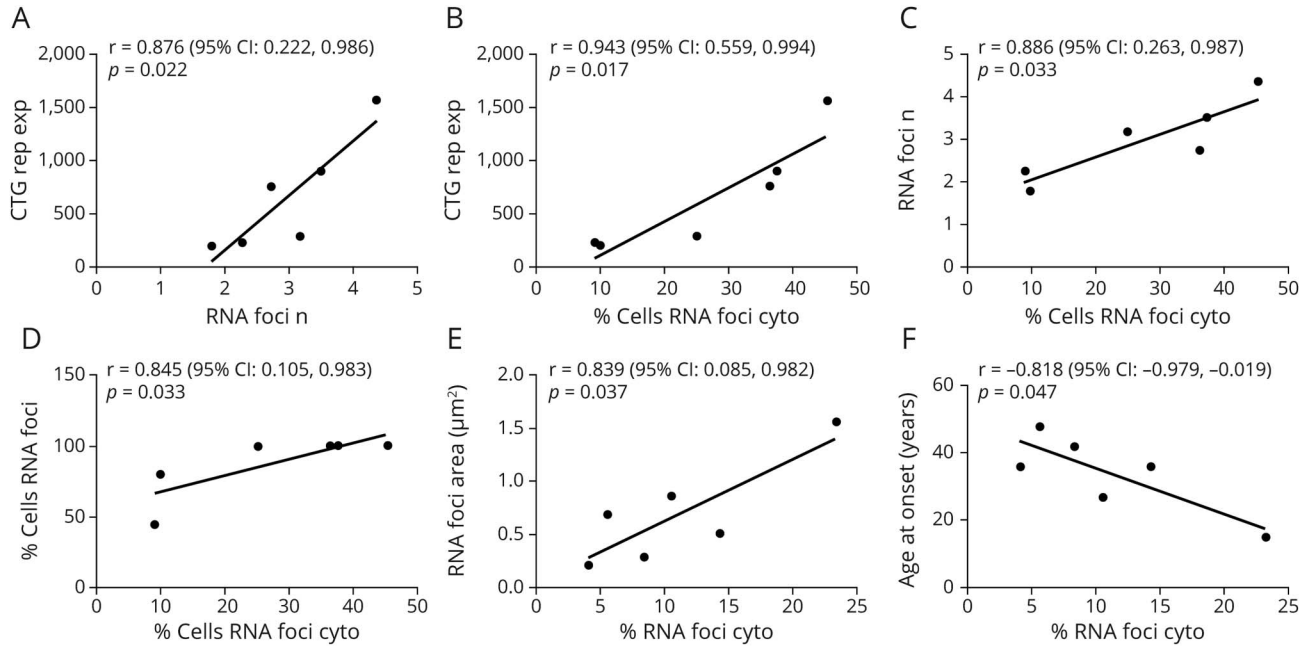
RNA foci were mainly present in the nuclei of DM1 cells. Nevertheless, all 6 DM1 myoblast lines also showed RNA foci outside this organelle. The prevalence of cytoplasmic RNA

Table 2 CTG expansion size and 3D analysis data in DM1 myoblasts

Derived DM1 cell line	CTG repeats	No. of cells analyzed	Minimum no. of RNA foci/cell	Maximum no. of RNA foci/cell	RNA foci/ cell	RNA foci area average (μm^2)	% of cells containing RNA foci	% RNA foci in the cytoplasm	% of cells containing both nuclear and cytoplasmic RNA foci
P1	758	11	1	6	2.7	1.6	100	23	36
P2	195	10	0	5	1.8	0.7	80	6	10
P3	899	16	2	6	3.5	0.5	100	14	38
P4	1,568	11	2	6	4.4	0.3	100	8	45
P5	292	12	1	6	3.2	0.9	100	11	25
P6	230	11	0	5	2.3	0.2	45	4	9

Abbreviation: DM1 = myotonic dystrophy type 1.

Figure 1 Significant correlations of 3D analysis data, CTG expansion size, and phenotype in DM1 myoblasts



(A) Correlation between CTG repeat expansion and RNA foci number. The 95% CI for the Pearson correlation coefficient was 0.222–0.986. (B) Correlation between CTG repeat expansion and percentage of cells presenting cytoplasmic RNA foci. The 95% CI for the Pearson correlation coefficient was 0.559–0.994. (C) Correlation between RNA foci number and % of cells presenting cytoplasmic RNA foci. The 95% CI for the Pearson correlation coefficient was 0.263–0.987. (D) Correlation between % of cells presenting RNA foci and % of cells presenting cytoplasmic RNA foci. The 95% CI for the Pearson correlation coefficient was 0.105–0.983. (E) Correlation between the RNA foci area (μm^2) and the presence of cytoplasmic RNA foci. The 95% CI for the Pearson correlation coefficient was 0.085–0.982. (F) Correlation between age at onset of the disease and the presence of cytoplasmic RNA foci. The 95% CI for the Pearson correlation coefficient was −0.979 to −0.019. rep exp = repeat expansion; n = number; cyto = cytoplasm.

foci was, however, heterogeneous—ranging from 4% to 23% of all RNA foci found (mean = 12%) (table 2). The percentage of myoblasts showing cytoplasmic RNA foci also varied among the different DM1 patient lines, ranging from 10% to 45% of cells. On the other hand, there were no cells showing cytoplasmic-only RNA foci.

We found a strong, positive correlation between the number of CTG repeats and the percentage of cells with cytoplasmic RNA foci (figure 1B). We also found a positive correlation between the average total number (nuclear + cytoplasmic) of RNA foci per myoblast and the percentage of cells presenting RNA foci in the cytoplasm (figure 1C) and also between the percentage of DM1 myoblasts presenting RNA foci (nuclear and/or cytoplasmic) and the percentage of RNA foci in the cytoplasm (figure 1D). We further analyzed the area occupied by RNA foci (table 2) and found a positive correlation between this variable and the presence of cytoplasmic foci (figure 1E). Thus, those cell lines that contained RNA foci with larger areas had more foci in the cytoplasm.

Higher number of cytoplasmic RNA foci is associated with an earlier disease onset

When analyzing the relationship between the 3D molecular findings and the phenotype of patients with DM1, we found that the number of cytoplasmic RNA foci was inversely related to the age at disease onset (figure 1F).

RNA foci only represent a small fraction of the total nucleus volume

The portion of the total nuclear dimension occupied by RNA foci was analyzed by determining the volume of all the RNA foci inside the nucleus and by comparing this variable with the total nuclear volume. RNA foci occupied 0.34%–0.53% of the total nuclear volume in DM1 myoblasts.

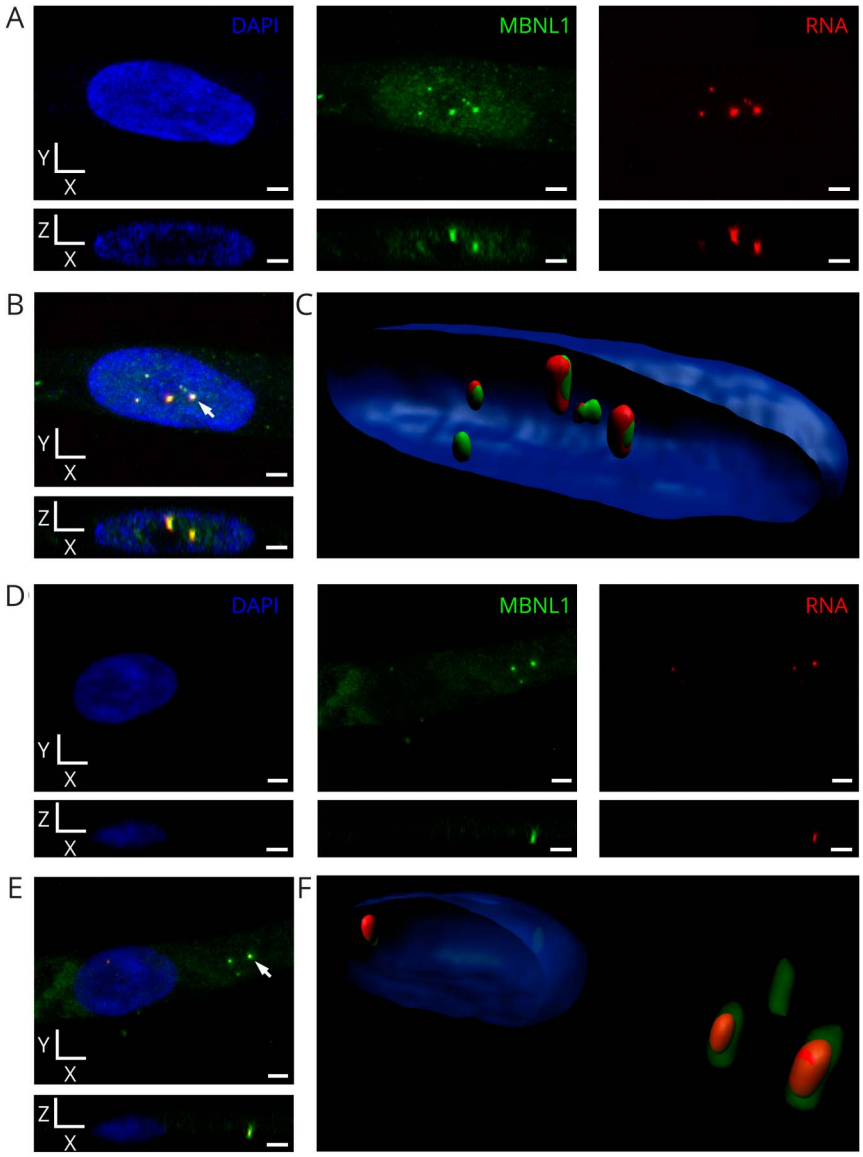
Trapped MBNL1 in RNA foci represent a negligible fraction of total MBNL1 expression

Given the main role that MBNL1 plays in DM1 disease, we aimed to analyze the trapping of this protein in the RNA foci. MBNL1 protein was present in control and DM1 myoblasts. In all analyzed myoblasts, MBNL1 was located both in the nucleus and in the cytoplasm (figure 2, B and E). Colocalization of RNA foci together with MBNL1 was found in all DM1 patients' lines. The trapped MBNL1 was not only found in the nucleus (figure 2, A–C), but also in the cytoplasm (figure 2, D–F), representing 9% of the total cellular colocalization (Video 1). Trapped MBNL1 represented less than 1% of the total MBNL1 expression in DM1 myoblasts.

RNA foci are associated with DMPK transcripts

As RNA foci are the product of *DMPK* gene transcription, we aimed to analyze DMPK transcripts in our myoblast cell lines. To this end, we chose qPCR as it is a more sensitive technique than RT-PCR and used a probe that was located outside the

Figure 2 Confocal microscopy of DM1 myoblasts



MBNL1 protein (green), RNA foci (red), nucleus (blue). Scale bars: 2.5 μm. (A) XY and XZ volume slices of individual channels corresponding to nucleus (blue), MBNL1 protein (green), and RNA foci (red). DM1 myoblast showing the nuclear colocalization between the RNA foci and the MBNL1 protein. (B) Merged image of XY and XZ volume slices of representative nuclear colocalization between RNA foci and MBNL1 protein in DM1 myoblast. White arrow indicates a single RNA foci aggregate colocalizing with MBNL1 protein. (C) Isosurface rendering of nuclear colocalization between RNA foci and MBNL1 protein in DM1 myoblasts. (D) XY and XZ volume slices of individual channels corresponding to nucleus (blue), MBNL1 protein (green), and RNA foci (red). DM1 myoblast showing the cytoplasmic colocalization between the RNA foci and the MBNL1 protein. (E) Merged image of XY and XZ volume slices of representative cytoplasmic colocalization between RNA foci and MBNL1 protein in DM1 myoblast. White arrow indicates a single RNA foci aggregate colocalizing with MBNL1 protein. (F) Isosurface rendering of cytoplasmic colocalization between RNA foci and MBNL1 protein in DM1 myoblasts. MBNL1 = muscleblind like 1.

CTG repeat for detecting expression of both wild-type and expanded *DMPK* alleles. We did not find significant differences between the *DMPK* expression of patients and controls myoblasts (figure 3A). In turn, we found a positive correlation between *DMPK* expression and (1) total (nuclear and/or cytoplasmic) RNA foci number (r = 0.967 [95% CI 0.079–0.999], p = 0.033), as well as with the RNA foci that were present in the nucleus only (r = 0.993 [95% CI 0.682–0.999], p = 0.008).

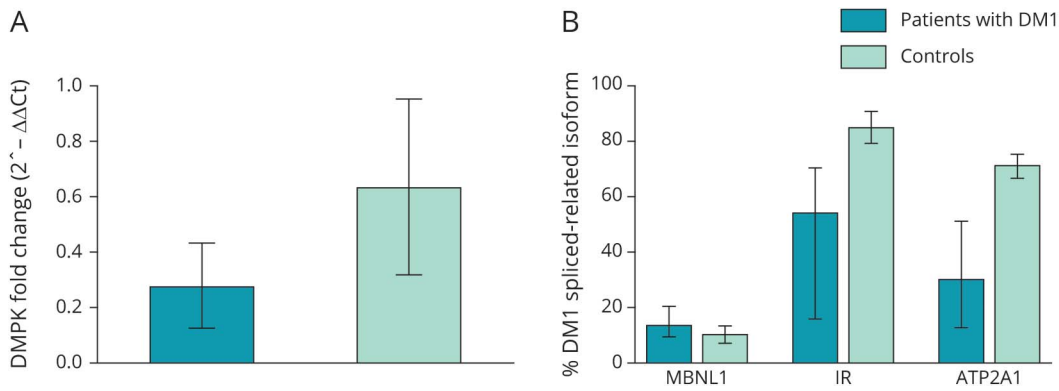
Absence of DM1 alternative splicing patterns in DM1 myoblasts

To study the extent to which was alternative splicing affected in DM1 myoblasts, we analyzed the expression of inclusion and exclusion isoforms—MBNL1 exon 7, INSR exon 11, and ATP2A1 exon 22—that have been previously reported to be altered in DM1.[4,8,9] No differences were found between controls and patients in the expression of MBNL1 or ATP2A1 isoforms. However, the expression levels of the INSR exon 11 exclusion isoform—the most expressed isoform in some DM1 models—showed a tendency of higher expression in controls than in patients (figure 3B).

Discussion

We studied the quantity and distribution of RNA foci and MBNL1 protein in DM1 patient–derived myoblasts using a novel 3D imaging approach. In addition, in the same myoblast cultures, we measured CTG expansion, DMPK expression, and some alternative splicing events. All the molecular data were correlated with the clinical characteristics of the patients with DM1. Thus, although more research is needed, the 3D

Figure 3 qDMPK expression and splicing analysis



Data are mean ± SD. (A) qDMPK expression in patients with DM1 vs controls. (B) Percentage of the DM1 spliced-related isoform in patients with DM1 compared with controls for MBNL1, IR, and ATP2A1 transcripts measured with RT-PCR. MBNL1 and ATP2A1 showed no significant differences, whereas IR showed a tendency of higher expression in controls compared with patients ($p = 0.057$; effect size [Cohen d] = 1.83). DMPK = dystrophia myotonica protein; IR = insulin receptor; MNBL1 = muscleblind like 1.

method we used appears as a potential tool to identify potential associations between molecular alterations and clinical manifestations in patients with DM1.

Our results indicate that the number of RNA foci per DM1 myoblast is regulated by the CTG expansion length. A previous study showed that RNA foci number differed between patients with DM1 carrying <500 or >1000 CTG repeats, respectively.[10] In this regard, we have analyzed patients carrying CTG expansions within the 500–1,000 range, with RNA foci related to CTG repeat number. On the other hand, our data indicate that there are a small number of RNA foci per DM1 myoblast, averaging only 3 per cell (range 0–6), which is consistent with the findings of previous research in muscle tissue.[3,10–12]

Of interest, not all the DM1 myoblasts derived from the same patient had RNA foci. Two lines of patient-derived myoblasts who carried the shorter expansions showed RNA foci only in 40% and 80% of the cells. Thus, our results suggest that CTG expansion not only regulates the amount of RNA foci per myoblast but also the number of myoblasts presenting RNA foci. In this regard, previous research has suggested that RNA foci can undergo dynamic changes during the cell cycle[13] and travel from one cell to another.[14] Thus, although we cannot discard a certain effect of the cell cycle phase on our results, we tried to minimize this potential confounder by seeding the different DM1 myoblast cell lines following a well-defined protocol and using consistently the same treatment method. Based on the expansion sizes that we studied, DM1 myoblasts carrying expansions close to—or larger than—300 CTG repeats would carry RNA foci in all their cells. To study whether 300 CTG repeats is an actual threshold for all patients with DM1 will require a challenging 3D study in muscle tissue of patients with DM1.

We found both nuclear and cytoplasmic RNA foci. Although we found myoblasts with cytoplasmic RNA foci in all the DM1 cell lines that we studied, no myoblast presented cytoplasmic-only RNA foci. CTG expansion size appears to regulate the formation of cytoplasmic RNA foci. Thus, larger CTG expansions will produce a higher number of nuclear foci, and therefore, more cells will contain cytoplasmic foci because the latter only appeared in association with nuclear foci. The presence and origin of cytoplasmic foci have not yet been studied in depth. Some authors have suggested that cytoplasmic RNA foci are a product of the cell cycle as well as of the breakdown of the nuclear membrane and as such are more likely to appear in dividing cells.[13] However, a study in an animal model of DM1 expressing CTG expansions in the heart tissue found RNA foci exclusively in the cytoplasm of cardiomyocytes, which are highly differentiated, nondividing cells.[15] On the other hand, our results show that cytoplasmic RNA foci colocalize with MBNL1. The role of cytoplasmic foci and whether they have a toxic effect should be studied in future research.

RNA foci represent less than 0.6% of the total nuclear volume in DM1 myoblasts. Although researchers in the DM1 area are aware of the small size of RNA foci and of the difficulty to identify them under the microscope by naked eye, the data presented here add some information on the impact that RNA foci have on the nuclear volume. In addition, when analyzing the area of RNA foci in relation to other molecular findings, we observed a correlation with the number of cytoplasmic foci. The fact that scarce data are currently available on cytoplasmic RNA foci makes it difficult to interpret our results. However, as previously described by other authors,[16] RNA foci can fuse or divide randomly with no apparent or known purpose.

Our analysis of MBNL1 revealed that the sequestration of this protein inside RNA foci represents less than 1% of the total MBNL1 myoblast expression. Although there are studies showing the pathologic role of MBNL1 in DM1,[17–21] our

results are consistent with a previous study by Coleman et al.[22] reporting that only a small proportion of MBNL1 protein (0.2%) was sequestered by RNA foci in DM1 lens epithelial cells. The study by Coleman and coworkers also showed that MBNL1 can freely travel from the nucleus to the cytoplasm or from the cytoplasm to the nucleus depending on the transcriptional levels and cell requirements. In this context, the low values of sequestration of MBNL1 would suggest that this phenomenon plays only a minor role in the pathophysiology of the disease, as 99% of total MBNL1 expression is free and DM1 myoblasts showed a distribution pattern similar to that of control myoblasts.

The levels of *DMPK* expression were related to the number of RNA foci per cell. This was an expected finding: indeed, if there are more RNA foci inside a cell, theoretically there should also be more DMPK, as for both RNA foci and DMPK determination, we used a technique, qPCR, which allows detecting expanded as well as wild-type alleles. How *DMPK* expression is affected in DM1 remains to be determined. *DMPK* expression levels have been analyzed in several studies with inconclusive or contradictory results. Some studies found that DMPK transcripts were increased in myotubes derived from patients with DM1,[23] but other authors found the opposite result.[10,24] In this regard, heterogeneity among studies with regard to assessment techniques and patient characteristics limits the ability to gain insight into the alteration of *DMPK* expression in DM1.

We did not find the expected altered DM1 splicing when analyzing IR, ATP2A1, and MBNL1 transcripts. As mentioned above, the trapped MBNL1 in RNA foci was a minor event in DM1 myoblasts, and thus, the cascade of molecular pathomechanisms in which the splicing alteration occurs due to MBNL1 protein sequestration does not seem to play a relevant role. These results could also be explained by the fact that the expression of the aforementioned aberrant isoforms is related to developmental stages,[4,9] and because we used undifferentiated cells (i.e., myoblasts), the expression pattern should differ from the pattern of fully differentiated myotubes, and especially of skeletal muscle fibers.

Our results indicate that an earlier age at disease onset is associated with a higher number of cytoplasmic RNA foci. By contrast, the age at onset was not correlated with the number of CTG repeats or RNA foci per myoblast or to *DMPK* expression levels. Several studies[25–28] have analyzed the possible association between CTG expansion size and the different DM1-related symptoms, but CTG instability adds heterogeneity to the disease, which further complicates to establish these associations.

Our study is limited by the low number of myoblast cell lines studied and by the complexity of 3D molecular imaging at the single cell level, which is a highly time-consuming approach. While keeping this limitation in mind, we were able to evaluate the molecular pathologic cascade in 6 patients with DM1 with different clinical manifestations of this disease.

Our 3D imaging study allowed us to analyze the role of potential DM1 players using a novel approach. Thus, our results would indicate that CTG expansion determines the number of RNA foci per myoblast and of myoblasts that contain RNA foci, as well as the appearance of cytoplasmic foci. An additional finding is that RNA foci only represent a small—and in fact negligible—part of the total nucleus volume and the sequestration of MBNL1 is an infrequent event. Thus, no DM1 splicing alterations were found. The *DMPK* expression levels are related to the number of RNA foci found per myoblast. Last, we found that cytoplasmic RNA foci are inversely related to the age at onset of the patients with DM1 studied. Cytoplasmic RNA foci should be considered in future studies because the role of these molecules in the pathobiology of DM1 needs to be clearly elucidated.

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