Ultrastructural characterization of the MBNL1-containing foci occurring in myoblast and myotube nuclei from patients affected by myotonic dystrophy type 2

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Summary
Myotonic dystrophy type 2 (DM2) is an autosomal dominantly inherited disease due to the CCTG repeat expansion in intron 1 of the zinc finger protein 9 (ZNF9) gene, and exhibiting multi-systemic clinical features. The expanded-CCUG-containing transcripts are retained in the cell nucleus and accumulate as discrete RNA-containing foci which specifically sequester the Muscleblind-like 1 (MBNL1) protein, a RNA binding factor involved in the regulation of alternative splicing. The knowledge about the nature of such foci is still largely incomplete; in particular, no information has been reported so far about their ultrastructural features. In this study, the nuclear foci occurring in cultured myoblasts and myotubes from DM2 patients were characterised at transmission electron microscopy by conventional morphology and immunocytochemistry. Our results demonstrate that the MBNL1-containing nuclear foci appear as roundish domains (100-200 nm in diameter) showing a rather homogeneous structure, with fine fibrils spreading out at their periphery. Based on the resemblance to HERDS (i.e. the RNP-containing nuclear aggregates forming upon transcriptional arrest), we hypothesize that the MBNL1-containing domains may represent accumulation sites of RNA processing factors, thus contributing to a general alteration of mRNA expression.

Keywords: cell nucleus, electron microscopy, myotonic dystrophy type 2, muscleblind-like proteins, RNPs.

Introduction

Myotonic dystrophy type 2 (DM2) is an autosomal dominantly inherited disease due to the CCTG repeat expansion in intron 1 of the zinc finger protein 9 (ZNF9) gene in 3q21.3 (Ranum *et al.*, 1998; Liquori *et al.*, 2001), and exhibiting multisystemic clinical features (Meola and Moxley, 2004). The expanded-CCUG-containing transcripts are retained in the cell nucleus and accumulate as discrete ribonucleoprotein (RNP)-containing foci (Liquori *et al.*, 2001). These foci specifically sequester the Muscleblind-like 1 (MBNL1) protein, a RNA binding factor involved in the regulation of alternative splicing, that therefore represents a good marker for the DM2-specific nuclear aggregates (Cardani *et al.*, 2006).

The knowledge about the nature of such foci is still largely incomplete: some biochemical and immunohistochemical studies suggested that proteins involved in RNA maturation may be sequestered therein, with a possible rebound on the overall nuclear function leading to the multiple pathological dysfunctions observed in dys-

trophic patients (Wheeler and Thornton, 2007); however, no information has been reported so far about the structural features of the nuclear domains appearing as foci at fluorescence microscopy.

In this study, the nuclear foci occurring in cultured myoblasts and myotubes from DM2 patients were characterised at transmission electron microscopy by conventional morphology and immunocytochemistry, in the attempt to elucidate their fine organization and their possible relationship with the usual nuclear structures involved in RNA transcription and processing.

Materials and Methods

Sample collection and cell culture

The biopsies for this study were used after informed consent from patients. The histological diagnosis was performed on serial sections processed for routine histological or histochemical staining, based on the clinical criteria set by the International Consortium for Myotonic Dystrophies (Moxley *et al.*, 2002). Myoblasts from two DM2 patients were isolated and cultured in HAM's F10 medium (Sigma-Aldrich) supplemented with 15% FBS (Gibco), 0.5 mg/mL albumin from bovine serum (BSA, Sigma-Aldrich), 0.5 mg/mL fetuin, 0.39 μg/mL dexamethasone, 10 ng/mL epidermal growth factor, 0.05 mg/mL insulin, 3 mg/mL glucose, 100 U/mL of penicillin and 100 μg/mL of streptomycin, as reported in Cardani *et al.* (2009). To induce differentiation into multinucleated syncytia (myotubes), some myoblast cultures were allowed to grow until 80% confluence and the proliferative medium was replaced with a DMEM medium supplemented with 7% FBS, in presence of 100 U/mL of penicillin and 100 μg/mL of streptomycin.

Transmission electron microscopy

For conventional morphology, cells were fixed with 2.5% glutaraldehyde and 2% paraformaldehyde in 0.1 M Sörensen phosphate buffer at 4°C for 1 h, washed, post-fixed with 1% OsO_4 at 4°C for 30 min, dehydrated with acetone and embedded in Epon. For immunoelectron microscopy, cells were fixed with a mixture of 4% paraformaldehyde and 0.2% glutaraldehyde in 0.1 M Sörensen phosphate buffer at 4°C for 1 h, washed, treated with NH_4Cl 0.5 M in phosphate buffer saline (PBS), dehydrated with ethanol and embedded in Unicryl resin.

Ultrathin sections from Epon-embedded samples were conventionally stained with uranyl acetate and lead citrate.

Ultrathin sections from Unicryl-embedded samples were placed on nickel grids and processed for immunocytochemistry by using a rabbit polyclonal anti-MBNL1 antibody (kind gift of Prof. C.A. Thornton, University of Rochester, New York, USA). Sections were floated on normal goat serum (NGS) diluted 1:100 in PBS and incubated for 17 h at 4°C with the primary antibody diluted 1:50 in a solution containing 0.1% bovine serum albumin and 0.05% Tween 20 in PBS. After rinsing, sections were floated in NGS and then reacted for 20 min with the specific gold-conjugated secondary antibody (Jackson ImmunoResearch, West Grove, PA) diluted 1:10 in PBS. Finally, sections were rinsed and air dried. As controls, some grids were treated with the incubation mixture without the primary antibody and then processed as described above. To reduce chromatin contrast and selectively reveal nuclear RNP constituents, the sections were bleached by the EDTA method (Bernhard, 1969).

All samples were observed in a Philips Morgagni TEM operating at 80kV and equipped with a Megaview II camera for digital image acquisition.

Results

At electron microscopy, myoblast nuclei showed small clumps of condensed chromatin mainly distributed at the nuclear periphery, abundant perichromatin fibrils (PF), perichromatin granules (PG) preferentially located at the hedge of the condensed chromatin, and small clusters of interchromatin granules (IG) in the interchromatin space; the nucleoli were large with a few fibrillar centers surrounded by the dense fibrillar component, and a prominent granular component (Figure 1a). The cell nuclei of myotubes showed general features similar to those of myoblasts, apart from a lower presence of heterochromatin clumps (Figure 1b).

In addition to the usual nuclear structural constituents, roundish domains (100-200 nm in diameter) were observed in the sections of about 20% of the myoblast nuclei and 10% of the myotube nuclei. Their morphological appearance was sim-

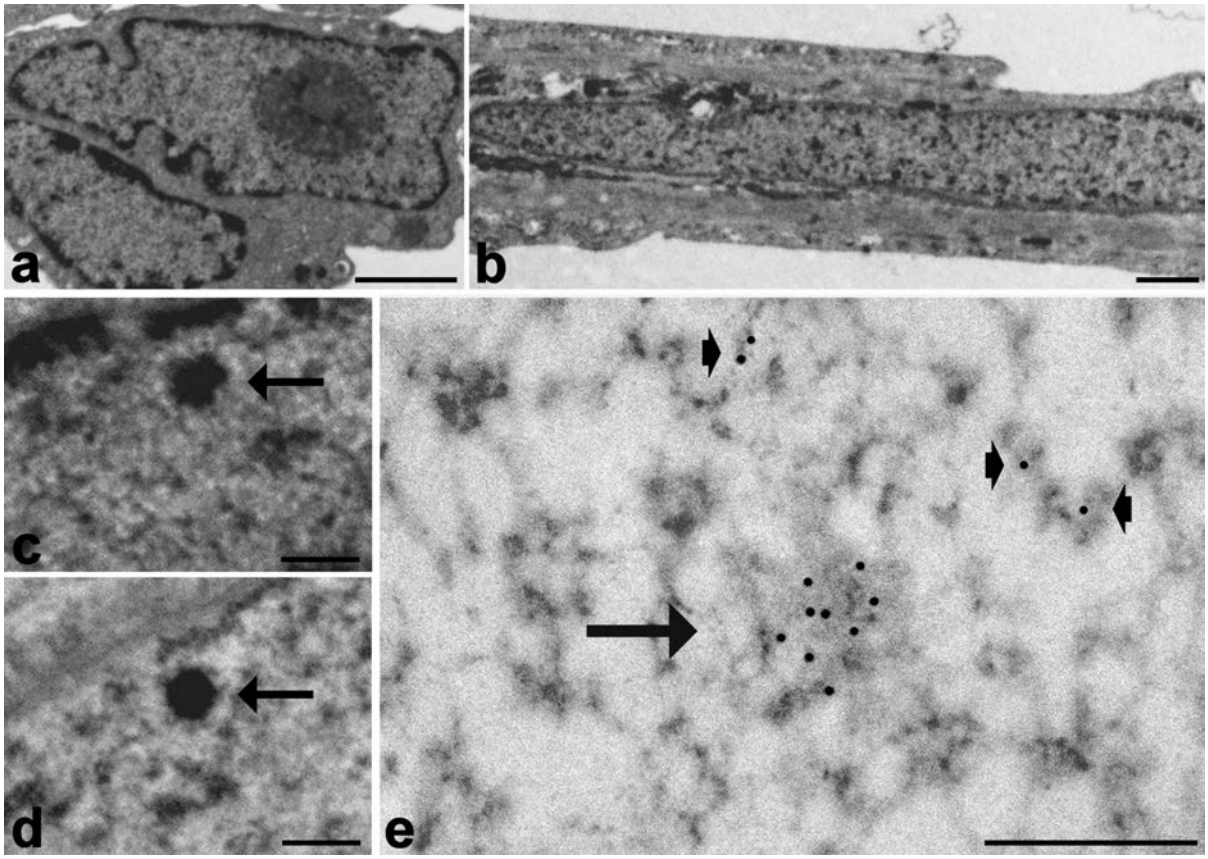

Figure 1. Conventional ultrastructural morphology of myoblasts (a,c) and myotubes (b,d) from muscle biopsies of DM2 patients. Both myoblast (c) and myotube (d) nuclei show roundish electron-dense nuclear domains (arrows). e. Detail of a myoblast nucleus immunolabelled with anti-MBNL1 antibody and counterstained for RNPs with the EDTA bleaching procedure: the gold grains accumulate in the RNP nuclear domain (arrow) and also associate with PF (arrowheads). Bars: 1 µm (a,b); 0.2 µm (c-e).

ilar in myoblasts and myotubes: in OsO_4-postfixed Epon-embedded samples, these domains resembled round black spots due to their strong and homogeneously electron-dense structure (Figure 1c,d); in the Unicryl-embedded samples after EDTA bleaching, they were positive for RNPs and appeared moderately electron-dense with fine fibrils spreading out at their periphery (Figure 1e).

The immunocytochemical analyses allowed us to unequivocally identify these roundish nuclear domains as the DM2-specific RNA-containing foci, since the anti-MBNL1 labelling specifically accumulated therein (Figure 1e). PF were also positive for MBNL1 (Figure 1e).

The background level was negligible in all samples (not shown).

Discussion

The electron microscopic analyses of the MBNL1-containing foci occurring in myoblast and myotube nuclei from patients affected by DM2 revealed the structural features of these nuclear domains as well as their spatial relationships with the usual nuclear constituents involved in mRNA transcription and processing.

In eukaryotic cells, transcription and splicing take place inside nuclear RNP-containing structures recognized, at electron microscopy, as PF, PG, and IG (Fakan, 2004; Spector, 1996). PF represent the structural counterpart of pre-mRNA transcription and co-transcriptional splicing, while IG are a storage site for snRNP and non-snRNP splicing factors, and PG are involved both

in the storage and the nucleus-to-cytoplasm transport of mRNA (recently reviewed in Biggiogera *et al.*, 2007). All these components have specific intranuclear location, PF and PG at the periphery of condensed chromatin (i.e. the perichromatin region), and IG in the so-called interchromatin space (for a review, see Puvion and Puvion-Dutilleul, 1996). Whenever transcription and/or splicing are altered, the organization, composition, and intranuclear location of RNP-containing structures are affected (Biggiogera *et al.*, 2004, 2007).

The morphological features and the RNP nature of the DM2-specific nuclear domains strongly remind the so-called amorphous bodies, i.e. the RNP-containing nuclear aggregates accumulating in several Vertebrate tissues during hibernation (a natural hypometabolic state during which nuclear activities are drastically reduced) and found to be transitory storage sites for many protein factors involved in pre-mRNA processing (e.g. Malatesta *et al.*, 1994, 1999, 2006). More generally, the DM2-specific nuclear domains resemble HERDS, i.e. the heterogeneous ectopic RNP-derived structures (Biggiogera and Pellicciari, 2000) which occur in the nucleus whenever transcription is impaired or arrested (irrespective of the physiological or induced origin of this event), and contain two or more RNP components which normally do not co-locate in normally transcribing nuclei. The accumulation inside HERDS of RNA and protein factors involved in the processing of nuclear RNAs blocks the maturation and export of mRNAs. Similarly, the DM2-specific nuclear domains could sequester specific pre-mRNA processing factors, possibly inducing a general alteration in the expression of mRNAs. The accumulation inside these domains of MBNL1, involved in the regulation of alternative splicing (Osborne *et al.*, 2006; Pascual et al, 2006), is consistent with this hypothesis, and the presence of PF (also containing MBNL1) protruding from their periphery suggest the occurrence of structural/functional contacts with the RNP fibrils. Interestingly, hibernators' amorphous bodies have been found to disassemble upon arousal as RNP fibrils, thus releasing in the nucleoplasm the pre-mRNA processing factors accumulated during lethargy (Malatesta *et al.*, 2001).

In myoblasts and myotubes of DM2 patients the formation of MBNL1-containing nuclear domains is irreversible, but the observed similarities with the amorphous bodies and HERDS pave the way to further analyses on their molecular composition. Identifying the RNA processing factors sequestered in the MBNL1-contaning nuclear domains could allow to understand the molecular pathways impaired in the muscle nuclei of DM2 patients and to relate the accumulation of the expanded-CCUG-containing transcripts with the DM2-specific cell phenotype.

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