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Restoration of normal ultrastructure after expression of the α1 subunit of the L-type Ca^{2+} channel in dysgenic myotubes

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Abstract

Muscular dysgenesis (mdg) is a spontaneous mutation affecting the α1 subunit of the skeletal L-type Ca^{2+} channel. *mdg/mdg* mice suffer from a skeletal muscle disease characterised by low levels of the slow Ca^{2+} current, lack of contractile activity, and immature organisation of skeletal muscle. Microinjections of a cDNA encoding α1 into mutant myotubes restore excitation–contraction coupling. We checked here that dysgenic myotubes transfected with expression vectors, including a full-length α1 cDNA, also recover normal ultrastructural features. Transfection of α1 cDNA partially deleted on the 5′ end leads to the recovery of a good structural organisation without any improvement in the mutant physiological phenotype. These results suggest that: (i) the proper expression of α1 is required for the full muscle differentiation of muscular dysgenesis myotubes, and (ii) portions of the α1 molecule may be involved in the structural organisation of a muscle fiber, independent of its known functional properties.

Key words: Muscle; Muscular dysgenesis; α1 Subunit of the L-type Ca^{2+} channel; Excitation–contraction coupling; Ultrastructure

1. Introduction

Muscular dysgenesis (mdg) is a recessive mutation in the mouse which drastically affects muscle development. Mutants are mainly characterised by complete muscle inactivity in skeletal muscles resulting from a complete lack of excitation–contraction (E–C) coupling [1]. A number of studies have shown that *mdg/mdg* mice do not express voltage-gated slow Ca^{2+} channels (L-type), pharmacologically defined as 1,4-dihydropyridine (DHP) receptors [2–4]. Recently, it has been established that *mdg* corresponds to a point mutation in the gene encoding the α1 subunit of the L-type Ca^{2+} channel [5] mapped in the central region of the mouse chromosome 1 [6].

In skeletal muscle, it is now widely accepted that the α1 subunit displays a dual function [7,8]: it is responsible for slow Ca^{2+} conductance, and it constitutes the voltage sensor for E–C coupling. Biochemical [9] and electrophysiological [10] studies have located L-type Ca^{2+} channels in the tubular membrane of mature fibers. Ontogenetic and functional studies of Ca^{2+} channels in primary cultures of mouse myotubes in relation to morphological maturation have also shown a relationship between Ca^{2+} channel activity, the establishment of E–C coupling, and the progressive structural organisation of the myotubes [10].

In spite of their functional deficiency, dysgenic myotubes also display a huge structural disorganisation mainly characterised by abnormalities of the triadic junction, lack of correct sarcomeric organisation, defective basal lamina, and centrally located nuclei [11]. In previous studies we have shown that the calcitonin gene-related peptide (CGRP) and/or dibutyryl cAMP induce an apparently normal sarcomeric and fibrillar organisation in *mdg/mdg* myotubes in vitro, without establishing either L-type Ca^{2+} channel activity or E-C coupling [12]. These observations led us to suggest that the cAMP level in muscle fibers may be specifically involved in their morphological development. In light of these results, it is tempting to speculate that in normal myotubes the activity of the α1 subunit of the L-type Ca^{2+} channel is involved in intracellular changes in cAMP levels able to control the morphological organisation of the myotube.

The specific purpose of this work was to test whether transfections of plasmid vectors coding for α1 or part of α1 were able to restore a normal morphology in *mdg/mdg* myotubes. We show here that the expression of a rabbit full-length α1 cDNA restores both E–C coupling properties, as previously described by Tanabe et al. [8], and normal ultrastructure morphology in *mdg/mdg* myotubes. In contrast, when transfections were performed with partially deleted α1-cDNA (5′ first kilobase missing) the mutant morphology improved whereas the deficit in E–C coupling remained unchanged, suggesting that the two maturation processes may be dissociated.

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2. Materials and methods

The expression plasmids pSG5 (*Stratagene*), carrying the rabbit skeletal muscle α1-1234 (full-length DHP receptor), and α1-234 (DHPr with its first domain deleted) were made as follows: (i) by inserting the *Eco*RI (1,007 bp from ATG)–*Bam*HI (3′ terminal linker) fragment from a plasmid Bluescript-α1 (a gift from A. Schwartz and G. Varadi, University of Cincinnatti, OH). The resulting expression plasmid contained the SV40 early promoter, the rabbit β-globin intron II and a cDNA fragment coding for the last three transmembrane domains of α1 (pSG5 α1-234). (ii) The plasmid coding for the full-length α1 was obtained by insertion of the *Eco*RI (5′ terminal linker)–*Eco*RI (1,007 bp) fragment at the *Eco*RI site of the pSG5 α1-234 plasmid: it is called pSG5 α1-1234.

Primary cultures of skeletal muscles from new-born normal and dysgenic mice were prepared at low densities (5×10^3 cells/ml) as previously described [13].

Calcium phosphate precipitations were carried out for transfection [14] with 5–10 μg of DNA just before myoblasts fuse into myotubes. In some experiments, expression vectors encoding α1-1234 (full-length DHPr) and α1-234 (5′ deleted DHPr) were co-transfected with a second expression plasmid encoding the bacterial *lacZ* gene with a nuclear location signal under the control of the SV40 early promoter in an approximately 10:1 molar ratio. 24 h after transfection, serum concentration in the culture medium was changed from 10% FCS (fetal calf serum) plus 10% HS (horse serum) to 10% HS in DMEM (Dulbecco's modified Eagle's medium).

Resulting transfected myotubes were electrically stimulated 6 days after transfection. Pulses were applied through an extracellular platinum electrode bathed in the culture dish. We used a DI 6402 stimulator. The stimulating pulse lasted for 10 ms and its amplitude was adjusted to be suprathreshold for evoking contractions of transfected myotubes. The stimulation frequencies used varied from 0.5 to 5 Hz. An oscilloscope Tektronic 2211 monitored contractions as a change in the voltage drop across a small photoresistor that was placed at the edge of a myotube image on a television monitor. Data acquisition and analysis were performed by means of computer program written by Julio Garcia and run on an IPC microcomputer.

In some experiments, we used pCEP-modified vectors (InVitroGen) offering the advantage of confering resistance to hygromycin B when transfected into cells. pCEP α1-1234 and -234 were constructed, respectively by cleavage and insertion of a *Sal*I (5′ flanking end of SV40 promoter)–*Sal*I (3′ terminal end of the poly-A signal) fragments from pSG5 α1-1234 and pSG5 α1-234 coding for either the α1 full-length or the deleted form. Stable transfectants were obtained in the dysgenic muscle cell line (129 DA3) with pCEP α1-1234 and -234 by selection in the presence of 200 μg/ml of hygromycin B (Sigma). 129 DA3 myoblasts were grown and maintained as described by Pinçon-Raymond et al. [15].

For immunofluorescence experiments, the culture dishes were fixed for 6 min in methanol at −20°C, immersed for 15 min in PBS solution containing 0.1 M glycine and washed twice with 10% sheep serum and 1% BSA in PBS for 1 h to block non-specific binding sites. The cultures were then incubated for 2 h at room temperature in the presence of a mouse monoclonal antibody against α1 (rabbit type; provided by S. Froehner) diluted 1:100 in 1% BSA in PBS. This was followed by the incubation of an FITC-conjugated second antibody (Eurobio).

In some experiments, cells were fixed for 15 min in 4% paraformaldehyde plus 0.2% glutaraldehyde in PBS and β-galactosidase activity was assayed as described by Sanes et al. 1986. The staining solution contained 2 mM 5-bromo-4-chloro-3-indolyl-D-galactoside (X-gal; Sigma), 5 mM potassium ferrocyanide, 5 mM potassium ferricyanide, and 2 mM $MgCl_2$ in BPS. Staining was performed overnight at 30°C.

For electron microscopy, cultures were fixed for 2 h in 2.5% glutaraldehyde/0.5% tannic acid in 0.1 M phosphate buffer at pH 7.4 at 4°C, followed by 0.6% glutaraldehyde/0.5% tannic acid in the same buffer overnight. Post-fixation was performed in 2% osmic acid in 0.1 M phosphate buffer for 1 h at 4°C. Then, samples were dehydrated in graded alcohol and embedded in Epon resin. Ultra-thin sections of about 65 nm were stained with a saturated solution of uranyl acetate in 50% acetone followed by staining with 0.2% lead citrate for 4 min. The observations were made with a Philips EM 410 electron microscope (accelerating voltage 80 kV, objective aperture 20 μm). Only contracting pSG5 α1-1234-transfected myotubes were chosen for ultrastructural observations; β-galactosidase-labelled nucleus myotubes and cross-striated myotubes at, respectively, 7 days and 20 days, were chosen for observations of pSG5 α1-234-transfected myotubes.

3. Results

3.1. Expression vectors

We constructed two expression plasmids pSG5/pCEP carrying both the rabbit skeletal muscle α1 subunit and a 5′-deleted form. Restriction enzyme analysis showed that the two α1 variant constructions only differ by an *Eco*RI (5′ terminal linker between intron II of the β-globin gene and the α1-cDNA–*Eco*RI (1,007) fragment. The construction encoding the full-length α1 cDNA was

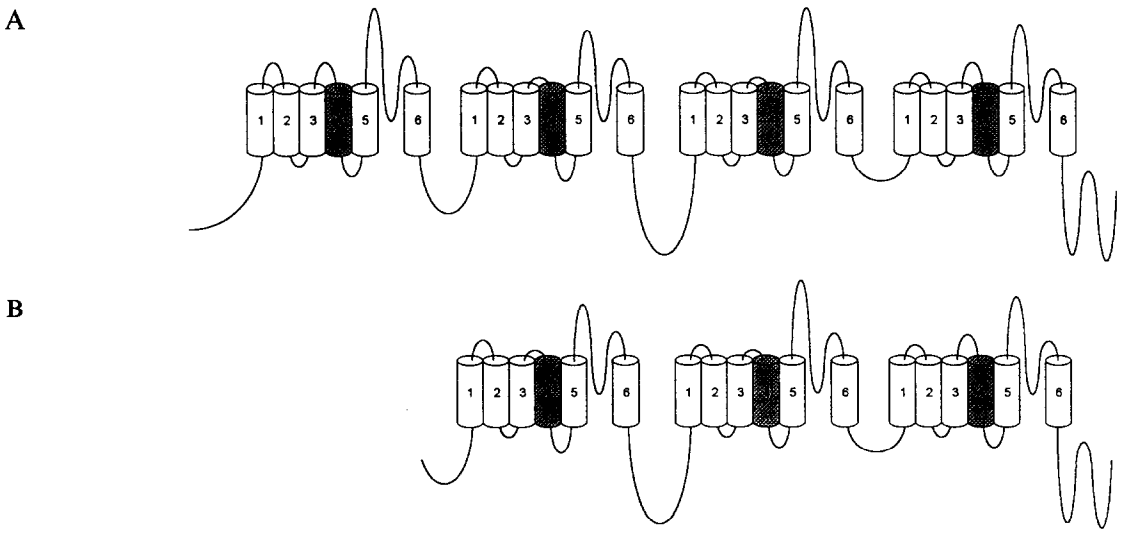

Fig. 1. (A) Putative structure of the α1 subunit. It contains the 4 main domains containing each of the 6 transmembrane segments (1234 form). (B) Putative structure of the protein generated after *Eco*RI fragment deletion: 3 of the 4 domains persist (234 form).

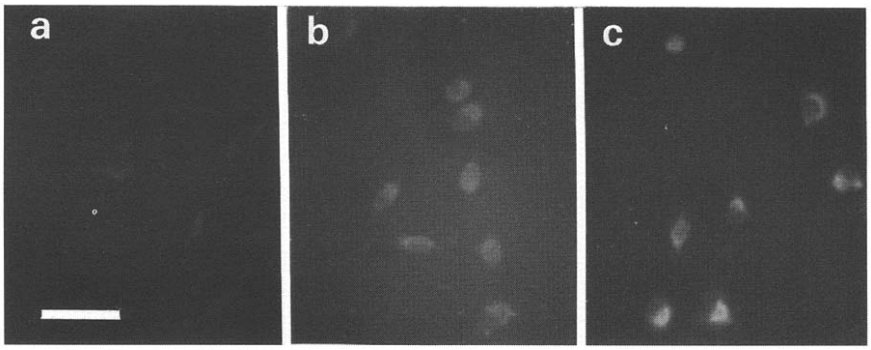

Fig. 2. Immunodetection of α1. Myoblasts transfected with pCEP1234 (b) and pCEP 234 (c) stain positive for α1 whereas non-transfected *mdg* myoblasts remain negative (a). (×210, bar = 50 μm).

called pSG5/pCEP α1-1234 as the resulting protein contains the 4 main transmembrane domains. *Eco*RI (link)–*Eco*RI (1,007)-deleted constructions were designated pSG5/pCEP α1-234 because the *Eco*RI site is located between the first and the second main domain at nucleotide position 1,007. Putative schematic structures of the resulting proteins are given in Fig. 1.

We checked the activity of these expression vectors (pCEP α1-1234 and -234) by stable transfection of myoblasts of the 129 DA3 *mdg* muscle cell line. After 2 weeks in the presence of hygromycin B (200 μg/ml) a few resistant clones were selected. The presence of α1 or deleted α1 was assayed for by using the anti-α1 subunit monoclonal antibody. Almost all of the resistant cells are revealed by positive immunofluorescent labelling (Fig. 2), indicating that both constructions (pCEP α1-1234 and -234) allowed the expression of proteins containing at least one epitope belonging to α1. These clones were not analysed further because they were all non-fusing clones.

3.2. Changes in E–C coupling

To maintain long-term cultures, we chose to work with primary myoblasts at low cellular concentration (5×10^3 myoblasts per ml). Under such conditions, the primary myoblasts can be transfected on the fourth day and allowed to fuse into myotubes after 7 days in cultures. In contrast to normal +/+ myotubes, control (non-transfected) *mdg/mdg* myotubes in primary culture never spontaneously contracted. When transfected with pSG5 α1-1234, functional changes occurred 5 or 6 days after fusion. Depending on the transfection efficiency, some myotubes displayed local spontaneous contractions (an average of 5 contraction foci per 35 mm culture dish) which were clearly not the local slow contractures sometimes observed in *mdg/mdg* cultures. At that stage, simultaneous recordings of electrical stimulation (7–10 Vpk) in the culture bath and associated contractions confirmed that transfection allows E–C coupling restoration in mutant myotubes (Fig. 3). However, pSG5 α1-234-transfected *mdg/mdg* myotubes in primary cultures never contracted, either spontaneously or in response to electrical stimulation.

3.3. Changes in myotube morphology

As frequently described, 6–7 days after fusion *mdg/mdg* myotubes lacked a normal organisation. Rare abnormal Z lines became visible in some myotubes but sarcomeres were never fully organised, as seen in Fig. 4a. When transfected with pSG5 α1-1234 or pSG5 α1-234, peculiar changes in their ultrastructure were observed. In almost all contracting areas (pSG5 α1-1234) we observed a local improvement in sarcomeric organisation (Fig. 4b). With pSG5 α1-234 (deleted α cDNA), since myotubes never contracted spontaneously nor in response to electrical stimulation, we identified transfected primary myotubes by co-transfecting pSG5 α1-234 with a second expression vector coding for nuclear β-galactosidase (nls-*lacZ* reporter gene under SV40 early promoter) (inset Fig. 4c). Ultrastructural morphology in a co-trans-

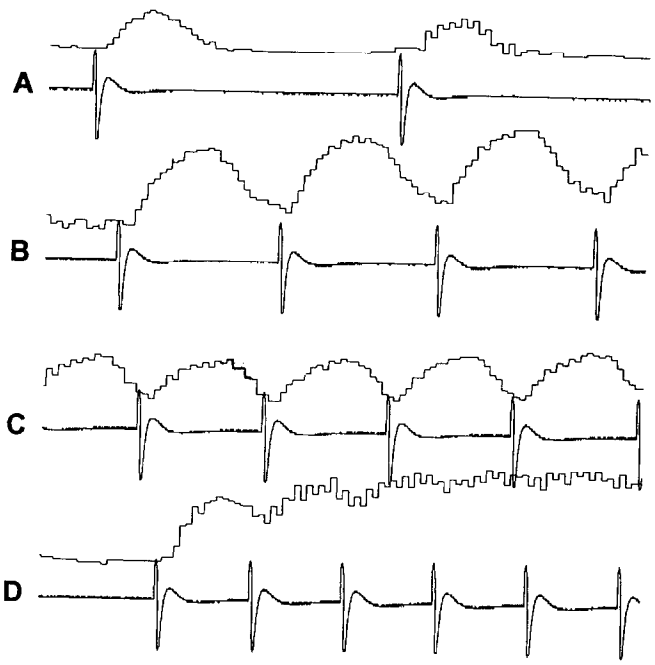

Fig. 3. Simultaneous recordings of electrical stimulation and associated contractions. Upper traces, evoked contractions; lower traces, spikes of stimulation. (A) 1 Hz, (B) 2 Hz, (C) 2.5 Hz, (D) 3.3 Hz. Evoked contractions are well coupled to electrical stimulation.

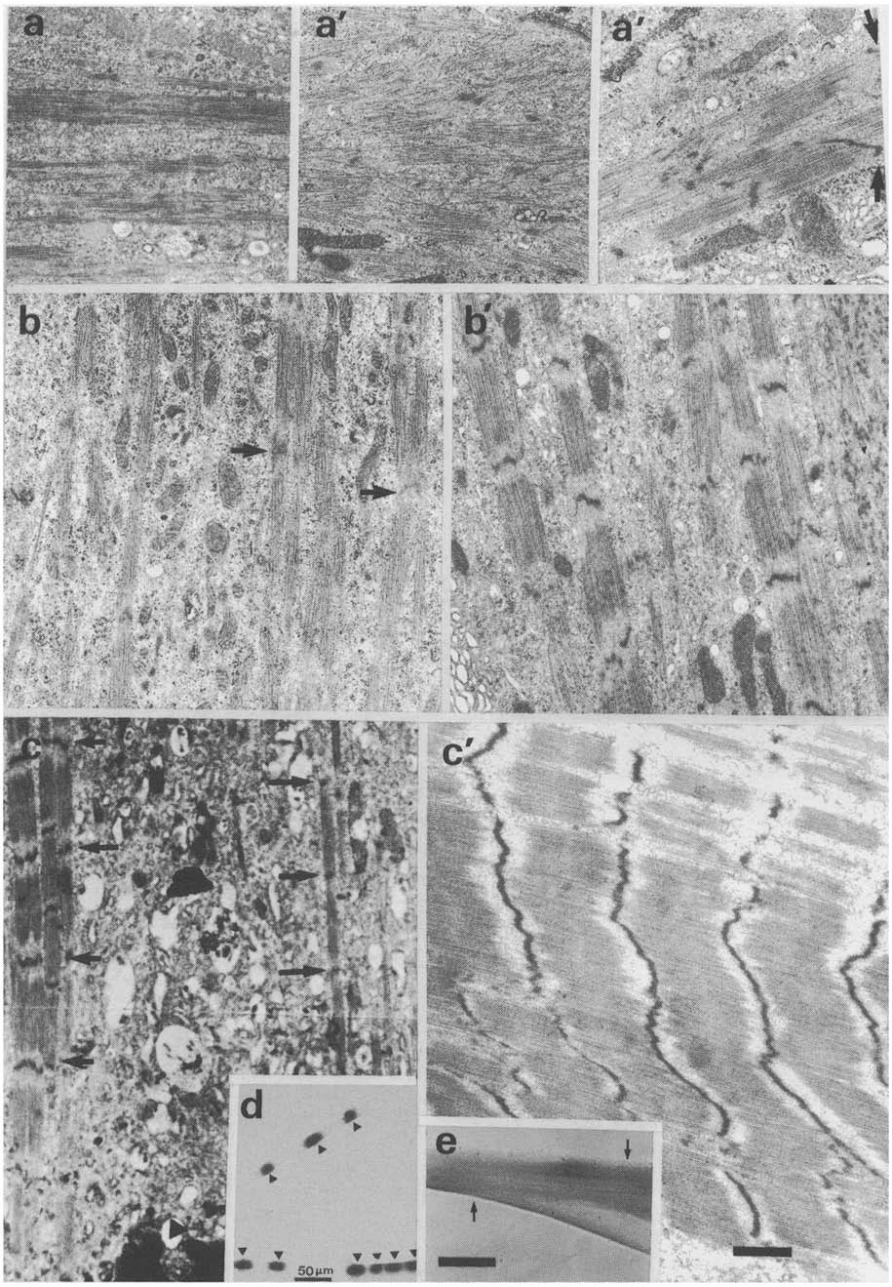

Fig. 4. Ultrastructural reorganisation of transfected *mdg/mdg* myotubes in primary cultures. (a–c) Electron microscopy after 13 days in culture. (a) Disorganisation of *mdg/mdg* myotubes. (b) In pSG5 1234-transfected myotubes, contracting areas show an improvement of sarcomeric organisation. (c) In pSG5 234-transfected myotubes, sarcomeres (arrows) are visible in the vicinity of β-galactosidase labelled nuclei (bar = 1 μm); (d) At the light level (arrowheads point to the stained nuclei). (a′–c′,e) Electron microscopy of myotubes after 20 days in culture. (a′) Non-organisation of *mdg/mdg* myotubes. Exceptionally, sarcomeres are visible in myotubes in old cultures (arrows). (b′) Distinct sarcomeric organisation in pSG5 1234-transfected myotubes and (c′) in pSG5 234-transfected myotubes (bar = 1 μm). (e) At the light level, transfected myotubes present cross-striations. (bar = 50 μm).

fected myotube in the vicinity of a β-galactosidase-labelled nucleus is shown in Fig. 4c. As previously observed for pSG5 α1-1234, transfected myotubes with pSG5 α1-234 displayed an improved sarcomeric organisation specifically around the β-galactosidase-labelled nuclei.

After 20 days in culture, the ultrastructural morphology of transfected myotubes continued to improve. Whilst control *mdg/mdg* myotubes were still disorganised (Fig. 4a′), pSG5 α1-1234 (Fig. 4b′) and α1-234 (Fig. 4c′) displayed large well-organised striated patterns and well-defined Z lines. This organisation is sometimes visible by light microscopy (inset Fig. 4c′). In addition to sarcomeric organisation, pSG5 α1-1234 transfected myotubes displayed few triads with regularly spaced densities (in an average of 3 out of 5 contracting loci). For pSG5 α1-234, at least 5 triads were found in the very large restored region presented in Fig. 4c′, whereas no triads

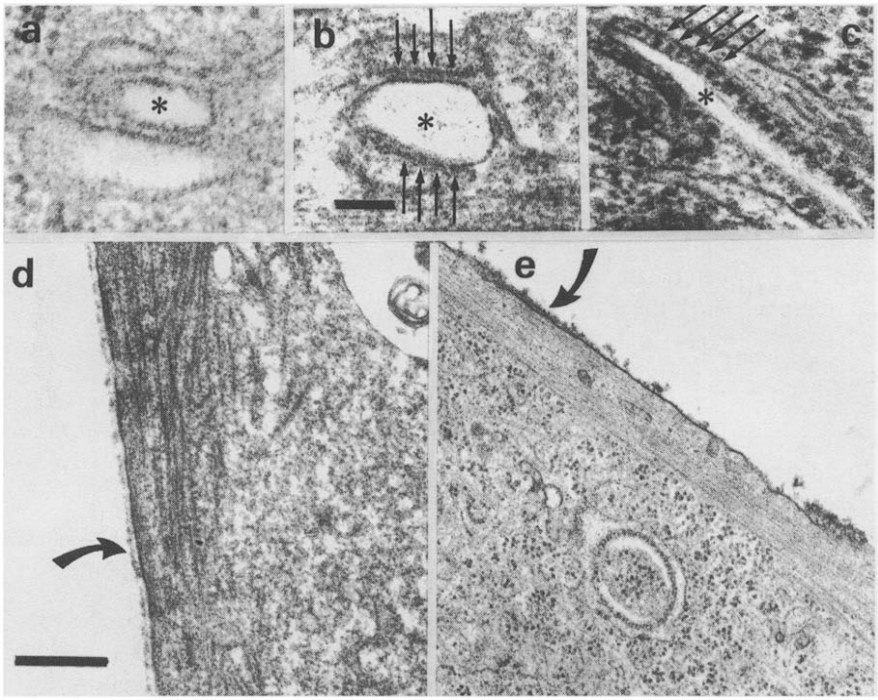

Fig. 5. Ultrastructural analysis of triadic junctions in *mdg/mdg* and transfected myotubes. (a) In *mdg/mdg* myotubes, triads are rare and spaced densities are not observed between T-tubule and sarcoplasmic reticulum membranes. After transfection with pSG5 234 (b) and pSG5 1234 (c), triads acquire a normal morphology with regularly spaced densities (arrows) (Bar = 0.1 μm). Basal lamina appears at the surface of transfected pSG5 234 (d) and pSG5 1234 (e) myotubes (Bar = 0.5 μm).

with junctional feet were found in non-transfected *mdg/mdg* myotubes (Fig. 5a–c). It is worth noting that both pSG5 α1-1234 and -234 elicit the accumulation of a well-defined basal lamina in *mdg/mdg*-transfected myotubes (Fig. 5d,e).

4. Discussion

The *mdg* mutation is expressed as a total uncoupling of excitation–contraction (E–C); recently it has been fully characterised at the molecular level, and can now be considered a natural α1-deficient mutation [5]. De novo expression of the rabbit skeletal α1 subunit in *mdg/mdg* myotubes gave conclusive information concerning two of its potential roles in skeletal muscle [8]: (i) it can work as an ionic channel, (ii) it constitutes the voltage sensor of the E–C coupling. However, it is relevant to stress that dysgenic myotubes express very low levels of cardiac α1 mRNA [16] and a peculiar slow-type Ca^{2+} current (I_{dys}) [17,18].

The first result of interest obtained in our study concerns the contribution of the α1 subunit to muscle organisation. As already described by Tanabe et al. [8], transfection of dysgenic myotubes with an expression vector encoding the rabbit α1 subunit allows a full restoration of E–C properties. We further show that concomitant with such a correction, myotubes improved very significantly in their ultrastructural level of differentiation. This result suggests that expression of α1 after transfection leads, in addition to E–C coupling, to improved structural organisation of skeletal muscle. We focused our attention mainly on the three structural components severely disorganised in the *mdg/mdg* myotube, i.e. sarcomeres, basal lamina and triads. New sarcomeric organisation was observed as soon as a few days after transfection. However, triads, although more numerous than in untransfected controls, were less frequent than expected. This can be explained by the decrease of expression capabilities of our α1 plasmid vectors as cultures grew older (data not shown estimated by the levels of *lacZ* expression during the time–course of the experiment). So, a faint and transient expression of α1 may be enough to promote sarcomeric organisation, whereas establishment of a full triadic network could need large, sustained levels of it.

The second result of interest concerns the striking effect of the expression of a 5′-deleted form of the α1 subunit on dysgenic muscle organisation. Although transfected myotubes could never contract, ultrastructural analysis shows a local improvement of the sarcomeric organisation, an increase in the number of triads and the accumulation of basal lamina. This result confirms that the organisation improvement previously described after transfection with the full-length α1 is not just a mere consequence of the acquisition by the dysgenic myotubes of either a functional contractile activity or slow Ca^{2+} voltage-gated permeation. Thus, it seems conceivable

that the α1 subunit might be directly involved in muscle differentiation via a molecular mechanism still to be defined. Two starting hypotheses can be proposed to explain these observations. In the first one, termed 'passive' α1 or part of α1, must be physically present to aggregate crucial components connected, for example, to the cytoskeleton. Absence of α1 would result in a missing physical link disrupting all the muscle internal architecture. This kind of direct coupling between α1 and other proteins, such as the Ca^{2+} channel from the sarcoplasmic reticulum, has already been described [19]. In the second hypothesis, termed 'active', we can imagine that the α1 subunit could be the voltage sensor of the molecular machinery in charge of an excitation–*differentiation* coupling. This coupling could use cAMP as a second messenger to promote muscle differentiation, since it has been shown that CGRP or dibutyryl cAMP induce normal ultrastructure in dysgenic myotubes [12].

The *mdg/mdg* muscle has been an important tool for demonstrating the dual function of the α1 subunit of the DHP receptor. It works as a slow-type Ca^{2+} channel, and it constitutes the silent voltage sensor of the E–C coupling. The present work shows, in addition, that proper expression of α1 is required for correct muscle differentiation and that α1 may be a structural organiser of the skeletal myofiber.

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