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RESEARCH ARTICLE OPEN ACCESS

A Novel Assay Reveals the Early Setting-Up of Membrane Repair Machinery in Human Skeletal Muscle Cells

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Keywords: fluorescence microscopy | limb girdle muscular dystrophy | membrane repair | muscular dystrophy | shear-stress assay | skeletal muscle

ABSTRACT

Defect in membrane repair contributes to the development of muscular dystrophies such as limb girdle muscular dystrophy (LGMD) type R2 or R12. Nevertheless, many other muscular dystrophies may also result from a defect in this process. Identifying these pathologies requires the development of specific methods to inflict sarcolemma damage on a large number of cells and rapidly analyze their response. We adapted a protocol hitherto used to study the behavior of cancer cells to mechanical constraint. This method is based on forcing the passage of cells through a thin needle, which induces shear stress. Due to size considerations, this method requires working with mononuclear muscle cells instead of myotubes or muscle fibers. Although functional sarcolemma repair was thought to be restricted to myotubes and muscle fibers, we show here that 24h-differentiated myoblasts express a complete machinery capable of addressing membrane damage. At this stage, muscle cells do not yet form myotubes, revealing that the membrane repair machinery is set up early throughout the differentiation process. When submitted to the shear-stress assay, these cells were observed to repair membrane damage in a Ca²⁺-dependent manner, as previously reported. We show that this technique is able to identify the absence of membrane resealing in muscle cells from patient suffering from LGMDR2. The proposed technique provides therefore a suitable method for identifying cellular dysregulations in membrane repair of dystrophic human muscle cells.

1 | Introduction

Cell membrane disruption is a physiological event that primarily occurs in cells submitted to mechanical stress, especially those of muscle, epithelial, or endothelial origin [1]. The downregulation of the membrane repair machinery leads to degenerative diseases such as muscular dystrophies [2], whereas its exacerbation in cancer cells promotes tumor

invasion and metastasis [3, 4]. To date, three out of the hundreds of muscular dystrophies have been formally identified as resulting from a defect of sarcolemma repair [2]. LGMDR2 (formerly 2B) [5] and LGMDR12 (2L) [6] result respectively from mutations in *dysferlin* and *anoctamin* gene, while facioscapulohumeral muscular dystrophy [7] is associated with dysregulation in the expression of DUX4, which hinders the membrane repair process. Nevertheless, many other muscular

Abbreviations: DIC, differential interference contrast; LGMD, limb girdle muscular dystrophy; PI, propidium iodide; TEM, transmission electron microscopy.

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dystrophies may result from a defect in membrane repair and remain to be identified. The development of methods able to quickly and reliably identify concerned pathologies is needed. The standard membrane repair assay involves inducing damage on a single cell using laser ablation [8]. This method requires complex equipment, including confocal microscope and infrared laser, and are time-consuming, as it necessitates the successive analysis of hundreds of cells, with each requiring several minutes.

Here we report a simple method that allows a large number of muscle cells to be submitted to mechanical constraints within a short timeframe. This method not only rapidly highlights muscle cells with membrane repair defect, but also geno- and pheno-typic consequences of sarcolemma damage. This method, called hereafter the muscle shear-stress assay, is based on forcing the passage of cell suspension through a thin needle inducing shear forces. We demonstrate that this method is able to identify muscle cells suffering from a defect in membrane resealing, notably those established from LGMDR2 patients, as previously reported [5]. This method enabled also to uncover that a functional membrane repair machinery is setting up early in myoblasts as soon as 24 h after differentiation has begun.

2 | Materials and Methods

2.1 | Cell Lines and Cell Culture

The control LHCN-M2 (referred to hereafter as LHCN) or KM1421 cell lines, and the dysferlin-deficient L578 cell line have been provided by the platform for immortalization of human cells from the Center of Research in Myology (Paris, France). These cell lines have been respectively established from satellite cells of the pectoralis major muscle of a 41-year-old subject [9], the paravertebral muscle of a 13-year-old subject, and the vastus lateralis adult muscle of a 25-year-old patient suffering from LGMDR2 [10]. Myoblasts were cultured in a skeletal muscle medium composed of one volume of Medium 199 with glutamax (Gibco by Life Technologies), four volumes of Dulbecco's modified Eagle Medium with high-glucose and glutamax, and without pyruvate (Gibco by Life Technologies) supplemented with 20% fetal bovine serum, 50- $\mu\text{g}/\text{mL}$ gentamycin and a commercial mixture of supplements (ref. C-39365, Promocell, Heidelberg, Germany), which included 12.5- $\mu\text{g}/\text{mL}$ fetuin, 2.5- ng/mL human recombinant epidermal growth factor, 0.25- ng/mL basic fibroblast growth factor, 2.5- $\mu\text{g}/\text{mL}$ insulin, 0.1- $\mu\text{g}/\text{mL}$ dexamethasone, as previously described [11]. Differentiated myoblasts were obtained by cultivating 80%-confluence myoblasts in a differentiation medium, composed of the skeletal muscle medium supplemented only with 10- $\mu\text{g}/\text{mL}$ insulin.

2.2 | Western Blotting

Preparation of protein extracts and western blot analysis were performed as previously described [12]. 40- μg protein extracts were separated on a 10% SDS-PAGE. Electrophoretic transfer (Bio-Rad, Hercules, CA, USA) onto PVDF membrane was

performed for 20 min at 60 V. The cellular content of caveolin-3 (20 kDa) and GAPDH (loading control, 37 kDa) was detected with mouse anti-caveolin-3 monoclonal antibody (C-2, Santa Cruz Biotechnology, Heidelberg, Germany) and rabbit anti-GAPDH polyclonal antibody (FL-335, Santa Cruz Biotechnology), respectively. Anti-caveolin-3 and anti-GAPDH antibodies were respectively diluted 1:100 and 1:5000 in saturation solution composed of Tris buffer saline (10-mM Tris, 150-mM NaCl, pH 8.0) supplemented with 0.1% Tween20% and 5% nonfat dry milk and incubated overnight at 4°C. Revelation was performed using secondary antibodies conjugated to horseradish peroxidase (GE-Healthcare) diluted 1:2000 in saturation solution and either Opti-4CN colorimetric kit (Bio-Rad) or Clarity Western Enhanced Chemiluminescent Substrate (Bio-Rad).

2.3 | Muscle Shear-Stress Assay

Adherent undifferentiated or 24h-differentiated muscle cells were collected at 80% confluence after incubation with 0.25% trypsin (Gibco) in 5% CO₂ at 37°C and 5 × 10⁵ cells were suspended in 1 mL of growth medium. At this stage either Ca²⁺ (Final concentration 2 mM), or 2-mM EGTA, was added to the medium. The suspension was slowly loaded into a 1-mL syringe (Becton Dickinson, NJ, USA), which was subsequently equipped with a 30 G needle (Terumo, Tokyo, Japan). Then, the suspension was gently expelled against the wall of a polystyrene tube at room temperature at a constant flow rate either via an automated NeMESYS syringe pump (200 $\mu\text{L}/\text{s}$, Cetoni GmbH, Korbussen, Germany) or manually. The load/expel cycle was repeated 10 times. 10 min after the treatment, 50 μL of the cell suspension were put into a 96-well plate and a mixture of propidium iodide (PI, final concentration 4 $\mu\text{g}/\text{mL}$) and Hoechst (40 $\mu\text{g}/\text{mL}$) was added to stain damaged cells. As a control condition, cells in growth medium with Ca²⁺ (final concentration 2 mM) were incubated with PI/Hoechst mixture without being subjected to shear stress. Cell imaging of Hoechst (all cells) and PI (unrepaired cells) was performed as described below. Images were analyzed with ImageJ software.

2.4 | Differential Interference Contrast (DIC) and Fluorescence Microscopy

Cell imaging was performed using a conventional fluorescence microscope IX81 (Olympus, Tokyo, Japan) equipped with a DIC acquisition system. Hoechst was observed using the U-MWU2 cube containing a band-pass excitation filter (330–385 nm), a dichroic mirror (threshold 400 nm) and a long-pass emission filter (threshold 420 nm). PI was observed using the cube U-MWG2 containing a band-pass excitation filter (510–550 nm), a dichroic mirror (threshold 570 nm) and a long-pass emission filter (threshold 590 nm).

2.5 | Membrane Repair Assay by Laser Ablation

Membrane repair assay was performed as previously described [13]. Cells were irradiated at 820 nm with a tunable pulsed

depletion laser Mai Tai HP (Spectra-Physics, Irvine, USA) of a two-photon confocal scanning microscope (TCS SP5, Leica, Wetzlar, Germany). Irradiation consisted of 1 scan (1.6 s) of a $1\ \mu\text{m} \times 1\ \mu\text{m}$ area with a power of $110 (\pm 5)$ mW. 512×512 images were acquired at 1.6-s intervals with pinhole set at 1 Airy unit. FM1-43 was excited by the 488 nm laser line (intensity set at 20% of maximal power) and fluorescence emission was measured between 520 and 650 nm. For quantitative analysis, the fluorescence intensity was integrated over the whole cell surface and corrected for the fluorescence value recorded before irradiation, using ImageJ software.

2.6 | Statistical Analysis

Data are presented as mean values (\pm SEM) from at least three independent experiments performed in triplicate. The statistical significance of data was assessed by a Mann–Whitney test using GraphPad Prism software. A probability value less than 0.05 is used for statistical significance. * $p < 0.05$, ** $p < 0.01$, and *** $p < 0.001$.

3 | Results

3.1 | Principle of the Method

We aimed to develop a method able to concomitantly damage a large cell population for statistical reasons. The muscle shear-stress assay was adapted from a protocol initially developed for cancer cells and based on forcing the passage of cells through a thin 30 Gauge needle [4, 14] (Figure 1). 10 min after the treatment, unrepaired cells were quantified by PI uptake assay and counterstained with Hoechst. PI and Hoechst are respectively membrane-impermeant and membrane-permeant nuclear dyes. Hoechst thus labeled every cell, while PI marked only damaged and unrepaired cells. We expected to be able to apply this method on muscle cells, in a novel way, to identify muscular dystrophies resulting from defective membrane repair. We also aimed to analyze molecular changes in stressed/damaged muscle cells. The muscle shear-stress assay required to work with mononucleated myoblasts instead of myotubes, which were too large to pass through the needle. Nevertheless, we previously demonstrated that myoblasts,

unlike myotubes, were unable to repair membrane damage [12]. Therefore, we needed to determine whether differentiating myoblasts acquired the ability to repair membrane damage before fusion into myotubes.

3.2 | Caveolin-3 Is Expressed Early During Differentiation of Human Skeletal Muscle Cells

Given that caveolin-3 was expected to be the membrane repair protein absent in myoblasts for membrane resealing [12], we studied its expression by western-blotting during muscle cell differentiation. LHCN muscle cells, either undifferentiated or differentiated for 1, 6, 16, 24, and 72 h were thus analyzed. Our observations indicated that only cells differentiated for 72-h formed myotubes (Figure 2A). It was confirmed that undifferentiated LHCN myoblasts weakly expressed caveolin-3 and uncovered that caveolin-3 was significantly expressed in LHCN cells as early as 24 h after differentiation induction (Figure 2B and Supporting Information S1: Figure S1A). This finding was further validated in the skeletal muscle cell line KM1421, which is another human cell line established from a healthy control individual (Supporting Information S1: Figure S1B).

3.3 | Membrane Repair Machinery Is Functional in 24-h Differentiated Myoblasts

We submitted both undifferentiated and 24-h differentiated myoblasts to the muscle shear-stress assay. Quantitative analysis of the membrane repair assay was performed using fluorescence microscopy after staining with Hoechst and PI (Figure 3). When submitted to the shear-stress assay in the presence of Ca^{2+} , undifferentiated myoblasts exhibited about 10% of damaged and unrepaired cells, a rate significantly higher than when untreated (Figure 3B, MB). This percentage dropped to $1.2 (\pm 0.9)$ % for 24-h differentiated myoblasts under similar condition (Figure 3B, 24-h-MB, +stress, + Ca^{2+}), confirming that the molecular machinery responsible for membrane repair is gaining early during the muscle cell differentiation process. As expected, membrane resealing was compromised in the absence of Ca^{2+} since about 25% of 24-h differentiated myoblasts were PI-positive under this condition (Figure 3B, 24-h-

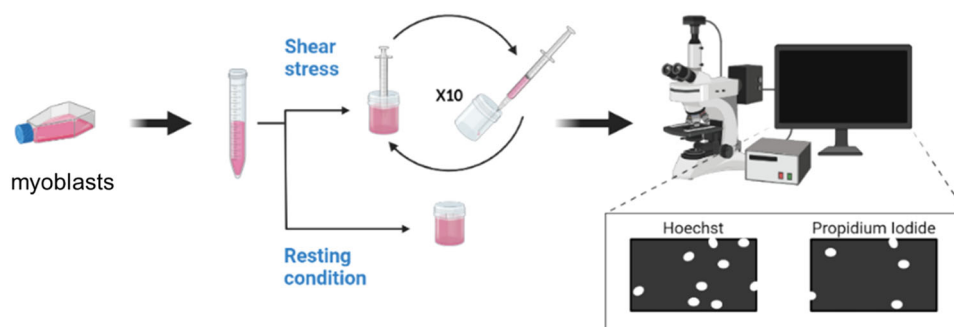


FIGURE 1 | Principle of the muscle shear-stress assay. Myoblasts were split into two populations: the stressed population was passed 10 times through a needle, while the other population remained in resting condition and used as a control. An aliquot of cell suspension was incubated in a mixture of Hoechst and PI. PI-positive (damaged) and Hoechst-positive (total) cells were observed and counted with a fluorescence microscope. Adapted from [4] using Biorender.com.

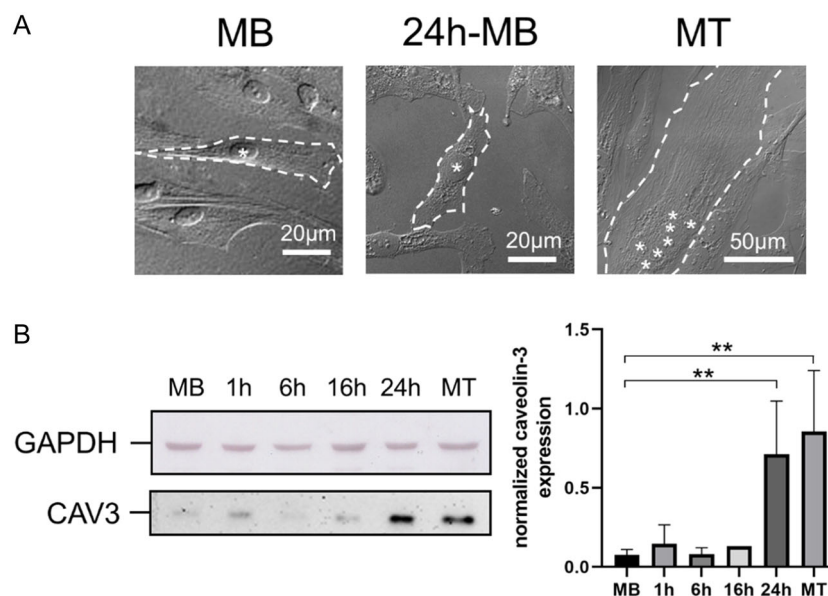


FIGURE 2 | Expression of caveolin-3 during myoblast differentiation. (A) Undifferentiated myoblasts (MB) or myoblasts differentiated for 24 h (24 h-MB) or 72 h (MT) were imaged by DIC microscopy. Myotubes were observed after 72 h of differentiation but not after 24 h. For the sake of clarity, the plasma membrane of one cell is drawn with dotted line on each image. Nuclei position is marked with a white asterisk. (B) Expression of caveolin-3 (CAV3) was analyzed by western-blotting in undifferentiated myoblasts (MB) or myoblasts differentiated for the indicated time. After antibodies incubation, the membrane was cut in half to reveal GAPDH (loading control) with a colorimetric detection kit and caveolin-3 by a chemiluminescent method. The entire membrane is presented in Supporting Information S1: Figure S1A. The histogram presents mean values (\pm SEM) of the ratio CAV3/GAPDH for MB ($n = 6$), 1 h-MB ($n = 2$), 6 h-MB ($n = 2$), 16 h-MB ($n = 2$), 24 h-MB ($n = 6$) and MT ($n = 4$) samples analyzed by the gel analysis plugging of ImageJ. Mann-Whitney test. $**p < 0.01$.

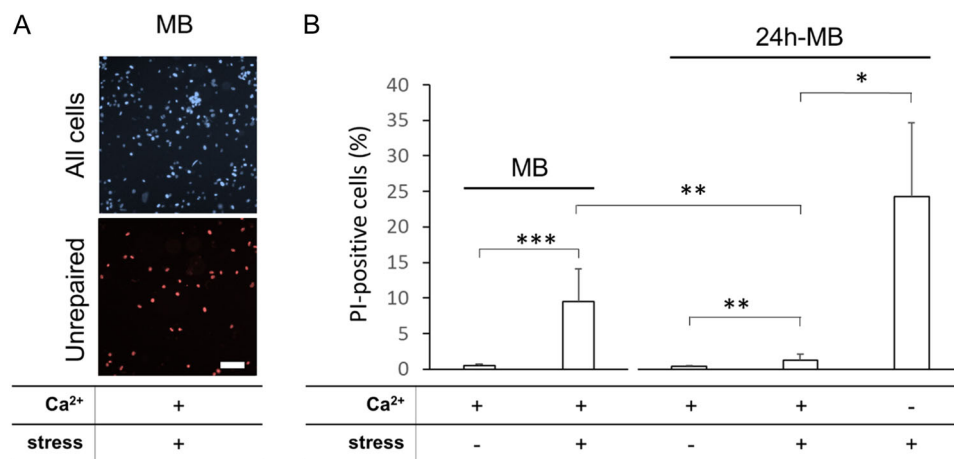


FIGURE 3 | Unlike undifferentiated myoblasts, 24-h differentiated myoblasts are able to repair membrane damage created by the muscle shear-stress assay. The shear-stress treatment was performed (+) or not (-) on undifferentiated (MB) or 24-h differentiated myoblasts (24 h-MB) in the presence (+) or absence (-) of 2-mM Ca²⁺, as indicated. (A) Representative images of undifferentiated myoblasts submitted to the shear-stress assay in the presence of Ca²⁺ and stained with Hoechst (all cells, blue) and PI (unrepaired cells, red). Images for the other conditions are presented in Supporting Information S1: Figure S2. Scale bar = 50 μ m. (B) Histograms represent mean values (\pm SEM) from at least three independent experiments performed in triplicate. Mann-Whitney test. $***p < 0.001$, $**p < 0.01$, and $*p < 0.05$.

MB, +stress, -Ca²⁺, reaffirming that membrane repair is Ca²⁺-dependent. To further validate the membrane repair ability of 24-h differentiated myoblasts, we used the standard membrane repair assay using laser ablation [8, 12].

Undifferentiated and 24-h differentiated LHCN myoblasts were damaged by infrared laser irradiation in the presence of Ca²⁺ and FM1-43. After laser injury, FM1-43 entered the cytosol where it

fluoresced upon incorporation into intracellular membranes. This fluorescence increased until the plasma membrane was resealed. In undifferentiated myoblasts, FM1-43 was observed entering the cells at the site of membrane irradiation within seconds post-injury, confirming the presence of membrane rupture (Figure 4A, +1.6 s, **arrow**). After 120 s, the fluorescent dye had diffused throughout the cell (Figure 4A, +120 s). The kinetics of the change in the fluorescence intensity revealed a continuous

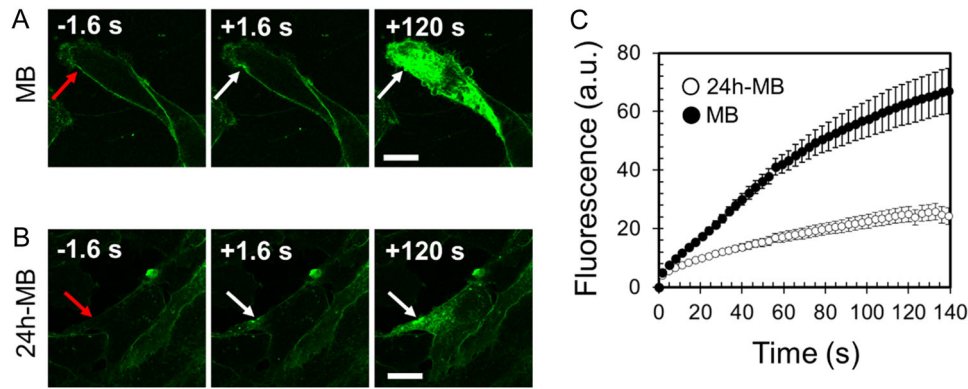


FIGURE 4 | Unlike undifferentiated myoblasts, 24-h differentiated myoblasts are able to repair membrane damage created by laser ablation. (A, B) Sequences of representative images showing the response of an undifferentiated (A) or 24-h differentiated (B) myoblast to a membrane damage performed by 110-mW infrared laser irradiation, in the presence of FM1-43 (green). In all figures, the area of membrane irradiation is marked with a red arrow before irradiation and a white arrow after irradiation. Scale bars: 10 μm . (C) Kinetic data represent the FM1-43 fluorescence intensity for undifferentiated (black-filled circles) or 24-h differentiated (empty circles) myoblasts, integrated over whole cell sections, averaged for about 30 cells (\pm SEM).

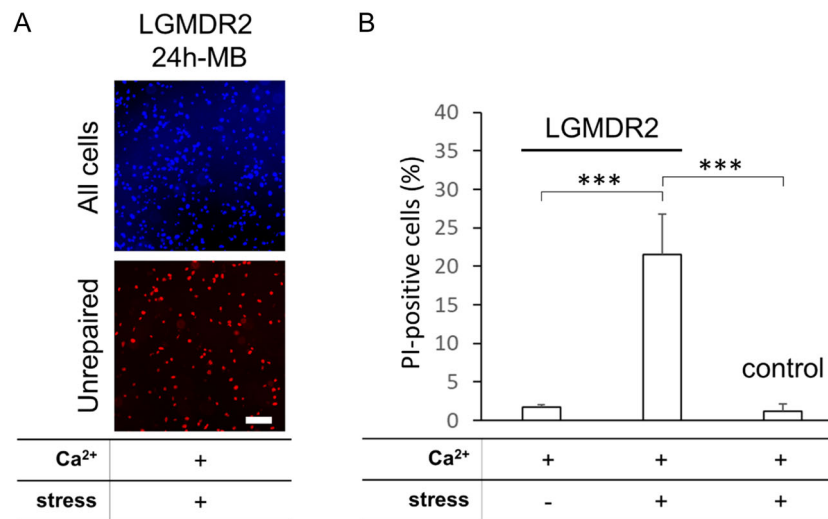


FIGURE 5 | Unlike control myoblasts, 24-h differentiated LGMDR2 myoblasts are unable to repair membrane damage created by the muscle shear-stress assay. The shear-stress treatment was performed (+) or not (-) on 24-h differentiated control or LGMDR2 myoblasts in the presence of 2-mM Ca²⁺, as indicated. (A) Representative images of 24-h differentiated LGMDR2 myoblasts submitted to the shear-stress assay in the presence of Ca²⁺ and stained with Hoechst (all cells, blue) and PI (unrepaired cells, red). Scale bar = 50 μm . (B) Histograms represent mean values (\pm SEM) from at least three independent experiments performed in triplicate. Mann-Whitney test. *** $p < 0.001$.

increase following membrane damage (Figure 4C, filled circles), underscoring the lack or a major delay in membrane resealing in undifferentiated myoblasts. Conversely, in 24-h differentiated myoblasts subjected to irradiation, most damaged cells exhibited an increase of intracellular fluorescence confined to the vicinity of the disruption site (Figure 4B). The kinetics of the change in the fluorescence intensity showed that intracellular fluorescence intensity increased for about 100 s and then tended towards a plateau (Figure 4C, empty circles), suggesting resealing of the plasma membrane. Time required for membrane resealing was longer for 24-h differentiated myoblasts compared to myotubes (100 s vs. 70 s [12]), reflecting a superior resealing capacity of myotubes. Actually, single-cell analysis within each experiment showed that the mean curve observed for 24-h differentiated myoblasts was negatively impacted by a few myoblasts incapable of repairing their membrane (Supporting Information S1:

Figure S3). Hence, we concluded that 24-h differentiated myoblasts possessed a functional membrane repair machinery.

3.4 | The Muscle Shear-Stress Assay Identifies the Defect in Membrane Repair of Dysferlinopathic Muscle Cells

Lastly, we assessed the effectiveness of the muscle shear-stress assay to detect membrane repair defects in pathological muscle cells. As a positive control, we used the L578 cell line, which has been established from a patient affected by LGMDR2. When submitted to the shear-stress assay in the presence of Ca²⁺, 24-h differentiated L578 myoblasts displayed significantly more unrepaired cells than control cell lines (Figure 5), mirroring the value seen in undifferentiated myoblasts (Figure 3B). We have

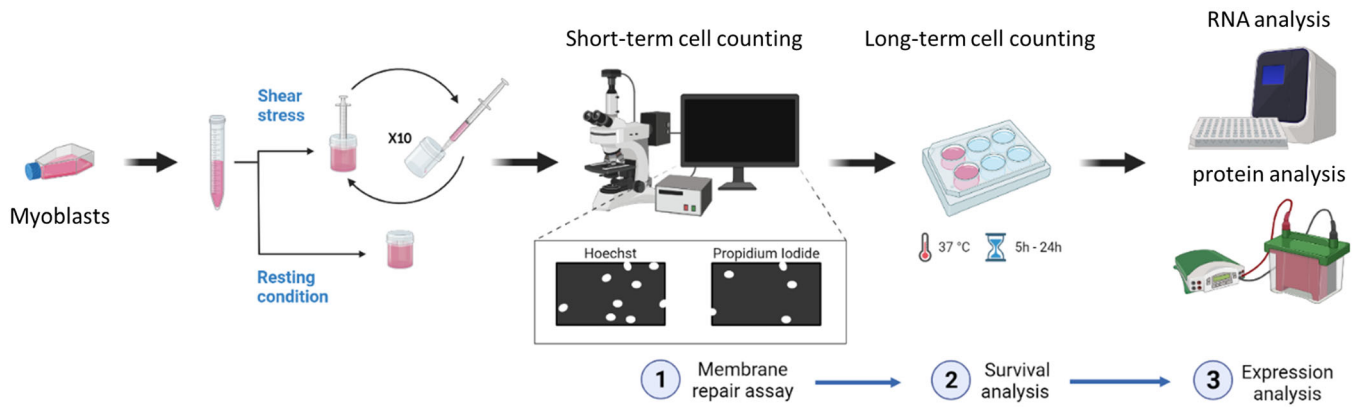


FIGURE 6 | Global insight of the applications based on the muscle shear-stress assay. Myoblasts are split into two populations: the stressed population is passed 10 times through 30 G needle, while the other remains in resting condition and used as a control. After performing the membrane repair assay (see legend of Figure 1), cells may be seeded back in growth condition for 5 or 24 h. The impact of mechanical constraints on cell survival rate may be assessed by counting adherent control (no stress) or shear-stressed cells 24 h after plating. Survival rate corresponds to the ratio of the number of living cells between the shear stressed population and the untreated population. Finally, the impact of mechanical stress on protein/RNA expression may be analyzed by western-blot or RTqPCR by comparing the expression of proteins or RNAs 5 or 24 h after shear-stress between stressed and untreated cells. Adapted from [4] using [Biorender.com](https://www.biorender.com).

previously shown that LGMDR2 L578 myotubes were indistinguishable from control LHCN cells in number and size after 72 h of differentiation [12]. To confirm that L578 myoblasts differentiated similarly to control cells, we observed using bright-field microscopy 24-h differentiated LHCN and L578 myoblasts submitted to shear-stress assay to validate the absence of multinucleated cells (Supporting Information S1: Figure S4). Besides, we also analyzed the expression of caveolin-3 during the differentiation process. We observed that caveolin-3 was significantly expressed in L578 cells as early as 24 h after differentiation induction (Supporting Information S1: Figure S5) as observed in control cells (Figure 2B). These results rule out the possibility that the differentiation of L578 myoblasts may be delayed compared to control cells and may explain the absence of membrane resealing. We concluded that the muscle shear-stress assay enables the detection of defective membrane repair associated with LGMDR2 muscle cells, as previously described [5].

4 | Discussion

In conclusion, our study demonstrates that the muscle shear-stress assay is a simple but efficient method for submitting a large number of cells to mechanical constraints in a brief period. Its application is limited to myoblasts, as myotubes or muscle fibers are cells too large to pass through the needle. Building on previous findings that undifferentiated myoblasts, unlike myotubes, cannot repair or present a major delay in repairing membrane damage [12], we investigated whether differentiating myoblasts acquire the ability to repair membrane damage before fusing into myotubes. Our results indicate that 24-h differentiated myoblasts exhibit a functional membrane repair machinery evidenced by expression of caveolin-3. At this stage, muscle cells do not yet form myotubes, revealing that the membrane repair machinery is set up early within the differentiation/fusion process. Sarcolemma repair in these cells depends on the presence of Ca^{2+} , as previously reported for myotubes [12]. Finally, we demonstrate that this method

effectively identifies the absence of membrane repair in muscle cells from patient suffering from LGMDR2, confirming earlier findings obtained using laser ablation assay [5]. This method not only rapidly highlights muscle cells with membrane repair deficiencies, but also geno- and pheno-typic consequences of sarcolemma damage. For instance, the muscle shear-stress assay facilitates the analysis of the impact of membrane damage on cell survival and gene/protein expression (Figure 6). Given its ability to affect a large number of cells quickly, this technique is arguably more appropriate than laser ablation for such studies. Our proposed workflow is as follows: after the muscle shear-stress assay, cells are immediately analyzed for membrane repair (application 1), and/or then plated for 24 h to evaluate survival rate (application 2) and/or gene/protein expression (application 3) analysis. Survival rate and gene/protein expression levels are assessed by quantifying number of cells and mRNA/protein levels (by RTqPCR or western-blotting) in myoblasts submitted or not to the muscle shear-stress assay.

Author Contributions

Léna d'Agata and Anthony Bouter performed the majority of experiments with assistance from Céline Gounou and Flora Bouvet (cell culture and western-blot analysis) and Phoebe Rassinoux (shear-stress assays). Dounia Bouragba and Kamel Mamchaoui established the L578 and KM1421 cell lines, respectively. Anthony Bouter coordinated the entire project and designed the experiments. Anthony Bouter wrote the manuscript with the help of Léna d'Agata.

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Conflicts of Interest

The authors declare no conflicts of interest.

Data Availability Statement

The authors declare that all data supporting the findings of this study are available within the article and its supplementary Information.

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Supporting Information

Additional supporting information can be found online in the Supporting Information section.