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Research Article

# GREG cells, a dysferlin-deficient myogenic mouse cell line

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ABSTRACT

The dysferlinopathies (e.g. LGMD2b, Myoshi myopathy) are progressive, adult-onset muscle wasting syndromes caused by mutations in the gene coding for dysferlin. Dysferlin is a large (~200 kDa) membrane-anchored protein, required for maintenance of plasmalemmal integrity in muscle fibers. To facilitate analysis of dysferlin function in muscle cells, we have established a dysferlin-deficient myogenic cell line (GREG cells) from the A/J mouse, a genetic model for dysferlinopathy. GREG cells have no detectable dysferlin expression, but proliferate normally in growth medium and fuse into functional myotubes in differentiation medium. GREG myotubes exhibit deficiencies in plasma membrane repair, as measured by laser wounding in the presence of FM1–43 dye. Under the wounding conditions used, the majority (~66%) of GREG myotubes lack membrane repair capacity, while no membrane repair deficiency was observed in dysferlin-normal C2C12 myotubes, assayed under the same conditions. We discuss the possibility that the observed heterogeneity in membrane resealing represents genetic compensation for dysferlin deficiency.

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## Introduction

Limb girdle muscular dystrophy 2b (LGMD2b) and Myoshi Myopathy (MM) are late-onset muscular dystrophies caused by point mutations and deletions resulting in reduced levels, or absence, of the protein dysferlin [1–4]. In dysferlinopathy patients, the sarcolemma displays characteristic abnormalities, including 0.1–2.0 μm discontinuities, thickened basal lamina, and accumulations of small vesicles at the sarcolemma [5]; these features suggest that dysferlin is required for maintenance of sarcolemmal integrity. Dysferlin is a large (~200 kDa) membrane-anchored protein with six C2 domains, having sequence similarity to synaptotagmins. By analogy to synaptotagmin's function as a possible calcium sensor in exocytosis, it has been proposed that dysferlin may serve as a calcium sensor for membrane repair [6–8]. Isolated wild-type mouse muscle fibers can reseal sarcolemmal wounds in the presence of ~1 mM $[Ca^{2+}]_{free}$, but fibers from a dysferlin knock-out mouse are defective in resealing, based on the unimpeded uptake of FM1–43 fluorescent dye following laser wounding [6].

Studies of muscle damage and repair in vivo suggest that dysferlin may have other functions in addition to membrane resealing. The A/J mouse strain has a spontaneous dysferlin mutation, due to a retrotransposon insertion in intron 4 of the dysf gene, and no detectable dysferlin protein expression [9]. A/J mice exhibit a progressive muscular dystrophy, appearing ~2 months in the proximal muscles and spreading to the distal muscles by 5 months. A/J mice exhibit a defect in recovery from muscle injury caused by a single large strain lengthening contraction [10,11]. Large strain lengthening contractions produce microtears in muscle fibers which spontaneously reseal, based on retention of fluo-

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rescent dextran. A/J muscle fibers appear to reseal normally following injury, but become necrotic a few days later, and must be replaced by new myogenesis [10,11].

Myogenic cell lines derived from mouse models for muscular dystrophies have proven to be of great value in understanding the pathobiology of these diverse diseases. Efforts to define the function(s) of dysferlin in membrane repair have been hampered by a lack of dysferlin-deficient myogenic cell lines. In order to study membrane resealing using a model cell system, we have developed a dysferlin-deficient myogenic cell line (GREG) from the A/J mouse strain. These cells differentiate into morphologically typical myotubes which do not express detectable levels of dysferlin. GREG myotubes exhibit a heterogeneous membrane resealing deficiency which varies in severity between individual myotubes, suggesting they possess both dysferlin-dependent and independent modes of membrane repair. Dysferlin-independent membrane repair could represent a compensatory process operant in the presence of dysferlin deficiency.

## Materials and methods

### *Isolation of GREG cells*

Thigh muscles from a nine day old A/J strain mouse were excised, minced and digested with a mixture of 0.25% trypsin–0.1% collagenase/dispase in phosphate buffered saline (PBS) to obtain a suspension of mononucleated cells [12,13]. The cells were resuspended in Ham's F-10 medium (Invitrogen, 11550) with 20% fetal bovine serum (FBS) (Gemini Bio Products, 100–106), 0.5% chick embryo extract (CEE) (Accurate Chemical & Scientific, CE650TL), Penicillin–Streptomycin liquid (Invitrogen, 15070) and L-Glutamine 200 mM (Invitrogen, 25030), and incubated in (uncoated) 100 mm tissue culture dishes for 1 h to deplete contaminating fibroblasts (which selectively adhere to the dishes), and then were transferred to collagen-coated dishes for myoblast attachment.

Primary myoblast cells were cultured in F-10 medium (with 20% FBS and 0.5% CEE, as above) for three weeks to suppress fibroblast cell growth. To further select for myoblasts and deplete fibroblasts, cells were lifted in PBS without $Ca^{2+}$, $Mg^{2+}$, trypsin or EDTA. Growth medium was aspirated, cells rinsed with PBS and a small amount of PBS was added to the dish. Cells were placed in a 37 °C incubator until they became rounded and could be knocked off the plastic. This procedure was used for the first month of culture expansion until most of the fibroblasts were depleted. During this time the cells grew slowly. The growth medium was changed every other day and cells were lifted and transferred to fresh collagen coated dishes every 5 days regardless of cell density. After approximately 6 weeks of growth, a majority of the initial cell population died but the proliferation rate of the remaining myoblasts accelerated, possibly representing spontaneous transformants, allowing selection for a pure myoblast culture. At this point F10 medium was replaced by Dulbecco's modified Eagle medium (DMEM) containing GlutaMAX instead of L-Glutamine (Invitrogen, 10569). This medium supported faster myoblast growth; media was changed every two days and a strong precaution was taken to avoid cell overgrowth. Cells now were able to grow routinely on regular tissue culture plastic; collagen coated dishes were reserved for experimental work.

For routine culture, GREG myoblasts are maintained in growth medium: DMEM plus 20% FBS and 0.5% CEE (Accurate Chemical & Scientific, CE650TL), plus antibiotic Primocin 100 mg/ml (InvivoGen, ant-pm-2). For passage, the cells are lifted using 0.25% Trypsin/EDTA (Invitrogen, 25200), at intervals of 3 days (when the cells are 80% confluent), and seeded at a density of $2\times10^4$/ml in T-25 flasks. For differentiation into myotubes, myoblasts are plated in growth medium on collagen-coated plastic dishes (e.g. BD Biocoat, 354456) and allowed to reach 80% confluency (1–3 days). The growth medium is replaced with differentiation medium (DMEM plus 5% horse serum) with 4 washings; cells are refed daily. Differentiation into myotubes occurs over a period of 2–5 days; >90% of the myoblasts fuse into myotubes. The cultures appear to be depleted of contaminating fibroblasts. Fibroblasts, when present, will overgrow cultured myotubes in a few days. We have not observed any fibroblast proliferation in myotube cultures maintained for up to 100 days.

### *C2C12 cells*

Parental C2C12 cells and an shRNA-transduced dysferlin "knock down" cell line were generously provided by Dr. Michelle Maxwell, Harvard Medical School [14]. These cells were cultured and differentiated as described for GREG cells (see above).

### *Genomic analysis*

Genomic DNA was prepared from GREG and C2C12 myoblasts using the Wizard genomic DNA purification kit (Promega, A1120). PCR primers for detection of the retrotransposon (ETn) insertion in the dysferlin gene were as described in Ref. [9]: dysf-F, dysf-R, ETn-oR, ETn-R2. 25 µl reactions contained 50 ng DNA, 0.4 µM primers, 20 mM Tris (pH 8.4), 50 mM KCl, 1.5 mM $MgSO_4$, 200 µM each dNTP, and 1 U Taq DNA polymerase (Invitrogen). Reactions were denatured for 3 min at 94 °C; then subjected to 35 cycles of 30 s at 94 °C, 30 s at 55 °C, 60 s at 72 °C; followed by a 10 min. extension at 72 °C. PCR products were analyzed by electrophoresis in a 1.5% agarose gel run in 1X TAE buffer, and visualized by staining with ethidium bromide.

### *Detection and quantification of muscle fiber proteins*

The expression of specific proteins in cultured cells was detected using fluorescent-labeled ligands or antibodies. Cells were washed with serum-free medium, fixed with 3.7% paraformaldehyde in PBS for 20 min, permeabilized with 0.1% triton X100 for 2 min. F-actin was detected by staining with 0.88 µM rhodamine-labeled phalloidin (Molecular Probes, R415) for 20 min at room temperature. Nicotinic acetylcholine receptor (nAChR) was detected by staining live cells with fluorescein-conjugated α-bungarotoxin (1 µg/ml in PBS; Molecular Probes, F-1176) for 90 min.

For detection of dysferlin and myoferlin we used an antigen retrieval procedure as described in Robinson, et al. [15]. Following Triton-X100 treatment (see above), cells were treated with 0.5% SDS for 5 min, washed with PBS and blocked with 5% normal goat serum in PBS for 1 h. Cells were incubated with Hamlet mAb to dysferlin (NCL-Hamlet, Leica Biosystems) diluted 1:40, or myoferlin (Cov-7D2, Novus Biologicals) diluted 1:50, for 1 h, washed with PBS, and incubated with Alexa Fluor-labeled goat anti-mouse IgG (Invitrogen A11029) diluted 1:1500 for 1 h. Cells were imaged using a Zeiss Axiovert 25 with a 10× objective and images were recorded using a digital camera (Hamamatsu Orca ER) controlled by IPLab (Version 3.61, Scanalytics, Fairfax, VA,