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MINI-JAG1 PEPTIDE TREATMENT ENABLES PATIENT-DERIVED IMMORTALIZED MYOBLAST CULTURES TO RESIST CALCIMYCIN-INDUCED MORTALITY

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ABSTRACT

Duchenne muscular dystrophy (DMD) is one of the most common childhood-onset, severe, life-threatening diseases. The absence of functional full-length isoform of dystrophin from the causal X-linked gene *DMD/DYS,* progressive loss of muscle function, cardiac weakness, and premature death. The patient-derived immortalized myoblast cultures recapitulate many diseases' specific pathological features and provide an excellent *in-vitro* model for therapy development. One of the previous studies indicated complete rescue in the Golden Retriever DMD model due to Jagged1 overexpression. However, the potential of the Jagged1 ligand as a future of DMD therapy remains unknown. Here, the effect of Jagged1 as a peptide was tested in patient-derived myoblast cultures. The findings raise hopes for therapy for this debilitating condition.

***Keywords**: Duchenne Muscular Dystrophy, Jagged1, Myoblast, Calcimycin*

INTRODUCTION

Duchenne muscular dystrophy (DMD) is a degenerative condition that progressively affects the skeletal muscles, heart muscles, and, in some cases, cognition. The disease is linked to mutations in the X-linked *DMD/DYS* gene, which disrupts the reading frame and inhibits the production of functional dystrophin (Dp427) in the cell membranes of the brain, heart, and skeletal muscles (Monaco *et al.,* 1986; Hoffman *et al.,* 1987; Koeing *et al.,* 1987; Tennyson *et al.,* 1995*;*). The absence of dystrophin in these cell membranes renders them highly vulnerable to damage during periods of increased activity (Ervasti *et al.,* 1991; Ohlendieck *et al.,* 1991; Mokhtarian *et al.,* 1999). Consequently, the continuous cycle of damage, inflammation, and improper regeneration, all occurring without dystrophin, leads to irreversible and progressive weakness throughout the body's muscles, ultimately affecting the heart and leading to premature death.

Ongoing efforts to correct or replace mutated genes/mRNA offer promising approaches for addressing DMD. However, further refinement and cost-effectiveness are required to make these interventions truly beneficial for patients. While standard management practices such as steroid administration, spine correction surgeries, and ventilator support have shown some positive effects in improving the patient's condition and delaying disease progression by 2-3 years, they are not without their side effects.

Extensive research has provided valuable insights into the pathological processes underlying muscle damage in DMD, which arise from dysfunctions in otherwise normal muscle contraction mechanisms (Nesari *et al.,* 2023). The *mdx* model has played a crucial role in unraveling the molecular mechanisms behind the disease. However, establishing causal relationships has proven challenging. The mild phenotype observed in the *mdx* model can be attributed to small size, gait, telomeric length, myogenic potential, and utrophin upregulation (Partridge *et al.,* 2013). Utrophin has consistently emerged as a disease modifier in the *mdx* model (Soblechero-Martín *et al.,* 2021). However, despite its potential, utrophin upregulation as a therapeutic approach has yet to demonstrate positive outcomes in clinical trials (NCT02858362), indicating that the efficacy of specific disease modifiers may be species dependent.

While Jagged1 upregulation has shown promising results in improving disease outcomes in the GRMD model and zebrafish (Vieira *et al.,* 2015), its potential as a disease modifier in human DMD needs further investigation.

Expression of Dp427 serves as a marker for the maturation phase of terminal myotube formation, along with myozenin/s (Yoshimoto *et al.,* 2020), indicating that dystrophic myoblasts normally differentiate in *in-vitro* cultures (Blau *et al.,* 1983a). However, due to their limited lifespan, maintenance challenges, and genetic manipulation difficulties, primary cultures cannot be directly utilized for drug testing or experiments. Recent advancements in cell culture techniques have led to the development of immortalized myoblasts derived from patients or induced pluripotent cells, which demonstrate normal differentiation in vitro (Arandel *et al.,* 2017; Piga *et al.,* 2019). However, these immortalized myoblasts exhibit increased oxidative stress, premature differentiation (Salvadori *et al.,* 2021), impaired calcium handling (Al Tanoury *et al.,* 2021), and susceptibility to various forms of osmotic stress, including calcium ionophores (Vandebrouck *et al.,* 2002). Immortalized myoblasts have proven invaluable due to their ability to retain patient-specific characteristics and have been successfully employed in drug screening (Arandel *et al.,* 2017).

The previously made and characterized immortal cell lines were made via lentiviral transduction of hCDK4, with antibiotic resistance as a selection marker (Mamchaoui *et al.,* 2011). The mini-Jagged1 peptide, despite some controversy (Xiao *et al.,* 2013), has been shown to act as a Notch receptor agonist (Nickoloff *et al.,* 2002) and offers feasibility for therapeutic development. Calcium ionophores, such as calcimycin (A23187), increase calcium influx in intact cells and can replicate calcium-induced pathology similar to dystrophic muscles post-exercise (Duncan *et al.,* 1978).

Due to significant variations in growth kinetics and the intensity of pathology-associated markers among patient-derived cell lines, recapturing phenotype variability is seen in dystrophy patients. Hence, conducting a primary analysis was crucial. Cell lines exhibiting similar growth and differentiation kinetics were specifically selected for this study. Consequently, immortalized dystrophic and age-matched control myoblasts were treated with the mini-Jagged1 peptide during a 24–36-hour differentiation window, a critical time point when the expression of MyoG/myogenin begins to increase notably. Following this treatment, the cells were allowed to further differentiate for five days. A calcium ionophore was subsequently added to enhance calcium ion permeability and simulate the effects of exercise. A comparison was then made between healthy and dystrophic cell cultures treated with and without Jag1 peptide, evaluating parameters such as fusion index, myotube width, and induction of apoptosis.

MATERIALS AND METHODS

2.1 Culture of Patient-Derived Immortalized Myoblast lines:

The patient-derived immortalized myoblast cell lines used in this study were AB1023DMD11Q clone 1 (mutation: stop exon 59: c.8713C>T, p.Arg2905X; 47.82 division number) and age-matched control cell line AB1190 clone 1 (48.7 division number). These cell lines were established by Dr Anne Bigot and Dr Vincent Mouly at the Institut de Myologie in Paris, France, and have been previously described (Mamchaoui *et al.,* 2011).

The myoblasts were cultured in complete Skeletal Muscle Cell Growth Medium (Takara, C-23060) supplemented with 1% Glutamax (Invitrogen ref 35050-038) and 1% Gentamicin (Invitrogen ref 15750-037), following the protocol provided by the senders. Myoblasts with similar division numbers were initially seeded at 2×10^5 cells for the experiments. They were then rinsed with 10 ml PBS, trypsinized (using 2 ml of trypsin for 5-10 minutes at 37°C, 5% CO_2), and the collected cells were resuspended in 5 ml growth media. The cells were centrifuged at 1200g and resuspended in 3ml growth media before being transferred to Matrigel GFR-coated 6-well plates.

Once the myoblasts reached 70-80% confluency, the growth media was replaced with a differentiation medium containing DMEM, Gentamicin (50µg/ml), and insulin (10µg/ml). To mimic the effect of Jagged1 expression during the myogenin window, synthetic Jagged1 mini peptide "CDDYYYGFGCNKFCRPR" at a final dilution of 10^{-5}M in PBS (Nickoloff *et al.,* 2002) or only PBS was added 24 hours post-treatment.

2.2 Myogenic Fusion Index:
The myogenic fusion index was determined by calculating the multinucleated nuclei (≥3) ratio to the total number of nuclei. Five fields were randomly selected, and this process was repeated in three technical replicates. The mean and standard deviation (SD) were calculated for one technical replicate. Three technical replicates were analyzed for each treatment group as one biological replicate, and statistical analysis was performed.

2.3 Myotube Width Measurement:
Similarly, myotube widths were measured using Image J software. Measurements were taken from five random fields, and three technical replicates were used to calculate the mean and SD of myotube width for a single biological replicate. Three biological replicates were included for each treatment group, and statistical analysis was conducted.

2.4 Apoptotic Induction:
Apoptotic induction was assessed by staining the cells with a final concentration of 50µM of Propidium Iodide (PI) in the dark for 30 minutes. The stained cells were then imaged using a Zeiss LSM 880 microscope. Hoechst stain was used as a counterstain. To determine the percentage of cells with PI-stained nuclei, the number of PI-stained nuclei was divided by the total number of nuclei and multiplied by 100.

2.5 Statistical Analysis:
The Control + PBS, Control +Jagged1peptide, DMD+PBS, DMD+Jagged1 peptide, were compared with their respective calcimycin treated and nontreated groups. GraphPad Prism software analyzed mean and SD data for 2-3 independent experiments with two-way ANOVA with Tukey's multiple comparison test. A p-value of less than or equal to 0.05 was considered significant.

RESULTS

3.1 Analysis of Myotube Formation and Quality in Control and DMD Myoblast Lines Treated with Mini-Jag1 Peptide
Figure 1 demonstrates a time series capture of control (AB1190) and DMD (AB1023) myoblast lines during myotube formation from 24 hours to 7 days in differentiation media. Qualitatively, both control and DMD cell lines exhibit similar myotube formation patterns under all treatment conditions. To quantitatively assess myotube quality, fusion index, and width were compared at seven days of differentiation, corresponding to 2 days of calcimycin treatment.

In Fig. 2A, multinucleated fibres are observed with very few unfused myocytes in both control and DMD cell lines across all treatment conditions. The percentage fusion index in PBS-treated control (87±1.9), PBS-treated DMD (88.87±1.12), Jag1 peptide-treated control (86.05±0.75), and Jag1 peptide-treated DMD (87.2±1.56) did not show significant differences (Fig. 2B).

Furthermore, myotube widths were measured from the same images (Fig. 2C). DMD cells treated with PBS (175±79.69) and DMD cells treated with Jag1 peptide (148.8±68.65) exhibited a significant reduction in myotube width compared to PBS-treated control (247±137). The myotube width in Jag1 peptide-treated control (197.7±112) was slightly lower than that of PBS treatment but did not reach statistical significance. Thus, the treatment with Jag1 peptide did not significantly reduce myotube widths in either control or DMD cell lines.

3.2 Mini-Jag1 Peptide Treatment Reduces Susceptibility to Calcimycin-Induced Apoptosis in Myotubes
The mini-Jag1 peptide treatment reduces susceptibility to calcimycin-induced apoptosis in myotubes. Surprisingly, the DMD myotubes exhibit lower susceptibility to calcimycin-induced death compared to control myotubes under similar conditions (Fig. 3A). Although there is no significant difference between PBS-treated and Jag1 peptide-treated DMD myotubes after the addition of calcimycin, there is a significantly lower percentage of Propidium Iodide (PI)-stained nuclei in the DMD + PBS + Calcimycin group and DMD+Jag1-peptide+calcimycin group compared to the control+Jag1-peptide+calcimycin group. Additionally, the percentage of PI-stained nuclei in the untreated DMD myotubes is significantly lower than in the control+Jag1-peptide+calcimycin group (Fig. 3B).

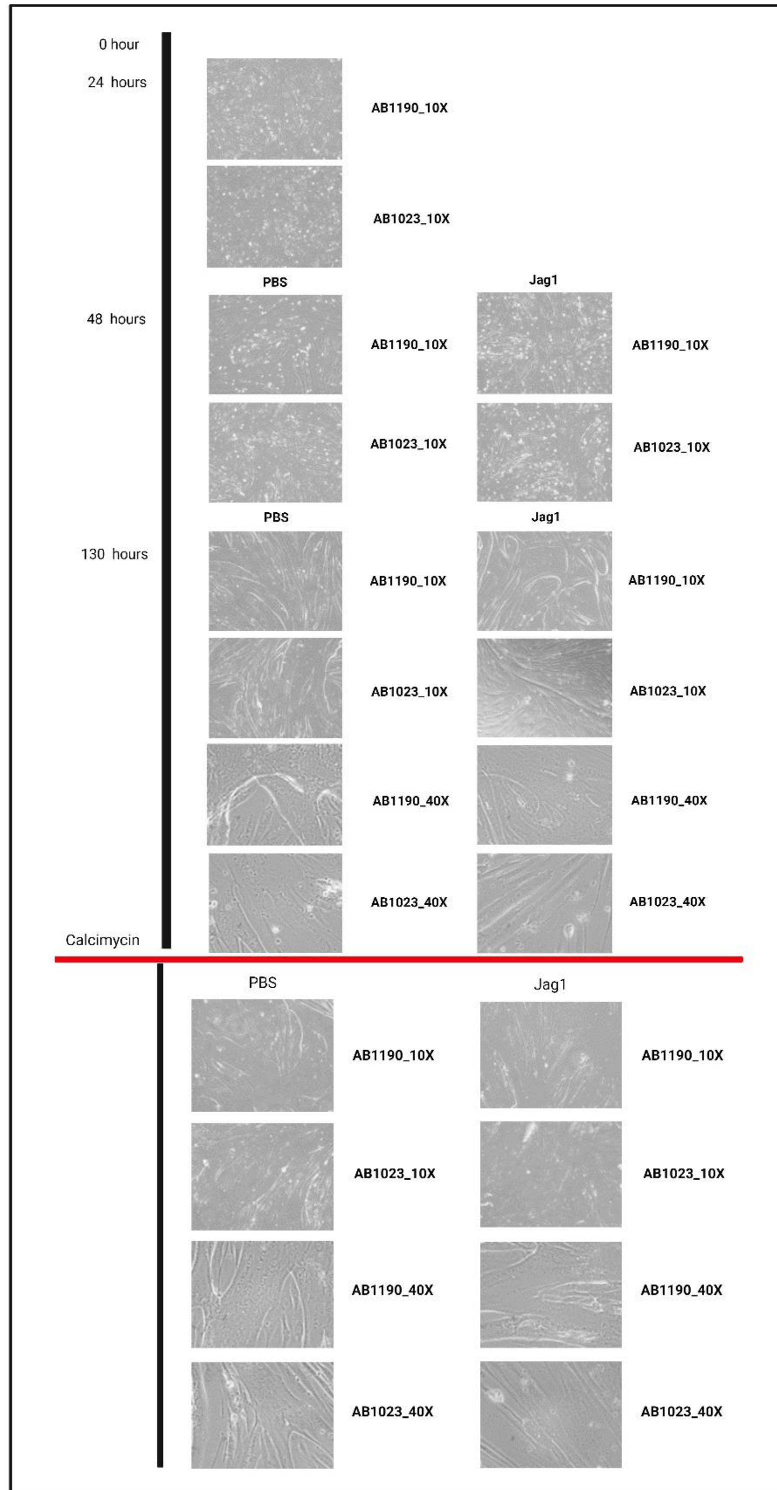
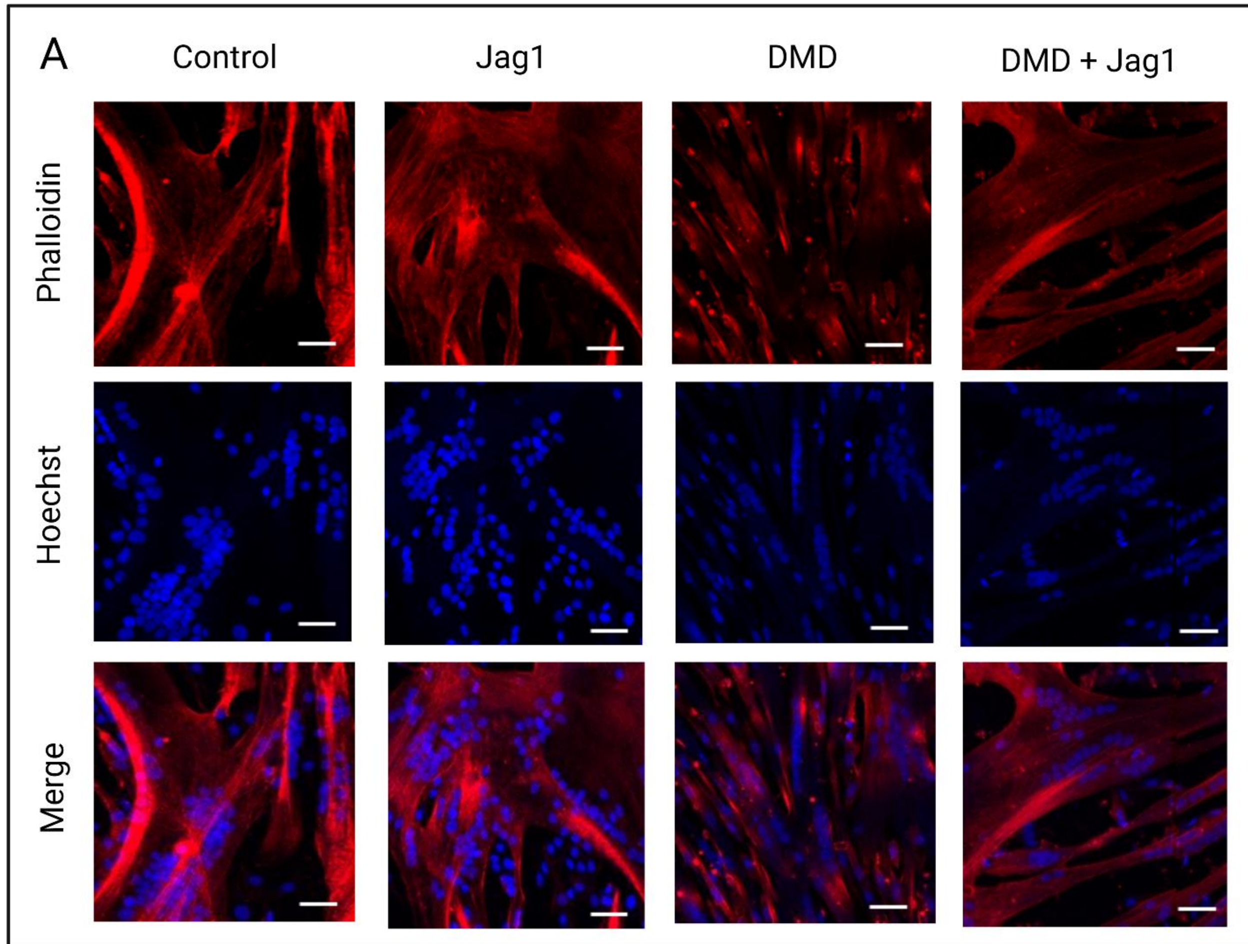
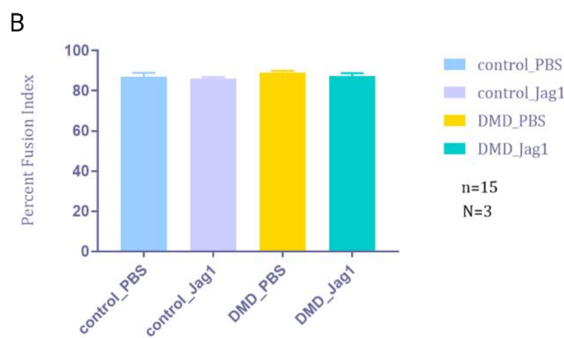


Figure 1: A Time Series of Differentiation of Control (AB1190) and DMD(AB1023) myoblasts.
Myoblasts were treated with PBS or mini-Jag1 peptide at 24 hours of differentiation. These myotubes were differentiated for 5 days before treating them with calcimycin, and images were captured 24 hours after calcimycin treatment.





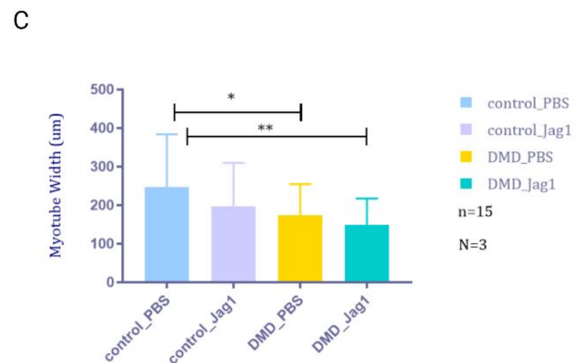


Figure 2: The mini-Jag1 peptide treatment does not affect the fusion index or myotube width. A: Representative Images of Hoechst and Phalliodin-633 co-staining (Scale bar: 50µm) in 7-day post differentiation myotubes in Jag1 peptide or PBS treated Control and DMD cell lines**. B:** Quantification of fusion index shows no difference between -Control and -DMD, Jag1 peptide treated, or PBS treated. **C:** Myotube width (um)is significantly lower in DMD than PBS treated Control, and remains reduced with Jag1 peptide treatment in DMD. N=3, n=15, significance: * p< .05, ** p<.02, *** p<.001

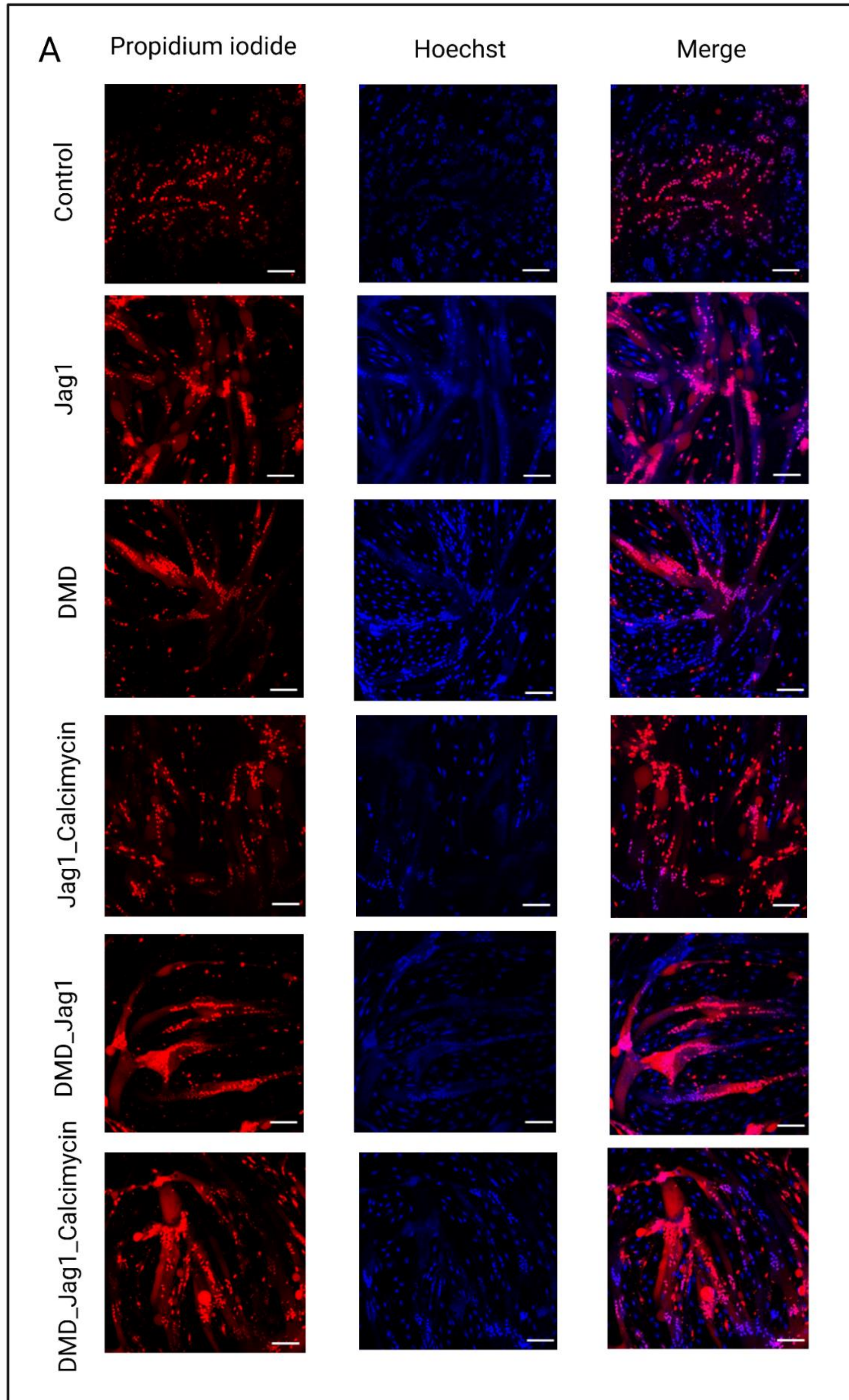


Figure 3A

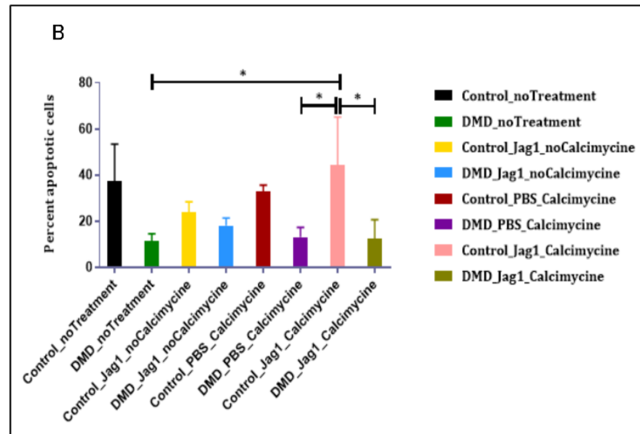


Figure 3 B

Figure 3: Calcimycin-induced apoptosis in control and DMD myotubes. A: Jag1 -peptide/PBS treatment 24 hours followed by calcimycin at 5 days of differentiation, myotubes were imaged at 7-day post - differentiation Control (AB1190) and DMD (AB1023) after live staining with Hoechst and Propidium Iodide (PI).
B: Quantification of percent of cells stained with PI in all treatment groups (Scalebar: 100um). Significant differences were only between pairs DMD no treatment vs. Control+Jag1+Calcimycin, Control+Jag1+Calcimycin vs. DMD+Jag1+Calcimycin, and DMD+ PBS+ Calcimycin vs. Control+Jag1+Calcimycin. N=3, n=15, significance: * $p < .05$, ** $p < .02$, *** $p < .001$

DISCUSSION

The immortalized cell lines derived from DMD patients (Mamchaoui *et al.,* 2011) exhibit variability in growth kinetics and pathological features, resembling the heterogeneity observed in DMD patients (Choi *et al.,* 2016). Two selected cell lines, AB1190 (control) and AB1023 (DMD) displayed similar growth and differentiation kinetics (Fig. 1) and were suitable for comparison. Patient-derived myoblasts are known to differentiate usually during in vitro culture without apparent defects (Blau *et al.,* 1983b), supported by the fusion index results indicating normal differentiation in the dystrophic line (Fig. 2A). However, the width of dystrophic myotubes was significantly reduced compared to the control (Fig. 2B). This variation in myotube width has been observed in other patient-derived cell lines and primary myoblast cultures from DMD patients (Nesmith *et al.,* 2016; Nguyen *et al.,* 2021).

Calcimycin, a calcium ionophore, increases intracellular calcium levels without permeabilizing cells. Due to the poor calcium handling capacity of dystrophic cells (Al Tanoury *et al.,* 2021), it is expected to induce cell death through calcium-activated proteases (Mareedu *et al.,* 2021). Surprisingly, we discovered that dystrophic myotubes were significantly less susceptible to detachment and cell death in culture. This could be attributed to the combination of reduced myofiber width and the known misalignment of dystrophic myotubes, resulting in a decreased force exerted against the culture surface and leading to delamination/detachment (Nesmith *et al.,* 2016; Barthélémy *et al.,* 2022). The inability of calcimycin to induce cell death in dystrophic myotubes in vitro may be attributed to its ability to stimulate glycolysis (Szibor *et al.,* 1981) and protein synthesis (Ionasescu *et al.,* 1976).

Furthermore, the reduced susceptibility of dystrophic myotubes to calcimycin-induced cell death suggests the presence of compensatory mechanisms or adaptations in the DMD cell line. These mechanisms might include altered calcium handling pathways, upregulation of cytoprotective factors, or enhanced cell survival signaling. Future studies should investigate these potential protective mechanisms to gain insights into the molecular pathways involved in the resistance of dystrophic myotubes to calcium-induced cytotoxicity.

CONCLUSIONS

The findings of this study contribute to our understanding of the complex phenotypic variability observed in DMD patient-derived cell lines. The divergent myotube width observed in different patient lines highlights the importance of considering inter-individual variation when using cell-based models for disease research and drug development. Moreover, the unexpected resistance of dystrophic myotubes to calcimycin-induced cell death challenges the conventional notion of heightened susceptibility of DMD cells to calcium-induced damage. This emphasizes the need for comprehensive investigations into the multifaceted cellular responses and adaptive mechanisms dystrophic muscle cells exhibit.

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DECLARATION OF CONFLICT OF INTEREST

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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