Chapter 5

Preliminary Studies on Immortalized Myoblast Cultures Treated with Mini-Jag1-Peptide Recapitulate the Temporal Effects of Jagged1 Overexpression

INTRODUCTION

Duchenne Muscular Dystrophy (DMD) is a progressive degenerative disease affecting skeletal and heart muscles and, in some cases, cognition. Mutations in the X-linked *DMD/DYS* gene that disrupt the reading frame and production of functional dystrophin (Dp427) in the cell membranes of the brain, heart, and skeletal muscles, are associated with DMD. The absence of dystrophin in the cell membranes makes them excessively susceptible to damage during increased activity. The continuous damage, inflammation, and improper regeneration in the absence of dystrophin snowballs into irreversible, progressive weakness in whole body muscles and the heart causing early death (Nowak & Davies, 2004).

The various approaches for mutated gene/mRNA corrections/replacements are being developed however, there is a need for further refinement in treatment at affordable price to benefit the patients. Standard management practices like steroids, spine correction surgeries, ventilators, etc., help improve the patient's condition and delay progression by 2-3 years despite some side effects (Markham et al., 2008; Parreira et al., 2010; Pardo et al., 2011; Schram et al., 2013; Kim et al., 2014; Gloss et al., 2016).

Extensive research has unraveled that muscle contraction associated molecular processes exhibit pathological attributes in dystrophic condition which leads to muscle damage (Nesari et al., 2023). The *mdx* model has been invaluable in deciphering molecular mechanisms of pathology, but causal relations have remained elusive. Factors like small size, gait, telomeric length, myogenic potential, upregulation of utrophin etc. (Partridge et al., 2013) contribute to the very mild phenotype in the mdx model. Utrophin has been consistently shown to be a disease modifier in the *mdx* model (Soblechero-Martín et al., 2021). Nevertheless, utrophin upregulation as therapy is yet to show positive outcomes in clinical trials (NCT02858362). This suggests that some disease modifiers are effective in a species-dependent manner. Though Jagged1 upregulation has shown positive disease outcomes in GRMD and zebrafish (Vieira et

al., 2015) supported by our findings stated in chapters 3 & 4, its disease-modifying potential must be tested for the human condition.

The Dp427 expression marks the maturation phase of terminal myotube formation and myozenins (Yoshimoto et al., 2020), and hence, dystrophic myoblasts differentiate typically in in vitro cultures (Blau et al., 1983). However, primary cultures cannot be used directly for drug testing or experiments because they are short-lived, difficult to maintain, or genetically manipulated. Recent advances in cell culture techniques have developed patient-derived immortalized myoblasts or induced pluripotent cell-derived myoblasts to differentiate in in vitro cultures normally (Arandel et al., 2017; Piga et al., 2019). Nevertheless, they show increased oxidative stress, premature differentiation (Salvadori et al., 2021), and poor calcium handling (Al Tanoury et al., 2021) hence, they are susceptible to a different type of osmotic stress, including calcium ionophores. The immortalized myoblasts have proven invaluable as they retain patient-specific features and, therefore, have been used for drug screening (Arandel et al., 2017). The patient-derived myoblast and age-matched control myoblasts were immortalized by lentiviral transduction of hCDK4 and hTERT genes (Mamchaoui et al., 2011), with antibiotic resistance as a marker for selection. The mini-Jagged1 peptide has been shown to act like a Notch receptor agonist (Nickoloff et al., 2002), though disputed. It has the advantage of feasibility for therapeutic development. Calcium ionophores like calcimycin (A23187) increase calcium ingress in intact cells, which can imitate calcium-induced pathology similar to post-exercise dystrophic muscles (Duncan et al., 1978). Hence, immortalized dystrophic and age-matched control myoblasts were grown and treated with mini-Jagged1 peptide during a 24-hour differentiation window.

The human Jag1 gene contains a microRNA between exons 9 and 10 in the intronic region, as shown in Figure 5.1. Though nucleotide sequence blasts using the NCBI tool did not reveal any similar sequence, it might still play a role in humans. Information is scant about miR6870 expression, tissue specificity, promoters, etc., and the UCSC genome browser did not show any potential promoter element which can independently express this microRNA.

Hence, along with host Jag1 gene expression during myoblast differentiation, we also checked the expression of mature derivative miRNAs called hsa-miR-6870-5p and hsa-miR-6870-3p. As Jag1 is a ligand for Notch receptors, the expression of known Notch target genes was also tested to determine if mini-Jag1 peptide can activate this pathway. Mitochondrial oxidative stress was compared as it was found higher in rescued dystrophic zebrafish larval muscles. The

differentiation index and calcimycin-induced cell death in myotubes which were differentiated for five days were then compared between peptide-treated or PBS-treated dystrophic or Control cell lines.

MATERIALS AND METHODS

Patient derived and age matched control immortalized myoblast cell lines were used. Synthetic Jagged1 mini peptide "CDDYYYGFGCNKFCRPR" final dilution in PBS (50µM) (Nickoloff et al., 2002) or only PBS was used to treat myoblasts in 24-hour post-differentiation as at this time myogenin expression increases in these cell lines. The cells were differentiated for five days and treated with calcimycin to induce pathology. At 48-hour post-calcimycin treatment, which is 7 days post-differentiation, cell death induction was checked. Mitochondrial oxidative stress was measured 24 hours post min-Jagged1 peptide treatment to check whether similar effects can be seen in human *in-vitro* culture to rescue dystrophic zebrafish larvae. Further, we found poorly characterized miRNA 6870 in the Jagged1 intronic region. In silico data from the UCSC genome browser found no promoter-like elements that can express this miRNA independent of the host Jagged1 gene. Additionally, a few of the predicted target genes of this miRNA6870-3p and -5p were seen in the "Escaper" GRMD transcriptomics data. Hence, Jagged1 and mi6870-3p/5p expressions were quantified along with canonical Notch target genes.

CULTURE OF PATIENT-DERIVED IMMORTALIZED MYOBLAST LINES

Immortalized myoblast cell lines AB1023DMD11Q clone1(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) used for this study were established by Dr. Anne Bigot and Dr. Vincent Mouly of Institute of Myology, France and described previously (Mamchaoui et al., 2011). The myoblasts were grown in complete Skeletal Muscle Cell Growth Medium (Takara, C-23060), 1% Glutamax (Invitrogen ref 35050-038) and 1% Gentamicin (Invitrogen ref 15750-037) according to protocol shared by senders. For experiments, similar myoblasts at similar division numbers were seeded at 2 x10^5 cells initially, rinsed with 10 ml PBS, trypsinized (with 2 ml of trypsin for 5-10min at 37°C, 5% CO_2 and then the cells were collected in 5ml growth media, centrifuged at 1200g and resuspended in 3ml growth media), and later transferred to Matrigel GFR coated 6 well plates. At 70-80% confluency, growth media was replaced with a differentiation medium containing DMEM + Gentamycin 50 µg/ml + 10 µg/ml of insulin. To

recapitulate the effect of Jagged1 expression during the myogenin window, 22-hours post differentiation, synthetic Jagged1 mini peptide "CDDYYYGFGCNKFCRPR" final dilution in PBS (50μM) (Nickoloff et al., 2002) or only PBS was added 24-hour post-treatment.

MYOGENIC FUSION INDEX

The fusion index was calculated as multinucleated nuclei (≥3)/total number of nuclei. Five fields were randomly chosen and repeated in 3 technical replicates and mean and SD were taken for 1 biological replicate. Three such biological replicates were considered for each treatment group and statistically analyzed.

MYOTUBE WIDTH

Similarly, myotube widths were measured in Image J from five random fields, and three technical replicates were used for the Mean and SD of a single biological replicate. Three such biological replicates were considered for each treatment group and statistically analyzed.

MITOCHONDRIAL OXIDATIVE STRESS

24 hours after mini-Jagged1 peptide treatment, which is ~48 hours post-differentiation, JC-1 dye (1μM) was added and incubated in the dark for 20 min. The images were taken in Zeiss LSM 880 confocal microscope. Fluorescence intensities of emission at 488nm (green) and 590nm (red) were quantified with Image J as arbitrary units (a.u.). The Green/Red ratios from 3 independent replicates were statistically analyzed.

NOTCH TARGET GENES EXPRESSION

Samples from control myoblasts (AB1190) proliferating for 24 hours in growth media were compared with DMD myoblasts (AB1023) in similar conditions. Myotubes from both cell lines were treated with mini-jag1 peptide at 24 hours in differentiation media. At 30 hours post differentiation (6 hours post Jag1 peptide treatment) was chosen for sample collection owing to rapid and transient notch receptor-mediated transcriptional activation as reported by Bray (2016). RNA was isolated with TRIzol (Sigma catalog number: T9424) reagent according to manufacturer instructions. Nanodrop was used to quantify RNA, and its quality was checked by agarose gel electrophoresis. Following manufacturer instructions, 1μg of RNA from all experimental groups was converted to cDNA with a BioRad kit (catalog: 1708890). The real-time PCR reactions were set using appropriate primers (refer to chapter 2) for known Notch

target genes. The β-actin and micro genes were normalizing "housekeeping" controls for calculating $^{\Delta}$CT values. The $^{\Delta\Delta}$ CT values based on Control (AB1190) myoblasts samples as Control were expressed as $\log_2$ fold change in the expression of Notch target genes. Three independent biological replicates were used to analyze gene expression statistically and represented as GraphPad Prism generated graphs.

MICRO RNA QUANTIFICATION

The simple method for miRNA quantification developed by Sharbati-Tehrani and co-workers (2008) was employed. The total RNA was used for reverse transcription. 50 ng/µl of the miRNA-specific DNA-oligonucleotide (RT6-miR-x) with 5' overhang and the Thermo Scientific™ Verso cDNA Synthesis Kit (Catalogue number: AB1453A) were employed to transcribe miRNA into cDNA. A mixture of 50 ng total RNA and RT6-miR-x was prepared in a 12 µl volume. The mixture was incubated at 70°C for 5 min and chilled on ice for 1-2 min. Then, the volume was brought up to 19 µl by adding the 5X cDNA synthesis buffer (4 µl), RT Enhancer (1 µl), and 5 mM dNTPs (2 µl). The reaction was incubated at 25°C for 5 min, and then, the Verso Enzyme Mix (1 µl) was added, followed by 25°C for a further 10 min and 42°C for 60 min. The enzyme was inactivated by heating at 70°C for 10 min.

Primer Sequences

Table 5.1: Primer Sequences for miRNAs

Sr No	MiR primer names	Sequence
1.	>**hsa-miR-6870-5p** MIMAT0027640	UGGGGGAGAUGGGGGUUGA
2.	RT6-miR6870_5p	tgt cag gca acc gta ttc acc gtg agt ggt **tcaacc**
3.	short-miR6870_5p-rev	cgt cag atg tcc gag tag agg ggg aac ggcgTGGGGGAGATGGG
4.	>**hsa-miR-6870-3p** MIMAT0027641	GCUCAUCCCCAUCUCCUUUCAG
5.	RT6-miR6870_3p	tgt cag gca acc gta ttc acc gtg agt ggt **ctgaaa**
6.	short-miR6870_3p-rev	cgt cag atg tcc gag tag agg ggg aac ggc gGCTCATCCCCATCTCC
7.	5S rRNA fw	gcc cga tct cgt ctg atc t
8.	5S rRNA rev	agc cta cag cac ccg gta tt
9.	MP-fw	tgt cag gca acc gta ttc acc
10.	MP-rev	cgt cag atg tcc gag tag agg

QUANTITATIVE PCR

The cDNA was quantified using the StepOnePlus™ Real-Time PCR System (Applied Biosystems, Darmstadt, Germany). For this purpose, triplicate measurements of 2 µl cDNA obtained from the RT reaction were made in 10 µl final reaction volume. SYBR Green qPCR was performed using 50 ng/µl short-miR-x-rev, 50 ng/µl MP-fw, and 50 ng/µl MP-rev using the 5 x FIREPol® Master Mix (Solis BioDyne, Tartu, Estonia). The amplification was carried out via the first step at 95°C for 10 min, followed by 40 cycles with 15 s at 95°C, 10-30 s at the particular annealing temperature, and 10-30 seconds at 72°C. The fluorescence signal was acquired at 72°C, and the Ct values were obtained for further calculations. $^{\Delta\Delta}$CT values were similarly expressed as $\log_2$ fold change and statistically analyzed.

STATISTICAL ANALYSIS

The DMD+PBS, DMD + mini Jagged1 peptide, Control + PBS, and Control +mini Jagged1 peptide treatment groups were compared. GraphPad Prism software was used to analyze mean and SD data for 2-3 independent experiments with two-way ANOVA with Tukey's Multiple Comparison tests. A p-value of less than or equal to 0.05 was considered significant.

RESULTS

THE MINI-JAG1 PEPTIDE TREATMENT DOES NOT AFFECT THE *IN-VITRO* DIFFERENTIATION OF THE IMMORTALIZED MYOBLAST CULTURES.

Figure 5.2 shows the time series capture of Control (AB1190) and DMD (AB1023) myoblast lines during myotube from 24 hours to 7 days in differentiation media. On day 5, calcimycin was added. Qualitatively, Control, and DMD cell lines look similar, and myotube formation normally occurs under all treatment conditions. The quantitative measures of myotube quality like fusion index, and width were compared at 7 days of differentiation and 2 days post-calcimycin treatment for both control and DMD cell lines. Figure 5.3 (A) indicates multinucleated fibers and a few unfused myocytes in Control and DMD under all treatment conditions. As Figure 5.3 (B) shows, the percent fusion index in PBS treated Control (87±1.9), DMD (88.87±1.12), mini-Jag1 peptide treated Control (86.05±0.75), and DMD (87.2±1.56) do not differ significantly (Table 5.2). The myotube widths measured from the same images (Figure5.3C) show a significant reduction in DMD treated with PBS (175±79.69) and DMD treated with Jag1 peptide (148.8±68.65) as compared to PBS-treated Control (247±137). The myotube width in Jag1 peptide-treated Control (197.7±112) is lower than in PBS treatment and

is non-significant (Table 5.3). Hence, Jag1 peptide treatment does not significantly reduce myotube widths in Control or DMD cell lines.

THE MINI-JAG1 PEPTIDE TREATMENT REDUCES SUSCEPTIBILITY TO CALCIMYCIN-INDUCED APOPTOSIS IN MYOTUBES.

Surprisingly, the DMD myotubes are less susceptible to calcimycin-induced death in culture compared to the control myotubes in similar conditions. Though there is no significant difference between PBS-treated and Jag1 peptide-treated DMD myotubes after calcimycin addition, there is a significantly lower percent of Propidium Iodide-stained nuclei in DMD+ PBS + Calcimycin (13.13±4.15) and DMD + Jag1-peptide + Calcimycin (12.37±8.32) groups than Control + Jag1-peptide + Calcimycin (44.26±20.94) (Figure 5.4A, Table 5.4). The percentage of PI-stained nuclei in the no-treatment DMD myotubes is significantly lower than the Control + Jag1-peptide + Calcimycin (44.26±20.94) group (Figure 5.4B).

JAGGED1 PEPTIDE INCREASES MITOCHONDRIAL ROS PRODUCTION IN IDMD MYOTUBES AT 48 HOURS OF DIFFERENTIATION.

As Figure 5.5A-B shows, mitochondrial oxidative stress measured as green/red intensity ratio is similar in Control myoblasts (1.69±0.28) and DMD myoblasts (1.15±0.13) during 22-48 hours of differentiation time during which myogenin (MyoG) is expected to begin sharp upregulation in expression. Treatment with mini-Jag1 peptide during this period increases mitochondrial oxidative significantly only in DMD myoblasts (2.44±0.37), not in Control myoblasts (1.39±0.08) (Table 5.5).

Myotube widths got reduced significantly with mini-Jagged1 peptide treatment in Control myotubes (34.48±5.25) compared to PBS treatment (51.5±11.62). The myotube width decreased with Jagged peptide treatment in DMD myotubes (45.34±12.04) compared to PBS (52.79±9.91), however, it was not statistically significant. There was no statistical difference in myotube widths between PBS treated Control and DMD cell lines at 48 hours of differentiation (Figure 5.5C) (Table 5.6).

JAGGED1 PEPTIDE TREATMENT DOES NOT CHANGE THE NOTCH TARGET GENES OR -INTRONIC MIRNA 6870 EXPRESSION.

The mini-Jag1 peptide treatment does not affect MyoD or Myogenin/MyoG expression pattern (Figure 5.6A). As expected, MyoD expression remains high during 24 hours in differentiation media while MyoG expression is induced but still non-significantly higher than the myoblast

stage. There is no significant change in the expression of MyoD or MyoG between control and DMD myoblasts.

The intronic microRNA from the human Jagged1 gene, post-processing which is designated as hmiR6870_5p and hmiR6870_3p, shows very low expression and does not show significant differential expression in any of the treatment groups or during 48 hours of differentiation (Figure 5.6B). The microRNA expression was similar to the host Jagged1 gene and followed a similar pattern.

When checked, Hes1, among the few notch target genes, shows higher expression in DMD myotubes at 30 hours of differentiation compared to DMD myoblasts or control myotubes. However, statistical significance could not be reached due to insufficient biological replicates. Other genes like HeyL or Hey1 do not show a clear expression pattern (Figure 5.6C).

DISCUSSION

The immortalized cell lines (Mamchaoui et al., 2011) show variability in growth kinetics and intensity of pathological features recapitulating the variability seen in DMD patients from whom they were derived (Choi et al., 2016). The two cell lines AB1190 (Control) and AB1023 (DMD) showed similar growth and differentiation kinetics, as shown in Figure 5.2, and hence were suitable for comparison. Patient-derived myoblasts usually differentiate and have no apparent defects during in vitro culture (Blau et al., 1983). Similarly, the fusion index indicates dystrophic line differentiates normally (Figure 5.3B). However, the width of the dystrophic myotubes was significantly lower than that of the Control (Figure 5.3C). This feature varies between patient cell lines, though. Nguyen and co-workers (2021) found that one out of three DMD patient-derived immortal cell lines generated by the same group (Dr. Vincent Mouly and co-workers) showed a reduction in myotube width. Another group (Nesmith et al., 2016) found a severe decrease in myotube width in primary myoblast culture from DMD patients compared to healthy Control.

Calcimycin is a calcium ionophore that increases intracellular calcium levels without needing to permeabilize cells. Due to the poor calcium handling capacity of dystrophic cells (Al Tanoury et al., 2021), it would induce cell death due to calcium-activated proteases (Mareedu et al., 2021). Unexpectedly, we found dystrophic myotubes significantly less susceptibility to detach and die in culture. This could be due to a combination of both reduced myofiber width and known misalignment of dystrophic myotubes that produce less force against a surface that leads

to delamination/detachment (Nesmith et al., 2016; Barthélémy et al., 2022). The reason behind the inability of calcimycin to induce cell death in in vitro dystrophic myotubes could be its ability to stimulate glycolysis (Szibor et al., 1981) and protein synthesis (Ionasescu et al., 1976).

Mitochondrial oxidative stress from dystrophic fibers is known to be higher (Huges et al., 2019). Likewise, in the current study, we find a similar green/red intensity ratio as an indicator of oxidative stress between DMD and Control myotube cultures. This could be due to the early stage of differentiation (48 hours) while myotube formation is happening. Though not quantified here, figure 5.3 shows more red fluorescence in single-celled myoblasts than in myotubes. The 48 hours of differentiation were used for the non-signaling effects of Jag1-peptide stimulation. The result shows a significant increase in oxidative stress after Jag1-peptide treatment only in dystrophic lines. Mitochondrial oxidative stress has been considered an adaptive response in DMD (Bellissimo et al., 2022). In the early myotube formation stage used here, it could indicate metabolic rewiring, which needs further study.

The 24-hours of differentiation is expected to have higher MyoD expression than MyoG. The difference in expression between DMD and Control cell lines is in accordance with phenotypic similarity in growth and differentiation visible under a microscope. Jag1 peptide activating notch receptor signaling is controversial (Nickoloff et al., 2002), which could not be confirmed here due to insufficient replicates. Jag1 expression is known to be low during the early differentiation phase (Zhang et al., 2021), and the findings here agree with the pattern. The intronic miR6870 was expected to follow the host gene (Jag1) expression pattern based on in silico absence of promoter-like elements in the proximity. The qPCR confirms this supposition, though its importance still needs to be ascertained.

CONCLUSION

The immortalized myoblasts from DMD patients as well as age-matched healthy Control, proliferate and usually differentiate in in vitro culture. Though myoblast fusion defects are not apparent, myotube width is reduced, indicating post-fusion growth deficiency. The DMD myotubes are less susceptible to detachment and death, which could be due to reduced force generation known in dystrophic muscles. The reduced myotube size could also contribute to the less force generated against the surface of the culture dish. DMD myotubes had higher mitochondrial oxidative stress than control myotubes, similar to the findings in the tail muscles of the zebrafish model (chapter4). The mini-Jag1 peptide does not affect the proliferation or fusion of myoblasts during differentiation nor reduce myotube width in dystrophic myocytes in

in vitro cultures. Since the notch target gene expression showed high variability, increasing the number of replicates would produce more reliable gene expression data. Though original "escaper" DEG data also did not indicate notch receptor signaling involvement in the rescue (Vieira et al., 2015), it is difficult to confirm as notch signaling response is transient, combinatorial, and pleiotropic. This is the first attempt to quantify miR6870 in human myoblasts or myotubes. There was no significant difference in the expression of Jagged1 or miR6870 between the Control or DMD at any time points of differentiation tested. Expression of both Jagged1 and its intronic miRNA6870 remains stably low, and expression of miR6870 followed the pattern of host gene Jagged1 expression during differentiation of immortalized human myoblast cultured in vitro. Due to technical limitations, the contribution of miR6870 could not be validated here by any other methods in the Jagged1-mediated rescue of DMD.

Further studies on the role of this microRNA are needed as it can have unpredictable consequences for patients, as this miRNA is not found in any of the commonly used animal models of DMD. Jagged1 could also be functioning through non-canonical pathways which are not well discerned. Hence, the freely available "escaper" DEGs data was reanalyzed in addition to the golden retriever, mouse models, and human patient data. The findings are discussed in the next chapter (chapter 6).

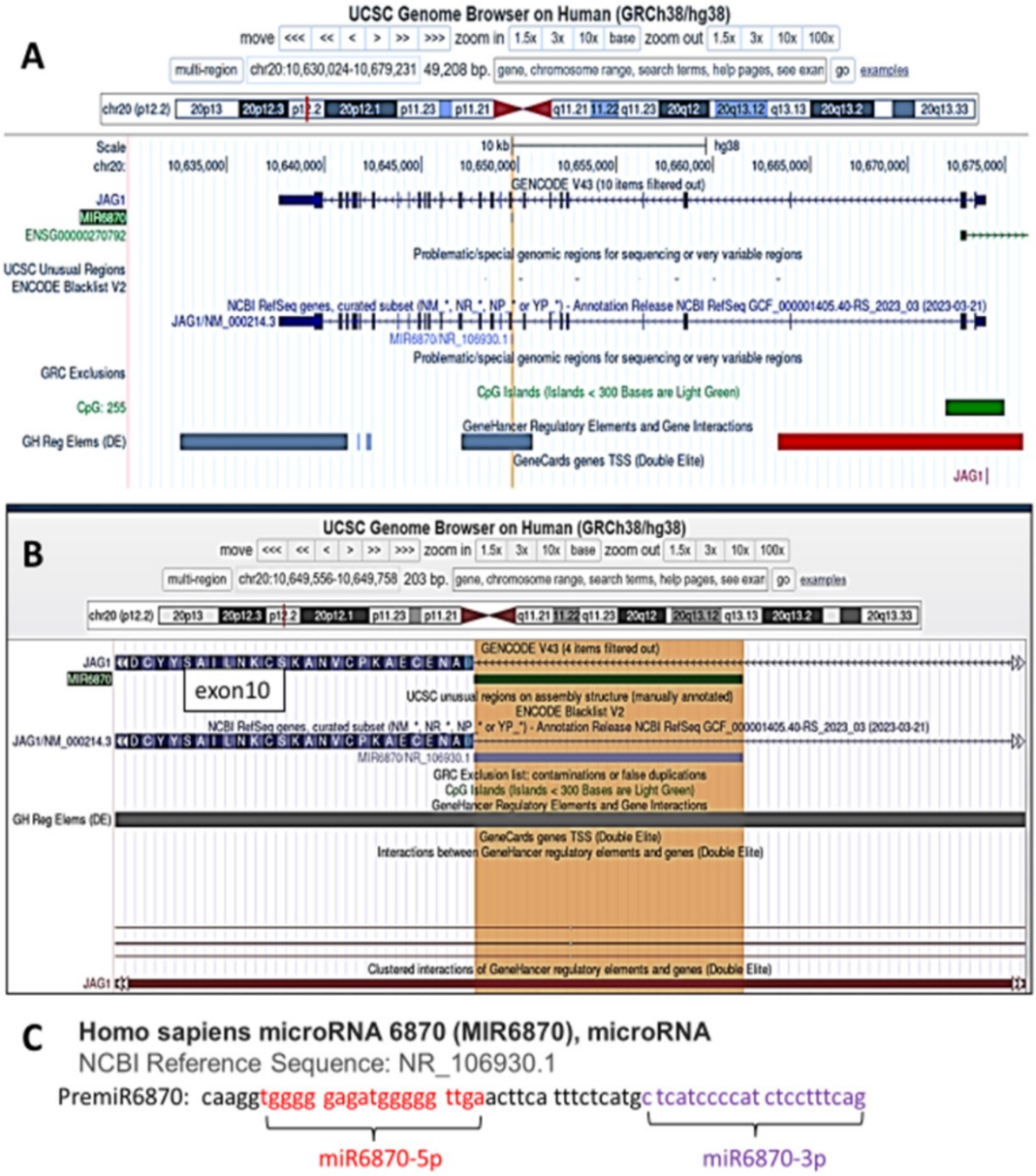

Figure 5.1: Jag1 gene contains intronic microRNA. A: As the figure shows, miR6870 is expressed from the intronic region between exons 9 and 10. It is highlighted in the yellow line. Gene regulatory regions and CpG islands are absent near the miR6870 site suggesting host gene-dependent expression. B: miR6870 region zoomed in with available information. C: sequences of pre-miRNA6870, mature miR6870-5p, and miR6870-3p.

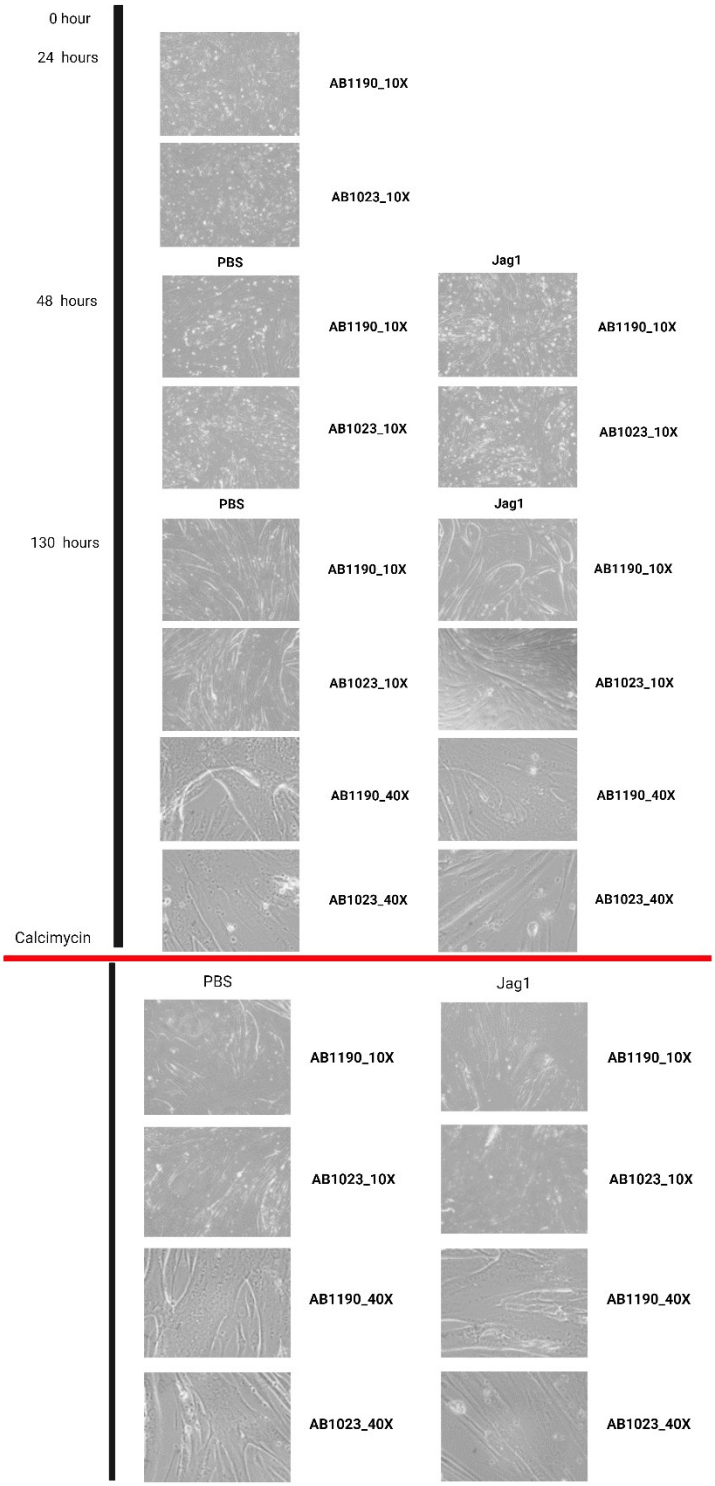

Figure 5.2: A Time Series of Differentiation of Control (AB1190) and DMD(AB1023) myoblasts. Myoblasts were treated with PBS or mini-Jag1 peptide at 24 hours of differentiation. These myotubes were differentiated for 5 days before treating them with calcimycin, and images were captured 24 hours after calcimycin treatment.

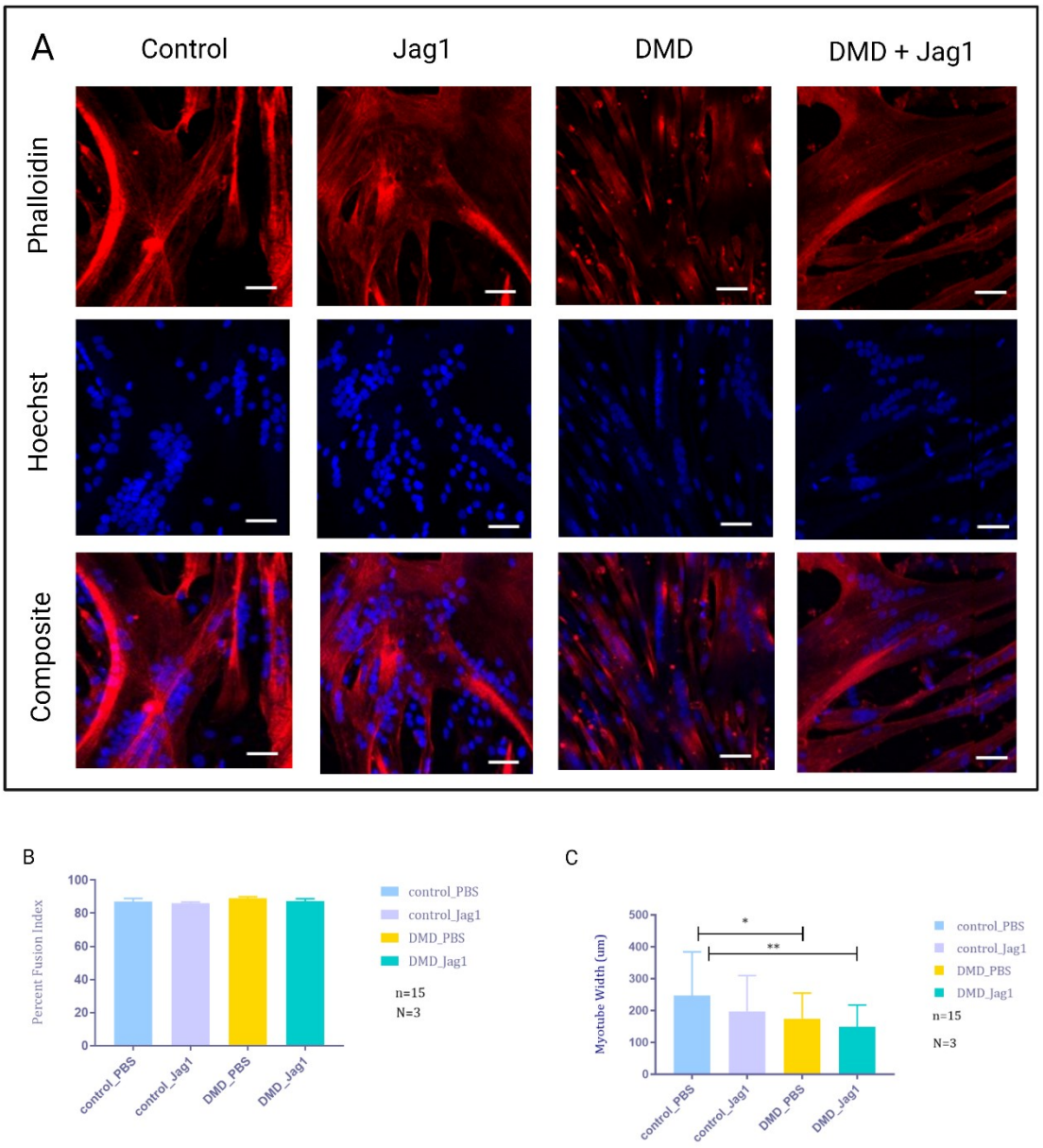

Figure 5.3: The mini-Jag1 peptide treatment does not affect the fusion index or myotube width. A: Representative Images of Hoechst and Phalliodin-633 co-staining (Scale bar: 50um) in 7-day post differentiation myotubes in Jag1 peptide or PBS treated Control and DMD cell lines. B: Quantification of fusion index shows no difference between -Control and -DMD, Jag1 peptide treated, or PBS treated. C: Myotube width (um)is significantly lower in DMD (175±79.69) than PBS treated Control (247±137) and remains reduced with Jag1 peptide treatment in DMD (148.8±68.65). N=3, n=15, significance: * p< .05, ** p<.02, *** p<.001

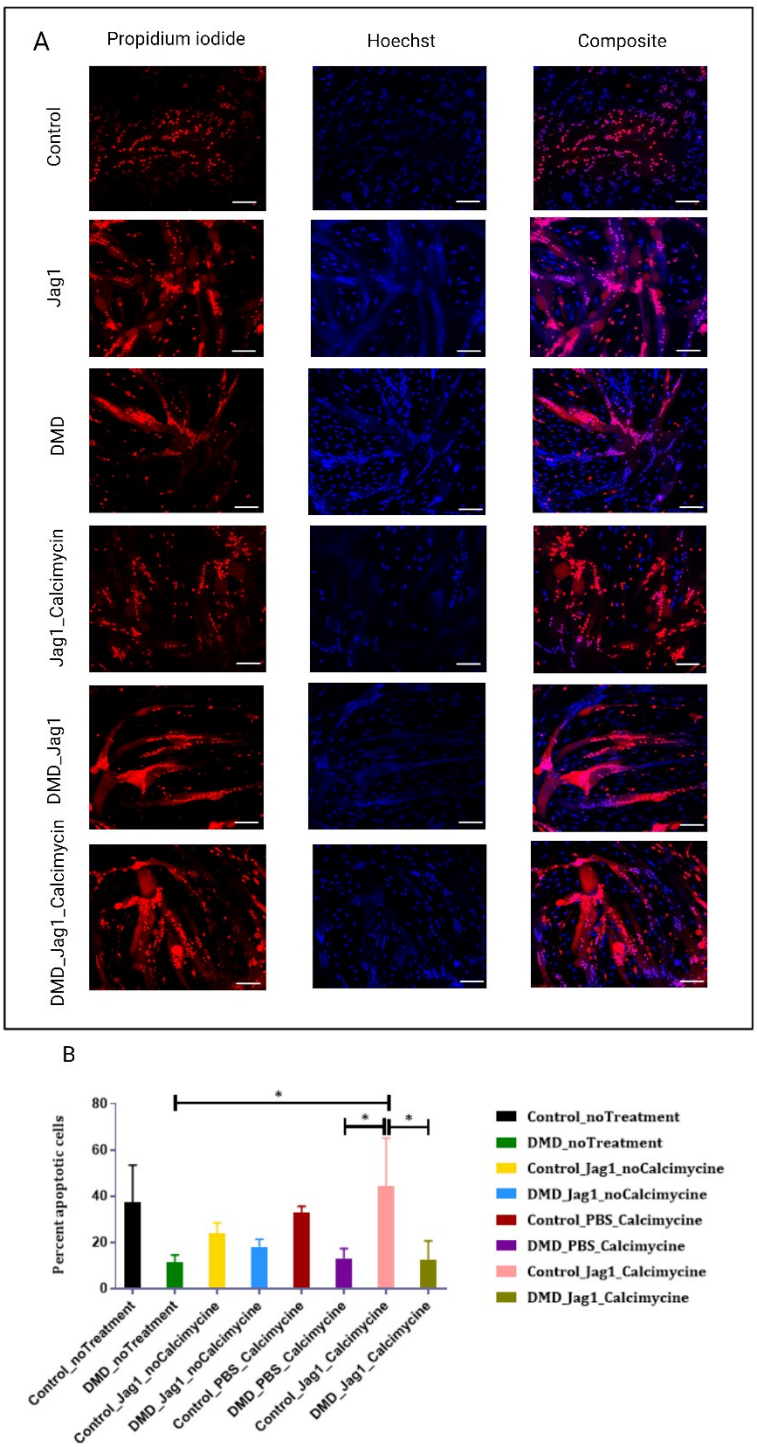

Figure 5.4: Calcimycin-induced apoptosis in control and DMD myotubes. A: Jag1 -peptide/PBS treatment 24 hour followed by calcimycin at 5 days of differentiation, myotubes were imaged at 7-day post -differentiation Control (AB1190) and DMD (AB1023) after live staining with Hoechst and Propidium Iodide (PI). B: Quantification of the percent of cells stained with PI in all treatment groups (Scalebar: 100um). Significant differences were only between pairs DMD no treatment (11.29±3.25) vs. Control+jag1+Calcimycin (44.26±20.94), Control+jag1+Calcimycin (44.26±20.94) vs. DMD+jag1+Calcimycin (12.37±8.32), and DMD+ PBS+ Calcimycin (13.13±4.15) vs. Control+jag1+Calcimycin (44.26±20.94). N=3, n=15, significance: * p< .05, ** p<.02, *** p<.001

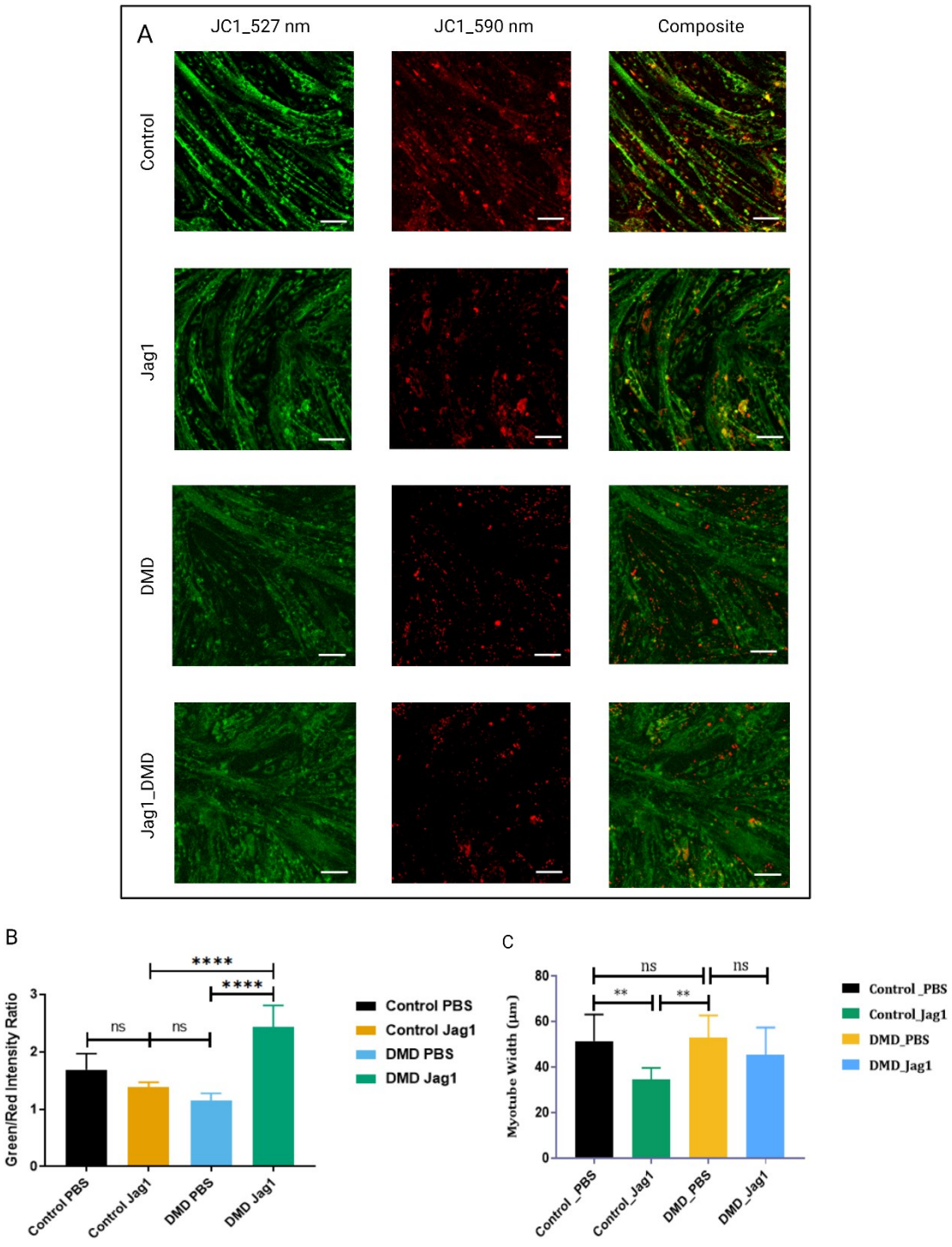

Figure 5.5: Mitochondrial Oxidative stress increases post-Jag1-peptide treatment. A: Representative images of JC1 dye staining (in 527nm/Green and 590nm/Red channels) at 48 hours of differentiation and 24 hours of mini-Jag1peptide treatment (Scalebar:100um). B: Quantification of the ratio of green/red intensities (arbitrary unit) shows a significant increase in the DMD +Jag1 peptide group (2.44±0.37) compared to Control +Jag1 (1.39±0.08) and DMD+PBS (1.15±0.13) groups. C: Myotube width (um) reduced significantly in Control +Jag1 (34.48±5.25) compared to PBS-treated Control (51.5±11.62) and DMD+jag1 (45.34±12.04) groups. N=3, n=15 p value ≤0.05-*, ≤0.01-**, ≤0.001-***

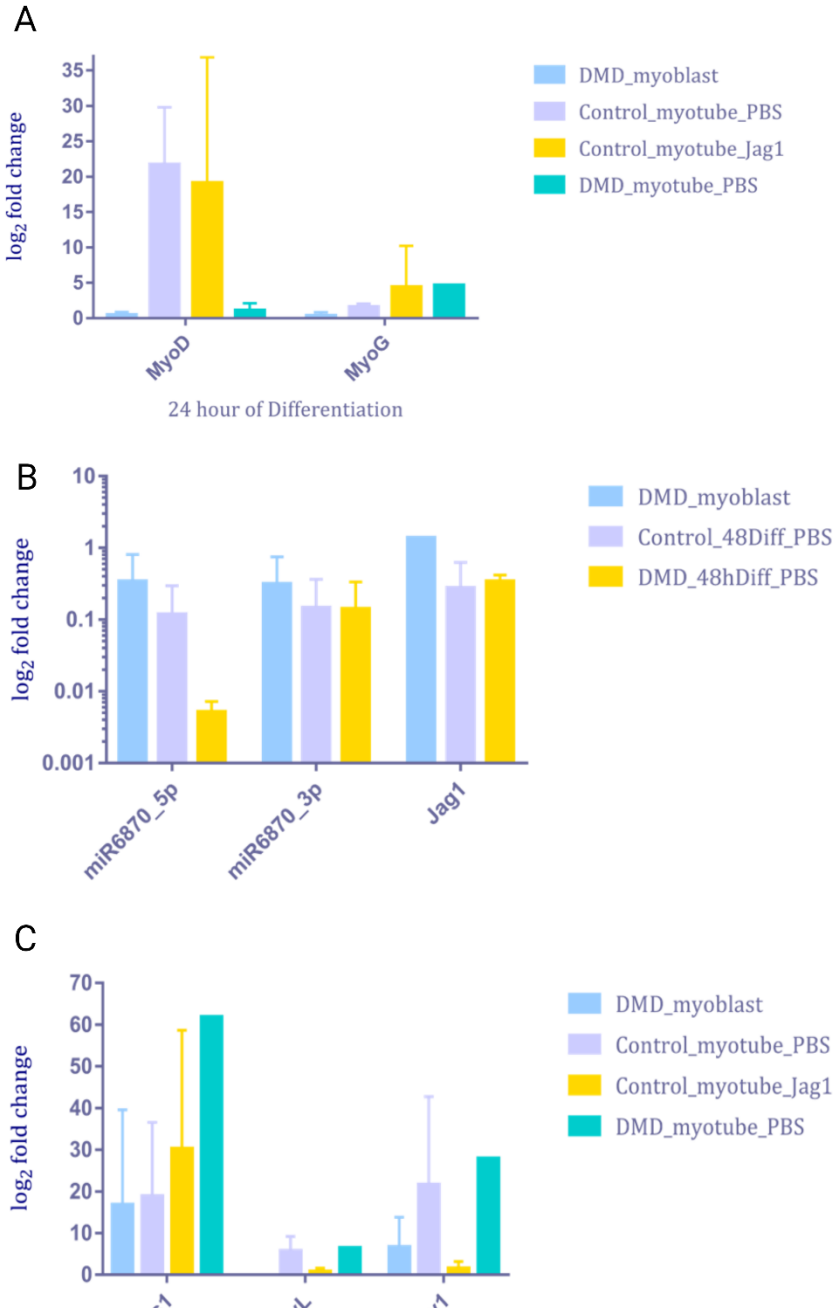

Figure 5.6: Notch target genes and intronic miRNA6870 during early differentiation. A: Log fold change in expression of MyoD and MyoG in 24 hours of differentiation is not significant between groups. B: Log fold change in expression of miRNA3870-5p and miRNA3870-3p and host gene Jag1 from DMD(AB1023) myoblast, 48hour differentiating Control (AB1190) and DMD (AB1023) myocytes compared to Control (AB1190) myoblasts. C: Log fold change in Notch targets: Hes1, HeyL, and Hey1 during 24 hours of differentiation show a significant increase in DMD myotubes compared to Control myotubes and DMD myoblast stage. N=2, n=15 p value ≤0.05-*, ≤0.01-**, ≤0.001-***

Table 5.2: Quantification of Myogenic Fusion Index of Immortalized Myoblast Cultures

Group	**% Fusion Index**
Control + PBS	87±1.9
DMD + PBS	88.87±1.12
Control + Jag1 peptide	86.05±0.75
DMD + Jag1 peptide	87.2±1.56

Table 5.3: Quantification of Myotube Width of Immortalized Myoblast Cultures

Group	**Myotube Width (µm)**
Control + PBS	247±137
DMD + PBS	175±79.69
Control + Jag1 peptide	197.7±112
DMD + Jag1 peptide	148.8±68.65

Table 5.4: Susceptibility of Immortalized Myocyte Cultures to Calcimycin-Induced Apoptosis in Myotubes.

Group	**% of PI Stained Nuclei**
Control + PBS + Calcimycin	32.793±2.859
DMD + PBS + Calcimycin	13±4.15
Control + Jag1 peptide + Calcimycin	44.26±20.94
DMD + Jag1 peptide + Calcimycin	12.37±8.32

Table 5.5: Quantification of Mitochondrial Oxidative Stress in Immortalized Myoblast Cultures at 48 hours of differentiation.

Group	**Ratio of 527nm/590nm Intensity**
Control	1.69±0.28
DMD	1.15±0.13
Control + Jagged1	1.39±0.08
DMD + Jagged1	2.44±0.37

Table 5.6: Quantification of Myotube at 48 hours of differentiation.

Group	**Myotube Width (µm)**
Control + PBS	51.5±11.62
DMD + PBS	52.79±9.91
Control + Jagged1	34.48±5.25
DMD + Jagged1	45.34±12.04