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Isotope Days 2024- Emily Canessa, BsC
Investigation of the Dystrophin Associated Protein Complex Using a SILAC Strategy

Andrew Percy, PhD (00:01):

Next speaker in this lineup is the penultimate one, Emily Canessa. Emily is a PhD student in Dr. Yetrib Hathout's proteomics lab at the School of Pharmacy and Pharmaceutical Sciences in Binghamton University. There she has been developing SILAC and SILAM MS methods to characterize the dystrophin associated protein complex in healthy and dystrophic skeletal muscle based samples, including ones in animal models and patients. This work, some of which will present here today aims to facilitate our understanding of Duchenne and Becker muscular dystrophy pathogenesis. Emily, the floor is yours. Thank you for the introduction.

Emily Canessa, BsC (01:08):

Thank you. So my name is Emily Canessa and I am a PhD student, PhD student in Dr. Yetrib Hathout's lab. Ours is a proteomic lab where the goal is to identify, quantify, and validate different protein biomarkers, Duchenne muscular dystrophy in order to study disease progression. Through my thesis, I'm studying lower bend protein biomarkers in biomarkers in muscle tissue and developing mass spectrometry methods to quantify them. In this case I'll be going over an investigation of the dystrophy associated protein conflicts and going over different silent strategies we use to study it. So first as an overview, Duchennen Muscular dystrophy or DMD is an X-linked disease caused by a mutation of the dystrophin gene. This is an outof frame mutation that results in no dystrophin protein being produced. It has an occurrence of one out of 5,000 live male births and results in loss of ambulation by ages 10 to 13. In later teens to twenties, different medical issues can arise such as cardiac arrhythmias and respiratory issues. Untreated DMD has an estimated lifespan of only 20 to 25 years. Treatments such as corticosteroids, which are anti-inflammatories, can help to extend lifespan by preventing a chronic inflammation from developing in patients. But overall, it's still a very debilitating disease with no true cure.

(02:44):

One of the other components that is essential to the disease is the dystrophin associated protein complex, which is my main area of study. This is a low budget protein complex and the members of this complex exist in about the same amount as the protein dystrophin, which has an occurrence of 0.002% of total protein and muscle lysate. The complex exists to maintain fiber I integrity during contraction and relaxation and different protein members such as Syntrophin and Dystrobrevin are also involved in important cell signaling mechanisms. Loss of the dystrophin protein destabilizes the complex as a whole, and our goal is to create therapies for DNT patients that allow them to produce their own dystrophin protein.

(03:41):

So when we talk about dystrophin replacement therapies from DMD, there are two main ones I'll discuss. The first one is exon skipping gene therapy where patients with an outof frame mutation that results in DMD phenotype are given a drug to restore the reading frame and allow a truncated dystrophin protein to be produced. There are also micro dystrophin gene therapies where an a v delivery vehicle containing a sequence including a micro dystrophin protein construct our administered

patients so that way their cells can produce a dystrophin protein. The goal of these treatments is to provide patients with therapies that will allow their cells to produce functional dystrophin protein. And what we'd want to know is how is protein is produced by these therapies and how effective is it as acting as a dystrophin protein? In order to do this, this requires sensitive quantification methods and in our case we use SILAC.

(04:35):

I will be going over those next. So there are a few different SILAC strategies that I'll be going over. The first is a standard SILAC spike in approach where you have an unlabeled sample that is spiked with a heavy standard that closely matches what is being studied. In our case, that is muscle. We are looking at muscle and we culture heavy labeled myo tubes spike them and we're able to quantify the relative abundance of our sample and compare to other samples. By using the SILAC spanking approach, we can access to accurately and specifically quantify low abundant proteins in skeletal muscle life.

(05:15):

Another approach is SILAC pulse chase, where a subject is fully labeled and over time the amount of labeling and after a certain point there is no more labeling and we can track the loss of heavy single over time measure it and use that to quantify protein turnover. I'll first be going over the Coles Chase example and going over previously published results where we looked at the protein turnover and exon non skipping treated mice. In this experimental model we had mice that we had wild type and MDX mice. They were initially went through an acclimation given unlabeled feed provided by Cambridge Isotope Labs. Then they were given heavy labeled feed over a 10-15 day and following that they were giving normal feed. And this was called our chase by looking at the relative isotope abundance, sorry. So relative isotope abundance increases over feeding of heavy lysine and when that feeding stops, that is our unlabeled chase period. Different proteins degenerate and regenerate at different rates and we can track this rate of degeneration and regeneration and determine individual turnover rates for different proteins. In this example, protein A has a longer turnover than protein C. Protein A has a higher half life as well than protein.

(07:00):

So these are the main questions that we're trying to answer with this study. One is, how does protein turnover of truncated dystrophin and exons giving treated mice compare to full length dystrophin and wild type mice? And two, how does the presence of truncated dystrophin affect the stability of dap seed proteins? In order to study this, we had developed a parallel reaction monitoring mass spectrometry assay. In this case lysates from Pulse-chase labeled mice were ran across different time points were fractionated on a gel and different bands containing proteins of instruments were excised. In this example we have dystrophin and dystroglycan. The bands were in gel digested with trypsin and peptides were extracted and ran on using our targeted LS method.

(08:01):

So for the proteins that we looked at, there was dystrophin laminate alpha two and alpha glycan, which are more central doxy proteins, titan, which is a more a protein to mark muscle degeneration and then

filament C as more of a control that isn't as related to muscle degradation. To do quantification, we targeted the following MS peptides and you can see that all peptides end with the lysine residue because we are giving the lysine the lysine feed heavy lysine feed. This allows us, we tracked each peptide over the 17 days of labeling and the 56 days of chase. And you can see representative skyline chromatograms here to explain the protein turnover experiment where from day from day 10 of labeling to day 17 labeling was still occurring. So the amount of heavy isotope is increasing, and then from day 17 to 73, heavy feed stopped and the amount of heavy label decreased over time.

(09:09):

We can plot this trend over time in order to see how different treatment and groups MDX treated and wild type have their proteins turnover. And when we normalize to the highest labeling point at day 17, we can calculate a exponential degree DK regression model in order to determine protein turnover. From this, we saw that truncated dystrophin in the treated MVX model has a longer turnover than wild type full length dystrophin protein. We then went to look at those DAPC proteins mentioned before, and in this case we saw the opposite where dystroglycan and laminin had double the half-life and the wild type as compared to the treated NDX. So while we saw improved turnover in the actual truncated dystrophin protein, this improvement was not reflected in other muscle related proteins. Our future goals are to try to use this experimental model to understand how doxy protein turnover is affected by the introduction of an AAV micro dystrophy gene therapy. Moving forward, we're going to look at a different topic, thick or muscular dystrophy. This is a mild form of muscular dystrophy where patients have an in-frame mutation that allows them to produce a truncated dystrophin protein. There is a high variability in disease severity in this disease as well as variable protein expression ranging from zero to over a hundred percent of normal expression. So what we want to understand in order to understand this disease more in depth, we need to develop a sensitive method for quantifying just different protein over a large meta dynamic range.

(11:05):

Our main question is, does dystrphin expression and DMD correlate with disease severity? In order to study this, we cultured human SILAC myotubes. To do this, we have myotubes proliferated in SILAC media containing heavy arginine and heavy lysine, and we grew these cells until they were fully labeled. We did a quality control check to make sure that the cells were fully dated. We then differentiated these myoblasts into myotubes to create a heavy labeled batch of SILAC myotubes and ensured that they expressed full length dystrophin protein. From there we proceeded with the SILAC spike in approach where 30 micrograms of non dystrophic or BMD human skeletal muscle lysate spike with 30 micrograms of SILAC myotube lysate. We then fractionated the samples on gels, excised the bands as described before and ran a targeted M method. This method has previously been described in literature or we previously published this method and we were able to show that this method can quantify as low as 30 picograms of dystrophin protein proving that is a sensitive method and viable for this study.

(12:26):

So one of the key, so this data has yet to be published, so I'm not going too much into depth, but one of the key things we saw is that dystrophin expression does highly correlate with severity and ambulatory

status. In the first grasp, we have box blasts representing dystrophin levels, percentages detected in patients with DMD where no dystrophin protein is identified. Patients with BMD that exhibit weakness and patients with BMD, that exhibit no weakness and there is a clear significant difference between patients with weakness between BMD patients with weakness and with no weakness. This is also reflected in the second plot that shows the proportion of ambulatory patients patients with less than, with less than 50% of normal dystrophin expression, show loss of ambulation, a fraction of C lanis loss of ambulation by the age of 20 to 30, whereas patients with over 50% dystrophin expression do not start losing ambulation until the age of 40.

(13:32):

So in conclusion, SILAC pulse -chase labeling provides a powerful method for studying protein turnover. SILAC spike in methods allow for the quantification of low abundant proteins and clinical samples collected from patients. These novel approaches as described can provide a better understanding of current dystrophin replacement therapies and their efficacy. In our future work, we are going to use a SILAC pulse-chase method to quantify DAPC protein turnover in mice treated to produce micro dystrophin. And we're also going to use the SILAC spike method to quantify DAPC proteins in BMD Muscle biopsics. Here are references you can refer to if you would like to learn more about these studies in depths, and I would like to thank the following people for their health in developing these experiments. Thank you. Any questions?

Andrew Percy, PhD (14:21):

Thank you Emily for that nice talk. There are no questions yet from the audience, so I'll ask one off the hoc for the isotope rich chows and the Silam work. Why did you use 13C6 lysine over other essential amino acids or isotopically labeled precursors such as 59 spirulina?

Emily Canessa, BsC (14:45):

So because we used the enzyme trypsin, we adjust our proteins that generates peptides ending in lysine and arginine reus. For a cost effective measure, we chose only to label the proteins with heavy lysine instead of doing both lysine and arginine, but ensure that we would have peptides that we could track for quantification.

Andrew Percy, PhD (15:11):

Okay, thank you very much. In the interest of time, we'll move forward to the final speaker.