Chapter 8
Proteome Profiling of Muscle Cells and Muscle Tissue Using Stable Isotope Labeling by Amino Acids

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8.1 Theory and History of Stable Isotope Labeling by Amino Acids for Proteomic Studies

Comprehensive and accurate proteome profiling of skeletal muscle remains challenging owing to the large proteome dynamic range in this tissue and the increased sensitivity needed to detect low-abundant proteins. Sarcomeric and glycolytic enzymes are by far the most abundant proteins in muscle, masking detection and quantification of low-abundant proteins such as dystrophin, dystrophin-associated protein complex, cell signaling proteins, and transcription factors. About 5400 unique proteins have been identified so far in skeletal muscle using extensive pre-fractionation methods and mass spectrometry [1]. While this is good for cataloging the muscle proteome, pre-fractionation methods are often not compatible with quantification or comparative proteomics because of inherent technical variability from experiment to experiment leading to false and inaccurate quantification. To overcome these challenges, several stable isotope labeling strategies have been developed and tested in the past in different cell culture models and tissues. Typically, the two samples to be compared are tagged with heavy and light stable isotope-labeled moieties, respectively, either at the peptide level, after digestion of proteins with a protease (e.g., trypsin or endoproteinase Lys-C), or even at the cellular level before protein extraction and processing. These different stable isotope labeling techniques are described elsewhere [2], and for this book chapter, we will focus on the most accurate methods.

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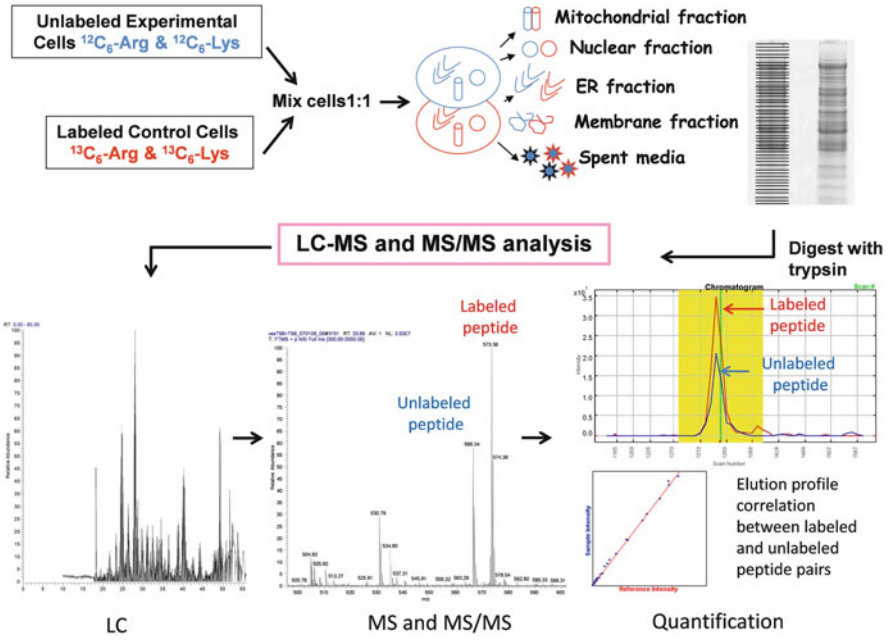

Fig. 8.1 Flowchart depicting the workflow of SILAC strategy. SILAC cells (most often control cells labeled with $^{13}C_6$-Lys and $^{13}C_6$-Arg) and unlabeled cells (most often experimental cells grown in $^{12}C_6$,$^{14}N_2$-Lys and $^{12}C_6$-Arg) are mixed at a 1:1 ratio for subsequent subcellular fractionation. Total proteins are extracted from each fraction of interest and further fractionated by SDS-PAGE to reduce complexity. Gel lanes are then sliced into 2–3 mm pieces. Each piece is in-gel digested by trypsin and the resulting peptides analyzed by LC-MS/MS. Labeled (red) and unlabeled (blue) peptide pairs co-elute, but their masses are resolved by mass spectrometry. The intensity ratio of unlabeled to labeled peptide reflects the amount of the corresponding protein in experimental relative to control cells. The bottom right panel shows the elution profile of labeled and unlabeled peptide pair

8.1.1 Stable Isotope Labeling by Amino Acid in Cell Culture (SILAC)

Stable isotope labeling by amino acid in cell culture (SILAC) is by far the most accurate method for quantitative proteomics when dealing with a cell culture system. SILAC was first introduced in 2002 using light unlabeled leucine (Leu-d0) and heavy deuterium labeled leucine (Leu-d3) to measure temporal changes in the expression of proteins over the course of C2C12 differentiation [3]. The SILAC technique was further improved by incorporating dual labeling using $^{13}C_6$-Arg and $^{13}C_6$-Lys, the two amino acids at which trypsin cuts. As depicted in Fig. 8.1, one set of cell culture, often control cells, is grown in media where normal Arg and Lys are replaced by heavy $^{13}C_6$-Arg and $^{13}C_6$-Lys, respectively, while the experimental cells are grown in classical unlabeled media with normal $^{12}C_6$-Arg and $^{12}C_6$-Lys. After passaging the cells five times (equivalent to seven cell doublings) in their respective

media, the proteome of SILAC control cells becomes fully labeled with heavy Arg and Lys residues, while the proteome of the experimental cells contains normal light Arg and Lys. A given tryptic peptide belonging to a given protein will have the exact same sequence in stable isotope-labeled control cells as its unlabeled homologue in the experimental cells. Typically, total protein extracts from SILAC labeled cells and unlabeled cells are mixed at a 1:1 ratio. The mixture is then digested by trypsin and the resulting peptides are analyzed on a reversed-phase liquid chromatography connected to a tandem mass spectrometry system with MS and MS/MS capabilities (LC-MS/MS). During chromatography, stable isotope labeled and unlabeled peptide pairs found in the same protein will co-elute since they have exactly the same amino acid sequence, and therefore the same hydrophobicity but their masses are resolved by the MS detector since the stable isotope-labeled peptide is 6 Da heavier than the unlabeled peptide. The MS/MS data of either labeled or unlabeled peptide is used to identify the protein, while the elution profile of labeled and unlabeled peptide pairs detected at the MS level is used to determine the ratio of that protein in experimental samples relative to the SILAC labeled control.

Because the light and heavy stable isotope-labeled sample pairs are mixed before protein digestion and sometimes even before subcellular fractionation and protein extraction (Fig. 8.1), variation due to sample handling and processing is minimized. Typical precision or CV using this technology is below 15%. The SILAC strategy has been widely used in a number of eloquent studies ranging from basic comparative proteomics [4] to more complex proteomics studies such as quantitative subcellular proteome profiling [5, 6], protein-protein interactions [7, 8], phosphoproteomics and cell signaling [9, 10]. As discussed below SILAC has been also implemented in studies using muscle cell culture as a model.

8.1.2 Stable Isotope Labeling in Mammals (SILAM)

More recently SILAC strategy has been extended to animal models where the whole animal proteome is labeled with a stable isotope-labeled essential amino acid such as $^{13}C_6$-Lys [11] or uniformly labeled with ^{15}N [12]. In this strategy, pregnant female mice are given $^{13}C_6$-Lys labeled mouse feed or ^{15}N-labeled Spirulina-based feed and the entire mouse colony is maintained under this regimen for a couple of generations. All proteins in the tissue and body fluids of the second-generation mice become fully labeled with the heavy Lys residue or ^{15}N. The proteome of the SILAM mouse is labeled with $^{13}C_6$-Lys and mixed at 1:1 ratio to the proteomes of unlabeled control and experimental mice. In this example only tryptic peptides that contain Lys residues can be used for quantification. But when the proteome of the SILAM mouse is labeled with ^{15}N and spiked into the proteomes of unlabeled control and experimental mice, all tryptic peptides can be used for quantification since all peptides contain several nitrogen atoms. In the SILAM $^{13}C_6$-Lys experiment, the mass difference between stable isotope-labeled peptide and its unlabeled peptide homologue is 6 Da, while in the SILAM ^{15}N experiment, the mass difference

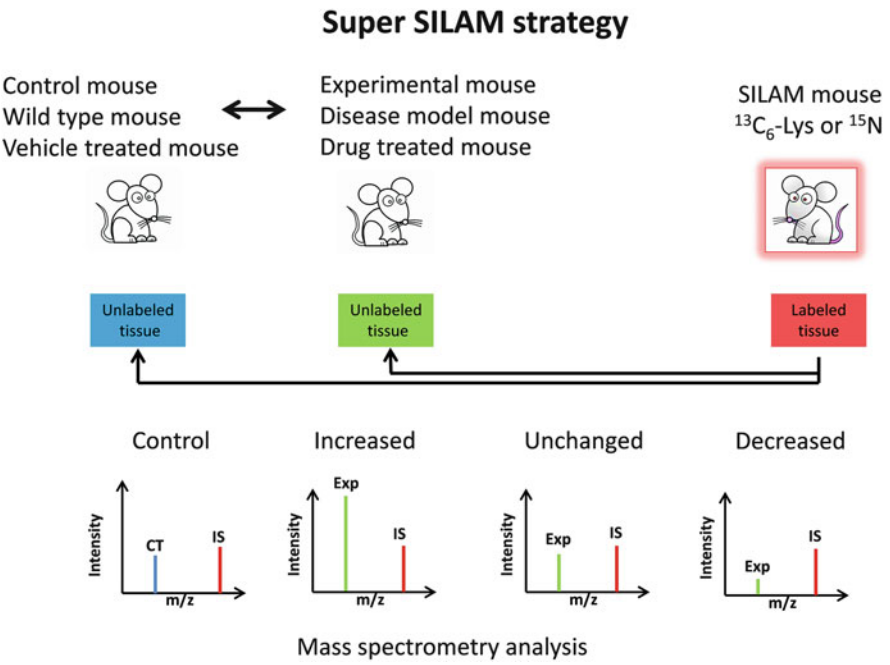

Fig. 8.2 Flowchart depicting the workflow of super SILAM strategy. Protein extracts from tissue or body fluids of experimental and control mice are spiked with known amount of protein extract from matching tissue or body fluids. Spiked samples are fractionated by SDS-PAGE and processed for in-gel digestion and mass spectrometry analysis. Depending on the requested experiments, replicate mice are used ($n = 3$ to 6 mice per group). Proteins are then identified and quantified using our IP2 software. The lower panel shows an example of a peptide from a protein that was 1:1 ratio in control (blue) relative to internal standard (red). Then a scenario where a peptide from a protein was 2:1 ratio, 1:1 ratio, or 1:2 ratio in experimental mice (green) relative to internal standard (red). Note that the amount of spiked internal standard is the same for this peptide of interest, allowing for accurate comparisons of protein levels across different samples

between stable isotope-labeled peptide and its unlabeled homologue depends on the number of nitrogen atoms in the peptide. In general, when using SILAM strategy, protein extract from a given tissue or body fluid from the SILAM mouse is used as an internal standard to quantify the same set of proteins in unlabeled experimental and control mice using intensity ratios of heavy and light peptide pairs (Fig. 8.2).

These innovative in vivo labeling strategies utilizing $^{13}C_6$-Lys and ^{15}N were successfully implemented in a number of impactful research studies. This includes a study on the role of specific genes using knockout mouse models [13], one defining alteration in cell signaling during skin carcinogenesis [14], one defining disease altered protein turnover in the brain of Alexander disease mouse model [15], studies on the development of the brain in rat models [16, 17], as well as a study on proteome turnover in different tissues and organs [18].

8.2 Implementation of SILAC and SILAM Strategies to Study the Proteome of Muscle in Health and Disease Conditions

8.2.1 In Vitro Studies Using SILAC Labeled Muscle Cell Cultures

Because of its accuracy and specificity, SILAC strategies have been implemented in a number of basic and translational studies including studies dealing with cultured muscle cell models. We highlight below some of the major applications in the field.

8.2.1.1 Study of Muscle Cell Differentiation and Myogenesis

One of the first applications of SILAC strategy was actually designed to define changes in protein expression during muscle cell differentiation using C2C12 cells as a model [3]. In this initial study, the author cultured undifferentiated C2C12 myoblasts in media containing unlabeled Leu-d0, while C2C12 undergoing differentiation were grown in media containing Leu-d3 and were harvested at days 0, 2, and 5 over the course of differentiation. Total protein extracts from Leu-d3 labeled C2C12 day 0, day 2, and day 5 were mixed at 1:1 ratio with total protein extract from undifferentiated Leu-d0 C2C12 and analyzed by LC-MS/MS. The authors identified several proteins that did not change over the course of differentiation, while other proteins either increased or decreased. This early study was performed using C2C12 cells, a transformed and immortalized mouse muscle cell line, and was done for only 5 days of differentiation.

To gain a better idea of how myogenesis occurs in human muscle, a recent study used human primary myoblast in combination with a triple SILAC strategy to define changes in the proteome dynamic during muscle cell differentiation [19]. Because the authors used light, medium, and heavy stable isotope-labeled Lys and Arg, as well as a more recent instrument, they identified key proteins involved in myogenesis but also unraveled several novel proteins never reported before. The authors identified 243 proteins that significantly changed during differentiation and grouped them into 5 different clusters: those that decreased upon initiating differentiation (clusters 1 and 3) and those that increased during differentiation (clusters 4 and 5). Cluster 2 rose at day 1 of differentiation and then returned to normal levels. This is one of the most comprehensive studies of the timecourse of protein changes during myogenesis in vitro and an excellent example of how versatile the SILAC strategy can be.

8.2.1.2 Study of the Muscle Cell Secretome

It is well accepted that muscle tissue secretes a large number of biologically active proteins such as myokines which regulate the secretion of a number of other biologically active molecules that have a key role in metabolism [20]. It is challenging to study the muscle secretome in vivo owing to difficulty in accessing the interstitial space between muscle fibers. Thus, growing differentiated myotube cell cultures was a first step in cataloguing the muscle cell secretome. In the past few years, a number of muscle cell secretome studies have published a list of proteins that were detected in the conditioned media of cultured myoblast and myotubes using C2C12 cells or primary human muscle cells [21–23]. Besides myokines, differentiated muscle cells secrete a large number of other biologically relevant proteins. About 554–954 nonredundant proteins have been identified so far in the conditioned media of these cultured myotubes. Only 25% of these proteins had a signal peptide and were secreted through the classical pathway [23]. The remaining 75% of proteins in the conditioned media were either released by leaking cells or via exosomes [23]. Some of the key secreted proteins identified in the conditioned media of differentiated myotubes are listed in Table 8.1 with their Swiss-Prot accession number and potential function relevant to muscle.

A key challenge in studying cell secretome is distinguishing between proteins that are truly released by the cells and protein contaminants that originate from the 2% bovine serum added in differentiation culture media or gelatins used to coat culture flasks. Indeed, several bovine sera proteins have high sequence homology with human or mouse proteins and might confound the true origin of the proteins identified in the conditioned media. However, SILAC strategy can overcome this background noise and distinguish proteins that are truly synthesized and secreted by SILAC labeled cells from proteins that might be contaminants which are not labeled by heavy isotope $^{13}C_6$-Arg or $^{13}C_6$-Lys residues.

Our group implemented SILAC strategy to define differential exosomal proteins secreted between dystrophin deficient mouse muscle cells and healthy normal mouse muscle cells [24]. This study demonstrated that dystrophin-deficient muscle cells exhibit disrupted vesicle trafficking and protein secretion.

8.2.2 In Vivo Studies Using SILAM Labeled Mouse Model for Muscle Diseases

8.2.2.1 Use of SILAM Strategy to Define Alterations in the Proteome of Dystrophin-Deficient Skeletal Muscle

SILAM was successfully implemented by our group to elucidate molecular events involved in muscle pathogenesis in the dystrophin-deficient mouse model mdx-52 [25] and in a mouse model of inflammatory myopathy [25]. Using this strategy, thousands of proteins were identified and 789 were quantified, allowing for their

Table 8.1 Partial list of proteins identified in the conditioned media of differentiated human primary myotubes (selected from [23])

Accession number	Protein name	Potential function relevant to skeletal muscle
Q15063	Periostin	Extracellular matrix (ECM) organization and response to muscle activity
P15502	Elastin	Skeletal muscle development
Q14118	Dystroglycan	Sarcolemma anchoring and skeletal muscle tissue regeneration
P35052	Glypican-1	Positive regulation of skeletal muscle cell differentiation
P07585	Decorin	Skeletal muscle tissue development
O14793	Myostatin or GDF8	Negative regulation of muscle hypertrophy, negative regulation of myoblast proliferation and differentiation
Q12841	Follistatin-related protein 1	A myokine involved in muscle fiber regeneration
P01137	TGFB1	Negative regulation of skeletal muscle tissue development
Q15389	Angiopoietin-1	Regulation of skeletal muscle satellite cell proliferation
P17936	IGFBP3	Positive regulation of myoblast differentiation
Q99988	GDF15	Positive regulation of myoblast fusion
P78504	Protein jagged-1	Myoblast differentiation
P25391	Laminin	Involved in myoblast fusion
P09382	Galectin-1	Involved in myoblast fusion
P09486	SPARC	Positively regulated in ECM remodeling
P08253	Matrix metalloproteinase-2	Positively regulated in ECM remodeling
P02751	Fibronectin	Collagen-/actin-binding protein, ECM remodeling
P08123	Collagen alpha-2 (I) chain	Upregulated as differentiation occurs, ECM remodeling
P55291	Cadherin-15	Positive regulation of muscle cell differentiation
P19022	Cadherin-2	Positive regulation of muscle cell differentiation

levels of expression to be compared between muscle disease mouse models and wild-type mice. Figure 8.3 shows the dystrophin protein which is absent in the skeletal muscle of an mdx mouse model compared to that of wild-type mouse, GAPDH (glyceraldehyde-3-phosphate dehydrogenase) which remains unchanged, and talin which is significantly elevated in the skeletal muscle of a dystrophin-lacking mouse. Detailed data about altered levels in the proteome of skeletal dystrophin-deficient skeletal muscle obtained using SILAM is discussed elsewhere [25, 26]. The key finding from this SILAM mouse study was the identification of pathways involved in early-stage muscular dystrophy pathogenesis in the mdx mouse model. A subset of these findings were confirmed in human muscle tissue comparing DMD muscle tissue to healthy muscle tissue [25].

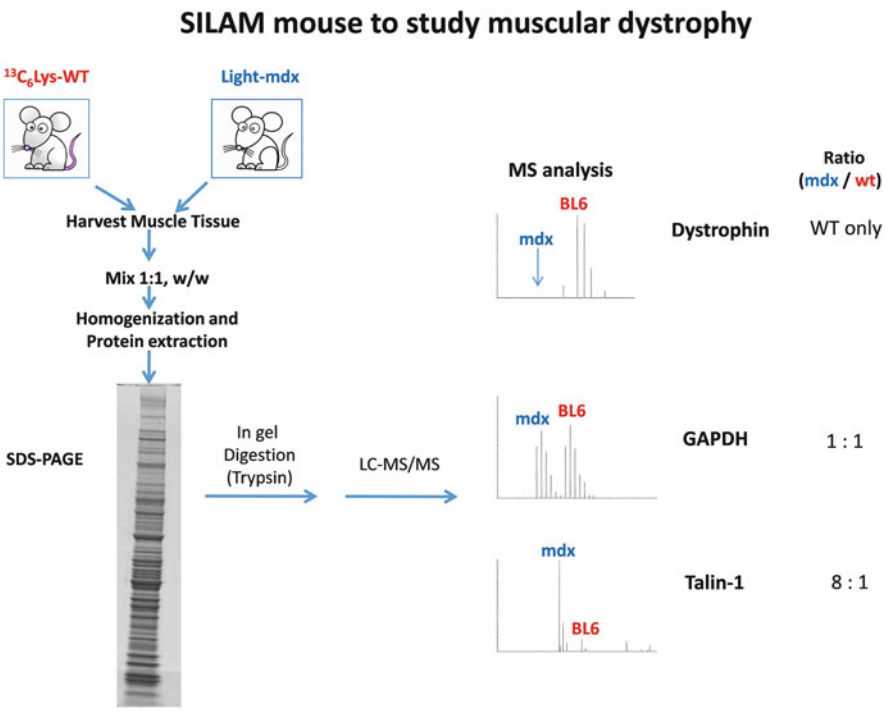

Fig. 8.3 Principle of SILAM mouse strategy to define the proteome signature of dystrophin-deficient skeletal muscle in the mdx mouse model. Protein extract from the muscle tissue of a SILAM labeled wild-type mouse is mixed at 1:1 ratio with protein extract from the muscle tissue of an mdx mouse. The protein mixture is then fractionated by gel electrophoresis. Bands are excised and in-gel digested by trypsin. The resulting peptides are analyzed by LC-MS/MS. Thousands of proteins were identified and quantified. Right panel shows examples of light and heavy peptide pairs detected for dystrophin, GAPDH, and talin-1 in mdx and SILAM wild type, respectively. Using the intensity ratio of light to heavy peptide will determine the ratio of corresponding protein in the muscle of mdx mouse relative to wild-type mouse

8.2.2.2 Use of SILAM Strategy to Accurately Quantify Levels of Dystrophin Protein in Skeletal Muscle

The need for accurate and precise dystrophin quantification in muscle biopsies and skeletal tissue collected from clinical and preclinical studies has become highly important since the introduction of dystrophin replacement therapies. Examples of such therapies are exon skipping using a phosphorodiamidate morpholino oligomer (PMO) [27], codon read through using ataluren drug, gene therapy using micro-dystrophin AAV vector, and stem cell therapy [28]. While these therapies hold promise because they repair the primary defect in DMD (e.g., restoration of the missing dystrophin in the skeletal muscle), the precise amount and function of the restored dystrophin remain a challenge to define which often delays approval by regulatory agencies and halts further clinical trials. Indeed, quantification of

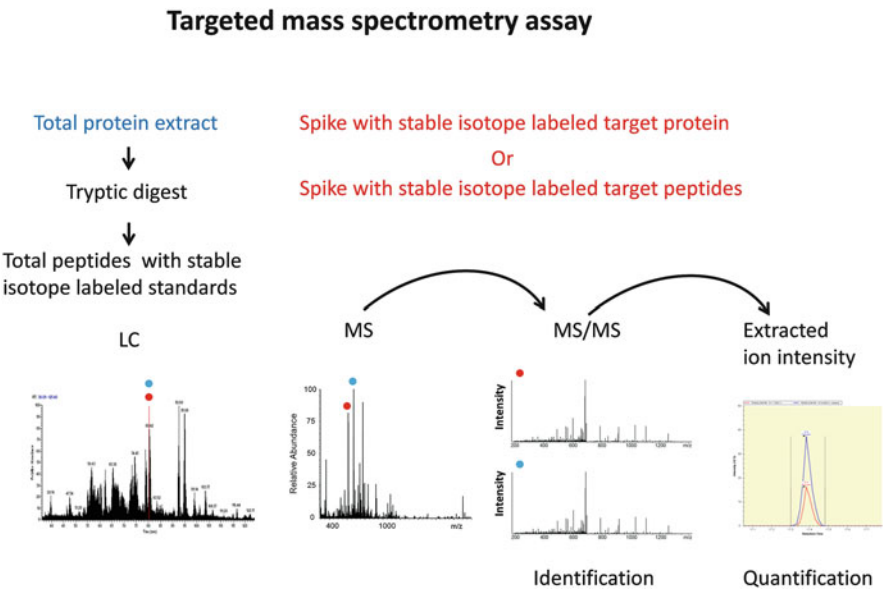

Fig. 8.4 Targeted mass spectrometry assay principle and flowchart. The quantification is done with MS/MS transition ions rather than *m/z* values at the MS level. This increases the sensitivity and specificity of the measurement because MS/MS data from modern high-resolution instruments often have little or no background noise. Quantification at the MS levels on anther hand can be noisier with a risk of false quantification due to co-eluting peptides that might have a very similar mass to the target peptide. Targeted mass spectrometry uses a combination of peptide (MS) selection followed by quantification of a "daughter" fragment ion (MS/MS). This greatly improves the specificity of the measurement because the likely hood of a co-eluting peptide having an identical fragment ion is low

dystrophin using classical antibody-based assays such as Western blot and immunofluorescence exhibited large variability when done on the same biopsies across different laboratories and even within the same laboratory [29]. Furthermore, different results can be obtained when using immunostaining depending on the muscle section, the antibodies, and image reading [30].

Stable isotope-labeled proteotypic peptide(s) can be used as internal standard(s) to quantify any given protein in a complex biological sample using targeted mass spectrometry methods. Figure 8.4 depicts the targeted mass spectrometry flowchart. Basically, the method focuses on selecting a peptide or peptides of interest based on their retention time and *m/z* value in a LC-MS/MS run. Then the sample to be analyzed is spiked with a known amount of stable isotope peptides that have the same sequences as the unlabeled peptides in the target protein. This allows quantification of the target peptides and therefore allows us to quantify the amount of protein of interest in the sample. The stable isotope peptides could be either custom synthesized and spiked into the sample after total protein digestion or generated from full-length stable isotope-labeled protein that is spiked into the sample before

digestion with trypsin. The full-length stable isotope protein could be generated by SILAC cultured cells that express the protein of interest or by using tissue samples from SILAM mouse that express the protein of interest.

We have successfully used this method to accurately quantify dystrophin in human muscle biopsies using full-length $^{13}C_6$-Lys labeled mouse dystrophin as an internal standard [31]. Indeed, since mouse dystrophin and human dystrophin share 91% sequence homology, several tryptic peptides had exactly the same sequence between human and mouse. These peptides were selected and used for quantification of dystrophin.

8.2.2.3 Use of SILAM Mouse to Define Serum Protein Biomarkers Associated with Dystrophin Deficiency

Fully ^{15}N-labeled wild-type mice were successfully used as surrogates to identify and quantify serum protein biomarkers associated with dystrophin deficiency using two independent mouse models, the engineered mdx-Δ52, and the naturally occurring mdx-23 [32]. Of the 355 screened mouse sera proteins, 23 were found significantly elevated and 4 significantly lowered in sera samples of mdx mice relative to wild-type mice (p value <0.001). Elevated proteins were mostly of muscle origin and included myofibrillar proteins, glycolytic enzymes, transport proteins, and other muscle proteins, while decreased proteins were mostly of extracellular origin. Furthermore, analysis of sera from 1 week to 7 months old mdx-23 mice revealed age-dependent changes in the level of these biomarkers. Most biomarkers acutely elevated at 3 weeks of age and then either remained increased or decreased in older mdx mice up to 7 months old. This data was validated in sera samples of DMD patients [32]. This study shows the utility of using SILAM mouse to define serum circulating biomarkers that are associated with muscle disease and could be extended to a number of other applications when looking at the response of these biomarkers.

8.3 Limitations and Extensions of the Technique

While the use of SILAC and SILAM strategies coupled with mass spectrometry enables accurate proteome profiling of cultured cells and animal tissues, respectively, these techniques have some limitations as described below.

SILAC and SILAM strategies suffer from low throughput because only pairs of samples can be compared at a time. To overcome some of the throughput challenges, investigators have introduced super SILAC strategy, where a SILAC labeled cell batch is prepared in large quantities and used as internal standard to quantify a series of experimental cells [33]. Similarly, super SILAM can be used in the same way to perform proteome profiling of different experimental mouse models. With improved sample preparation methods and state-of-the-art LC-MS/MS instruments with higher

speed, resolution, and sensitivity, the quantitative coverage of the proteome will dramatically improve and several samples can be processed in only 1 week instead of a month. But sample preparation still remains the limiting step in a number of proteomic studies, especially those implementing SILAC and SILAM strategies, because it takes on average 1 month to fully label cells with SILAC and up to 6 months to generate a SILAM mouse colony.

Another limitation of SILAC/SILAM strategies is they are restricted for use only in cell culture models and animal models. Although this is true, cultured SILAC labeled human myotube extract was successfully used as a surrogate to profile the proteome of human muscle tissue [34]. However, this strategy still suffers from the lack of perfect match between the proteome of cultured SILAC myotubes and the proteome of muscle tissue, resulting in several orphan proteins without an internal standard for quantification. Indeed, comparison of the whole proteome of human muscle tissue and cultured human myotubes in our laboratory revealed that 38% of the total identified proteins in muscle tissue extract were not detected in cultured human myotube extract (Fig. 8.5). This could be explained by the fact that muscle tissue is heterogeneous and comprised of a complex mixture of cells besides the muscle fibers, while cultured human myotubes are more homogenous. For example, several components of the neuromuscular junctions, endothelial cells, and conjunctive tissue were detected in muscle tissue but not in cultured human myotubes. Nevertheless, the use of SILAC labeled human myotubes as a surrogate can still be used to quantify a large number of key proteins in human muscle tissue.

A third limitation of super SILAC/SILAM strategy is leaky or missing data when dealing with the analysis of several samples for comparison. This missing measurable data is most often due to lack of MS/MS events and fragmentation of low-abundant peptides in the LC-MS/MS run in data-dependent acquisition

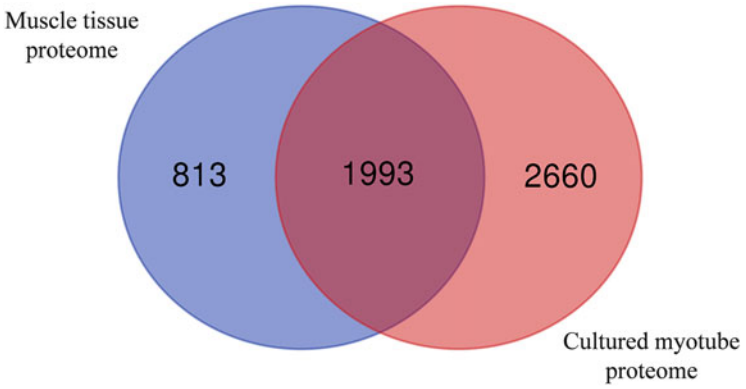

Fig. 8.5 Venn diagram depicting the number of overlapping and unique proteins identified by LC-MS/MS in human muscle tissue and cultured human myotubes. More proteins were identified in cultured human myotubes than in muscle tissue for several reasons. Cultured cells had a lower dynamic range than muscle tissue. Proteins are more easily extracted from cultured cells than from muscle tissue

(DDA) mode. Although the peptide is detected at the MS levels, there is no MS/MS data attached to it. To overcome this issue, a more sophisticated mass spectrometry method such as the sequential window acquisition of all theoretical mass spectra (SWATH-MS) analysis was introduced [35] and is being implemented in several proteomics studies and could be extended to muscle cells or muscle tissue.

8.4 Vital Future Directions

While metabolic labeling by stable isotope amino acids enables accurate quantitative proteomics in muscle cells, muscle tissues, and other tissues, there are still knowledge gaps that may require a combination of the SILAC/SILAM strategies with other additional proteomics methods.

Proteomic Data Interpretation in the Context of Muscle Tissue in Health and Disease Conditions Indeed, most of the time proteomic data is interpreted using knowledge databases that are gathered from proteomics data and literature generated on other tissues and disease conditions which often creates networks that are biased toward cancer and other prevalent diseases. Although muscle tissue expresses about 50% of the total human genome, only a handful of genes (~14%) were defined as being enriched in muscle using a combination of transcriptome data and antibody-based profiling [36]. While this is a great initiative, identifying true muscle-specific proteins using large-scale skeletal muscle proteome profiling will bring insight into the muscle proteome. The next step ideally is to determine the absolute amount of each mass spectrometry identifiable protein in muscle tissue. For example, several key proteins such as dystrophin, utrophin, dysferlin, and dystroglycan are all associated with certain muscle diseases, and knowing the precise levels of these proteins in skeletal muscle under healthy and disease conditions might help define therapeutic targets for gene replacement therapies. SILAC or SILAM strategy in combination with targeted mass spectrometry analysis can easily help determine the absolute amount of these key proteins in skeletal muscle. The next step is perhaps to extend targeted mass spectrometry analyses to absolute quantification of global proteins in skeletal muscle. This will help refine the skeletal muscle proteome atlas.

Protein-Protein Interaction in Muscle Another challenging aspect of working with the muscle proteome, and perhaps in a number of other tissues, is defining protein-protein interaction and networks. While several cell signaling studies and protein interaction studies were facilitated by the use of SILAC and/or SILAM strategies, in other cell models and tissues, only a few studies have been undertaken using muscle cells and muscle tissue. Such studies will bring insight to molecular mechanisms involved in a number of muscle diseases and might help identify novel and innovative therapeutic targets.

Quantitative phosphoproteomics using SILAC and SILAM strategy is another area of interest that could also bring exciting insights about the muscle biology in general but also about alterations in muscle cell signaling under disease conditions.

Qualitative and Quantitative Glycoproteomics of Muscle Tissue Glycoproteins play a crucial role in cell function and tissue integrity. Unfortunately, only a few glycoproteomics studies have been undertaken in skeletal muscle, and most of these studies were focused on the dystrophin-associated protein complex that plays a crucial role in muscle fiber integrity and function. Aberrant assembly and/or glycosylation pattern of this complex has been implicated in a number of muscle diseases and has been a target for thorough glycosylation studies [37]. Extending these analyses to other muscle glycoproteins in combination with SILAC strategy using stable isotope-labeled glutamic acid and SILAM strategy using ^{15}N might help understand the role of glycoproteins in the pathogenesis of dystroglycanopathies [38].

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