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The potential of proteomics for in-depth bioanalytical investigations of satellite cell function in applied myology

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ABSTRACT

Introduction: Regenerative myogenesis plays a crucial role in mature myofibers to counteract muscular injury or dysfunction due to neuromuscular disorders. The activation of specialized myogenic stem cells, called satellite cells, is intrinsically involved in proliferation and differentiation, followed by myoblast fusion and the formation of multinucleated myofibers.

Areas covered: This report provides an overview of the role of satellite cells in the neuromuscular system and the potential future impact of proteomic analyses for biomarker discovery, as well as the identification of novel therapeutic targets in muscle disease. The article reviews the ways in which the systematic analysis of satellite cells, myoblasts, and myocytes by single-cell proteomics can help to better understand the process of myofiber regeneration.

Expert opinion: In order to better comprehend satellite cell dysfunction in neuromuscular disorders, mass spectrometry-based proteomics is an excellent large-scale analytical tool for the systematic profiling of pathophysiological processes. The optimized isolation of muscle-derived cells can be routinely performed by mechanical/enzymatic dissociation protocols, followed by fluorescence-activated cell sorting in specialized flow cytometers. Ultrasensitive single-cell proteomics using label-free quantitation methods or approaches that utilize tandem mass tags are ideal bioanalytical approaches to study the pathophysiological role of stem cells in neuromuscular disease.

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Biomarker; muscular dystrophy; muscle stem cell; neuromuscular disease; regenerative medicine; single-cell proteomics



1. Introduction

Basic and applied myology is concerned with the biochemical, physiological, histological, and cell biological characterization of voluntary contractile fibers and their cellular and acellular environment. The proper integration and operation of skeletal muscles form the basis of essential bodily functions, such as directional movements, postural stabilization, breathing, thermogenesis, and metabolic regulation. Muscular dysfunctions are involved in a variety of acquired and genetic disorders, including various primary neuromuscular diseases, as well as serious co-morbidities that are associated with diabetes, sepsis, and cancer cachexia. Within the diverse class of muscle wasting diseases, the large grouping of muscular dystrophies are characterized by extensive muscle degeneration that is often highly progressive in nature [1].

Crucial mechanisms that help to counteract muscular dysfunction in disease or following injury include regenerative processes that involve regulation and activation of muscle stem cells, called satellite cells (SCs) [2], proliferation and differentiation, followed by myoblast fusion to form new multinucleated myofibers, and muscle self-repair [3]. A portion of SCs return into

quiescence to replenish the resident stem cell pool [4]. Thus, abnormal functioning of SCs in damaged skeletal muscles results in disturbed neuromuscular homeostasis, impaired myofiber maintenance, weakened muscle plasticity, and inadequate adaptation of muscles to changed physiological demands [5]. Neuromuscular disorders, where dysfunction of SCs plays a central pathophysiological role [6], have recently been classified as ‘satellite cell-opathies’ [7].

In order to better understand complex changes in myofibers and associated SCs in primary neuromuscular diseases, muscle-associated co-morbidities or the age-related functional decline of voluntary muscles, mass spectrometry (MS)-based proteomics is a highly suitable large-scale and high-throughput technology with an unbiased perspective in discovery mode [8]. Proteomic profiling can be used to establish novel biomarkers for improved differential diagnostics and therapeutic monitoring, especially in the case of therapies based on pharmacological intervention, stem cell transfer, gene substitution, exon skipping, or genomic editing. This article gives a brief overview of the role of SCs in skeletal muscle and outlines the future potential of proteomics, focusing especially on single-cell proteomics (SCP) for biomarker discovery in the field of neuromuscular pathobiology.

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Article highlights

- Mass spectrometry-based proteomics represents an ideal bioanalytical tool for the detailed characterization of satellite cell function in applied myology.
- The isolation of muscle-derived cells can be carried out by mechanical and/or enzymatic dissociation protocols, followed by fluorescence-activated cell sorting in specialized flow cytometers.
- Mass spectrometric analyses of satellite cells can be performed by ultrasensitive single-cell proteomics employing label-free quantitation methods or approaches that utilize tandem mass tags.
- Single-cell proteomics of satellite cells, myoblasts, and myocytes can help to better understand the complex process of myofiber regeneration in health and disease.

2. The central role of SCs in skeletal muscle regeneration

Skeletal muscle repair of damaged myofibers encompasses complex interactions and communications between various cell types within the muscle environment, including SCs, immune cells, and fibro-adipogenic progenitors, together with a large cohort of myokine signaling factors [9]. SCs represent crucial stem cells that confer a high degree of regenerative capacity to muscles [10]. SCs form the cellular basis for efficient muscular self-repair and provide skeletal muscles with a swift mechanism to cope with the detrimental consequences of acute tissue damage, chronic myofiber degeneration, and functional decline during the natural aging process [2,5].

Figure 1 gives a diagrammatic overview of the precisely regulated process of regenerative myogenesis following traumatic injury or disease.

Upon injury, the stimulation of the quiescent cell population of SCs, that is positioned between the basal lamina and the sarcolemma of contractile fibers, leads to their proliferation and differentiation into myogenic progenitor cells called myoblasts. They fuse to damaged fibers or to each other to form new multinucleated myofibers. Reliable markers of SCs, myoblasts, myocytes, and myofibers are listed in Table 1. The differential expression of a series of transcription factors include paired-box-7 (PAX7) and forkhead-box-O (FOXO), as well as the family of myogenic regulatory factors, including myoblast-determination-protein-1 (MYOD1), myogenic-regulatory-factor-4 (MRF4), myogenic-factor-5 (MYF5), and myogenin (MYOG) [11]. Figure 1 outlines the changes in the expression pattern of PAX7, MYOD1, and MYOG during the expansion of SCs and the differentiation and fusion of myoblasts. Following SC-mediated repair of matured muscles, fast versus slow myofiber populations can be differentiated by their expression of different types of myosin heavy chains (MyHC), that is, MyHC-1 (*Myh7* gene) in slow-oxidative type I fibers, MyHC-2a (*Myh2* gene) in fast oxidative-glycolytic type IIa fibers, and MyHC-2x (*Myh1* gene) in fast glycolytic type IIx fibers [12]. In rodent skeletal muscles, an additional fast-twitch myosin-4 isoform is present, that is, MyHC-2b (*Myh4* gene). The assumed network of myosins in mouse skeletal muscles is presented by the bioinformatic STRING analysis [13] shown in

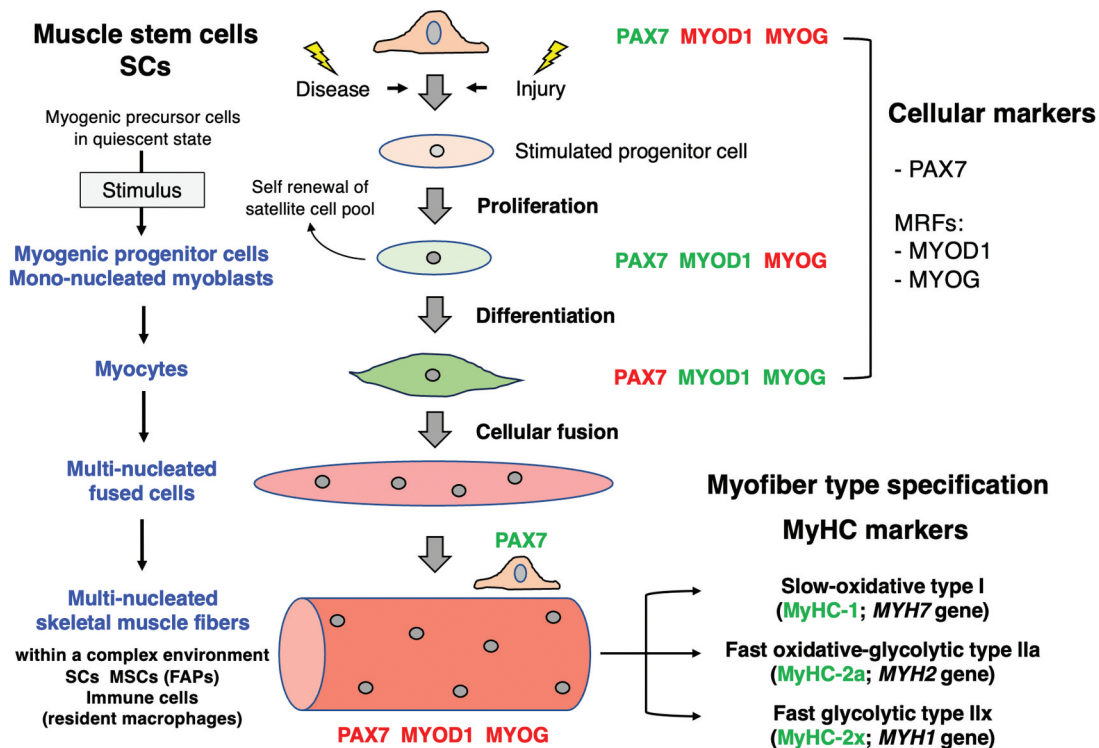


Figure 1. Overview of the involvement of muscle stem cells in regenerative myogenesis. The color of the transcription factors marks their presence (green) versus absence (red) in specific cell types. FAPs, fibro-adipogenic progenitors; MRFs, myogenic regulatory factors; MSCs, mesenchymal stem cells/multipotent stromal cells; MYOD1, myoblast determination protein 1; MyHC, myosin heavy chain; MYOG, muscle-specific transcription factor myogenin; PAX7, paired box 7; SCs, satellite cells.

Table 1. Listing of the main cell types that are involved in embryonic myogenesis versus adult regenerative myogenesis, and their markers.

Cell types	Markers
(i) Embryonic myogenesis	
Progenitor/stem cells	PAX3, PAX7
1st progenitor	MYF5, MRF4, MYOD1
2nd progenitor	MYF5, MYOD1
Embryonic/fetal myoblasts	MYF5, MYOD1
1st/2nd embryonic myofiber	MYOG
(ii) Adult regenerative myogenesis	
Quiescent satellite cells	PAX7, FOXO
Activated satellite cells	PAX7, MYF5, MYOD1
Myoblasts	PAX7±, MYF5, MYOD1
Myocytes	MYOD1, MYOG, MRF4
Myofiber	MYOG, MRF4, MyHC

*Abbreviations used: FOXO, forkhead box O; MRF4, myogenic regulatory factor 4; MYF5, myogenic factor 5; MyHC, myosin heavy chain; MYOD1, myoblast determination protein 1; MYOG, myogenin; PAX, paired box.

Figure 2 that outlines protein–protein interactions between light and heavy chains of myosins, as well as myosin-binding proteins [12].

3. SC dysfunction in muscular dystrophy

The dysfunction or exhaustion of SCs plays a central pathophysiological role in various neuromuscular disorders [6,7], including muscular dystrophies [14]. Here, we focus on the most frequently inherited muscle wasting disease of early childhood, which is Duchenne muscular dystrophy (DMD), a multi-system disorder that is triggered by mutations in the extremely large *DMD* gene [15]. Skeletal muscle abnormalities in DMD are related to the almost complete loss of dystrophin, which represents the full-length Dp427-M protein isoform of this membrane cytoskeletal component [16]. Deficiency in dystrophin causes the collapse of

a sarcolemmal glycoprotein complex, which weakens the linkage between the extracellular matrix and intracellular cytoskeleton of myofibers. This causes increased micro-rupturing of the plasma membrane during excitation-contraction-relaxation cycles. Increased calcium influx through the leaky sarcolemma and elevated levels of proteolysis render myofibers more susceptible to chronic degeneration. In conjunction with impaired function and exhaustion of SCs, decreased levels of muscle regeneration and progressive myonecrosis are followed by chronic inflammation, fat substitution, and reactive myofibrosis in DMD [16].

Dysfunction of SCs plays a central pathophysiological role in DMD and appears to be due to reduced levels of activated SCs that properly perform asymmetric cell division causing the disruption of cellular polarity [14]. In normal skeletal muscle, dystrophin supports asymmetric cell division and its unequal cellular allocation seems to be crucial for initiating the process of myogenic differentiation. Thus, the essential regulation of the polarity of SCs and mediation of asymmetric cell division is impaired by dystrophin deficiency [14]. Importantly, expression levels of mitogen-activated protein kinase MAPK12 and the microtubule affinity regulating serine-threonine kinase MARK2 are affected in Dp427-deficient SCs. Both kinases interact normally with dystrophin via α 1-syntrophin, so the lack of this signaling axis impedes delocalization of the partitioning defective protein PARD3, an important cell polarity regulator, to the opposite side of SCs [14,16].

This central role of stem cell dysfunction in DMD opens new avenues for the identification of novel therapeutic targets to restore the functionality of SCs. As outlined below, proteomics represents an ideal bioanalytical tool to further investigate the involvement of SCs in neuromuscular disorders.

STRING analysis of myosins in skeletal muscle

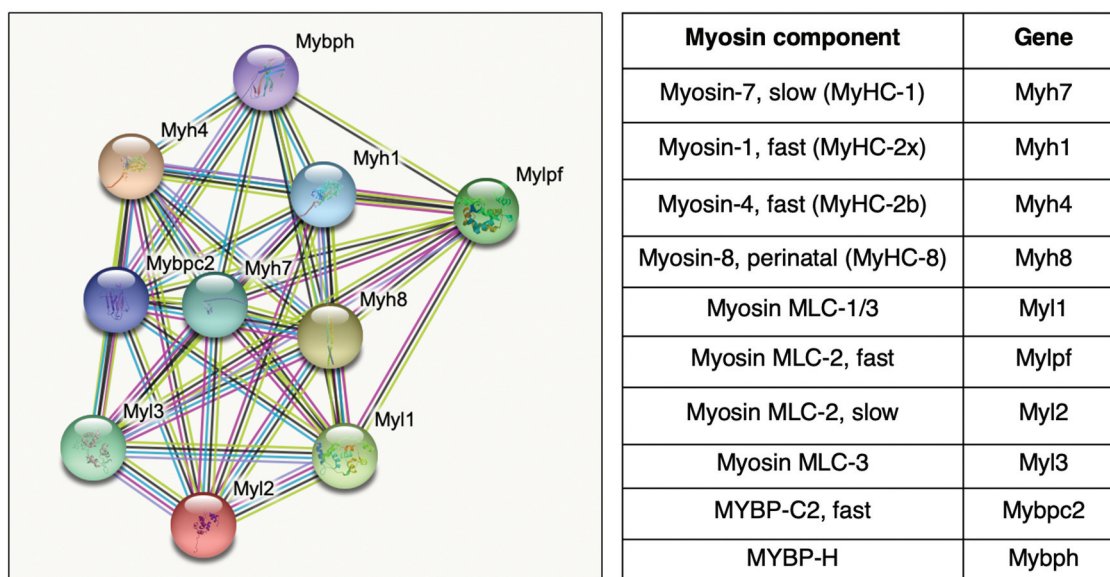


Figure 2. Bioinformatic STRING analysis of major myosin components in the sarcomere of mouse skeletal muscles. MyHC, myosin heavy chain; MLC, myosin light chain; MYBP, myosin-binding protein.

4. Proteomic approaches for studying muscle stem cells

MS-based muscle proteomics employs most frequently two main approaches, bottom-up analyses with a peptide-centric focus and top-down analyses with a proteoform-centric methodology [8]. Starting materials are usually tissues, cells, or subcellular fractions derived from patient biopsy specimens, surgical remnants, or muscle samples from genetic animal models that mimic neuromuscular disorders. Figure 3 provides an overview of proteomic studies that are based on the screening of total muscle protein extracts, muscle-derived cell types, or suspensions of single cells. The most commonly used MS techniques and data acquisition methods are listed in Figure 3, including both label-free quantitation (LFQ) techniques and muscle cell analyses using stable isotope labeling [8]. Table 2 provides an overview of major top-down and bottom-up proteomic approaches and their bioanalytical advantages versus technical limitations. Particular challenges are evident when analyzing proteoforms, introduced as a consequence of post-translational modifications (PTMs), alternative RNA splicing, and/or genetic variation [17]. Incomplete protein sequence coverage generated from bottom-up proteomics remains a significant disadvantage to comprehend proteoform complexity. Developments in top-down proteomic workflow are in continual development, and while this

approach is ideally suited to PTM profiling, challenges such as protein solubility remain an issue [18].

Studying low-abundance regulatory proteins or transcription factors by proteomics is also challenging [19]. To overcome this issue at least partially, subcellular fractionation strategies can be used to separate high-abundance sarcomeric proteins from low-copy number muscle proteins [20]. Combining the reduction in complexity and dynamic range of cellular proteins can be highly useful for enriching lower abundant protein fractions prior to MS analysis [21]. Efficient muscle protein separation can be achieved by exploiting various criteria, such as cellular location, solubility, hydrophobicity, protein size, charge and/or isoelectric point [22].

For MS-based analyses of individual cells, the SCP technique has been optimized over the past few years and presents an excellent tool for studying the dynamic nature of the proteomic profile of distinct cell types [23]. For SCP-based surveys of SCs, the flowchart in Figure 3 shows the main bioanalytical steps, starting with the mechanical and/or enzymatic dissociation of heterogeneous mixtures of muscle-derived cells, followed by incubation with fluorescently labeled antibodies prior to fluorescence-activated cell sorting (FACS) [24] in a specialized flow cytometer [25]. Detailed reviews on MS methods used for label-free SCP and approaches that utilize tandem mass tags (TMT) in

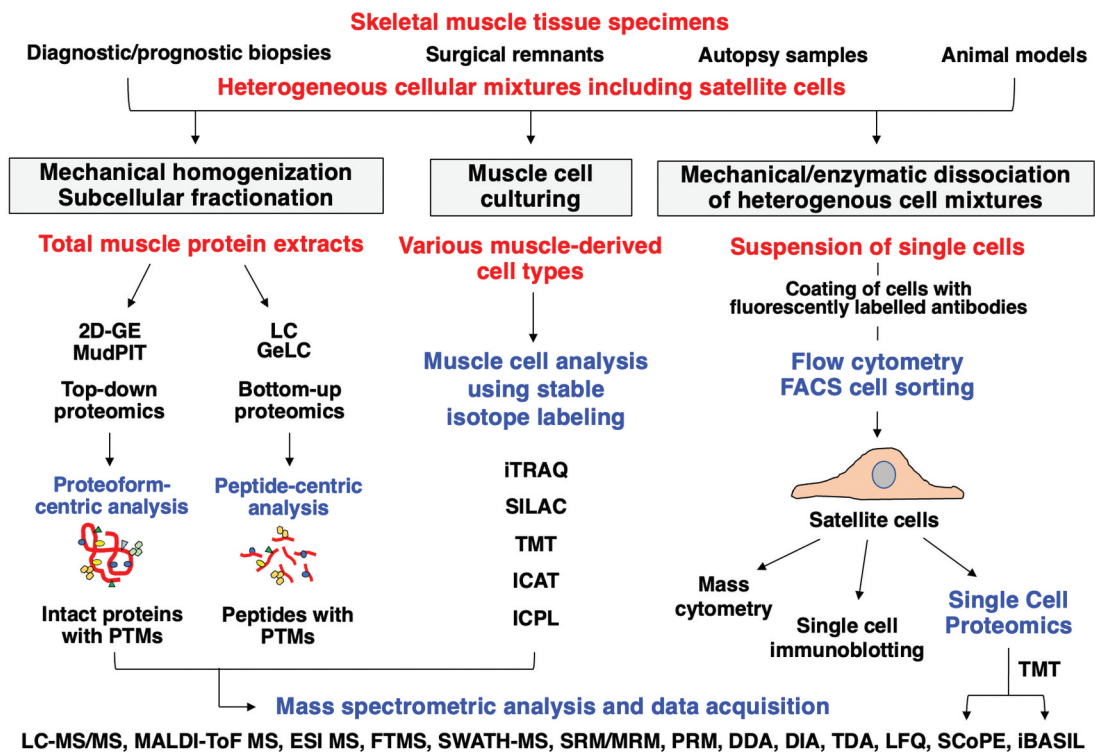


Figure 3. Proteomic profiling strategy to study skeletal muscles with a special focus on the muscle stem cell niche. Shown is the analytical workflow to study total muscle protein extracts from tissue sources, muscle-derived cell types following culturing and single-cell analysis with the help of flow cytometric sorting and separation. Frequently used methods for mass spectrometry-based protein identification and data acquisition, muscle cell analysis using stable isotope labeling and single-cell proteomics are listed. 2D-GE, two-dimensional gel electrophoresis; DDA, data-dependent acquisition; DIA, data-independent acquisition; ESI, electrospray ionization; FACS, fluorescence-activated cell sorting; FT, Fourier-transform ion cyclotron resonance; GeLC, gel electrophoresis-liquid chromatography; iBASIL, Improved Boosting to Amplify Signal with Isobaric Labeling; ICAT, isotope-coded affinity tags; ICPL, isotope-coded protein labeling; iTRAQ, isobaric tagging for relative and absolute quantitation; LC, liquid chromatography; LFQ, label-free quantification; MALDI-ToF, matrix-assisted laser desorption/ionization time-of-flight; MS, mass spectrometry; MudPIT, multi-dimensional protein identification technology; PRM, parallel reaction monitoring; PTMs, post-translational modifications; SCoPE, Single Cell Proteomics by Mass Spectrometry; SILAC, stable isotope labeling by amino acids in cell culture; SRM/MRM, selected/multiple reaction monitoring; SWATH, Sequential Window Acquisition of all Theoretical Mass Spectra; TDA, targeted data acquisition; TMT, tandem mass tags.

Table 2. Overview of major proteomic approaches and their bioanalytical advantages versus technical limitations.

Proteomic methodology	Advantages	Disadvantages/limitations
Gel-based and top-down proteomics		
Two-dimensional gel electrophoresis (2D-GE) coupled with mass spectrometry (MS)	Individual 2D protein spots displaying unique proteoforms are highly suitable for MS-based protein identification	High-/low-molecular weight proteins and hydrophobic proteins are difficult to separate and analyze; 2D-GE has low throughput
Fluorescence two-dimensional difference in-gel electrophoresis (2D-DIGE) coupled with MS analysis	Multiplexing of samples; greatly reduced gel-to-gel variations; highly sensitive fluorescent tagging of proteins of interest	Using CyDyes for differential tagging adds a significant cost to comparative proteomic experiments
Gel-free and bottom-up proteomics		
Stable isotope labeling by amino acids in cell culture (SILAC)	Multiplexing (pairwise comparisons); compatibility with complex fractionation procedures	Mostly limited to tissue culture models; not applicable to tissue/biofluid samples
Tandem mass tags (TMT)-based analysis	Multiplexing is compatible with any sample type; accurate quantitation	Co-eluting peptides with similar molecular weights can affect quantitation of reporter ions; variable efficiency in labeling reported
Label-free quantification (LFQ) analysis	LFQ approach avoids the usage of expensive labels; streamlined protocols	Multiplexed analysis is not possible in a single experiment; lower accuracy of quantification compared to labeling methods; high technical and experimental reproducibility needed (retention times)
Single-cell proteomics (SCP)	SCP allows cellular heterogeneity to be uncovered at single-cell resolution; enables comparison of SCP data to other single-cell platforms (transcriptomics)	High levels of technical challenges encountered; dedicated microfluidics and MS instrumentations necessary

SCP, such as Single Cell Proteomics by MS (SCoPE-MS), multiplexed SCoPE using isobaric carriers (SCoPE2) or Improved Boosting to Amplify Signal with Isobaric Labeling (iBASIL), have been published [26,27]. The application of TMT labeling in combination with suitable fractionation approaches can be routinely used to quantify between 7,000 and 10,000 proteins [28]. Using TMT in MS-based experiments allows more samples to be combined together (recent TMTpro-18 plex) and evaluated in a single MS run, with deeper analysis by off-line fractionation prior to MS analysis. This is a significant advantage resulting in a considerable increase in peptides detected compared to label-free methods. Recently, the improvement of ultrasensitive proteomic applications was reviewed for studying single cells [29], including pluripotent stem cell models [30].

Single myofiber proteomics has made great progress to characterize fiber type specification. Precise and unbiased fiber type assignments were used to define a myofiber-resolved proteome, including estimated expression values of distinct proteoforms in individual subtypes [31,32]. These analyses provided a baseline for providing the utility of single-cell type analysis in understanding heterogeneity associated with physiological and pathological conditions. Recent advances in cell sorting and MS analysis have made it feasible to determine changes in protein abundance between individual cells without the need for immuno-based approaches [33]. Proteomic analysis at the single-cell level still presents great challenges; however, the growing body of ultrasensitive single-cell approaches is rapidly propelling this area in understanding the protein-level cellular heterogeneity and capturing rare cellular populations, that is, muscle stem cells. In contrast to multinucleated and extremely large myofibers, SCs are small mono-nucleated cell types that can be conveniently sorted and separated from other cells by the FACS method. SCs can be sorted by flow cytometry and studied by SCP, single-cell immunoblotting and/or mass cytometry. This should lead to a better comprehension of the modulation or dysfunction of SCs in muscle wasting diseases with an impaired regenerative capacity, such as DMD [6,14–16].

Spatial proteomics, which involves the study of MS-based protein localization within cells [34], offers a complimentary

approach for comprehending the biological processes associated with regenerative myogenesis. The transition from self-renewal to differentiation of SCs allowing for adult myogenesis is associated with major changes in cell morphology, cellular signaling, adhesion processes, dynamic PTMs, and altered levels of protein synthesis/degradation. Understanding the subcellular localization of proteins, and how these properties change as cells differentiate, is vital for the detailed elucidation of regenerative myogenesis at the cellular level. Sequential immunofluorescence methodology for the spatial detection of proteins, cytometry by time-of-flight using heavy metals coupled to antibodies with pattern detection by MS, and localization of organelle proteins by isotope tagging (LOPIT) by MS, are examples of high-throughput approaches for interrogating the spatial proteome [35–37]. The future application of these types of spatial proteomic analyses promise to decisively improve our biomolecular insights into muscle stem cell biology.

5. Conclusion

Both embryonic and regenerative myogenesis are essential biological processes that are involved in the developmental establishment and continuous maintenance of the neuromuscular system in the human body. Adult myogenesis occurs throughout life in the matured organism to reduce the detrimental impact of tissue injury or the degenerative effects of chronic disease and aging. Following stimulation, specialized myogenic stem cells that are located between the muscle plasmalemma and basal lamina, proliferate and differentiate to support skeletal muscle self-repair. Abnormal functioning or exhaustion of the SC pool of stem cells is linked to a reduced regenerative capacity in diseased or senescent myofibers. In order to study SCs in isolation, muscle-associated stem cells can be prepared by cell biological dissociation procedures and then sorted by flow cytometry. The most promising biochemical approach to characterize isolated SCs is ultrasensitive SCP. MS-based analyses can be based on LFQ techniques or methods that employ tandem mass tags. The detailed characterization of myogenic stem cells will ensure the generation of

novel insights into pathophysiological mechanisms of neuromuscular disorders.

6. Expert opinion

Myofibers form the largest biomass in the human body and are intrinsically involved in a variety of essential cell biological and metabolic processes. Loss of muscle mass and contractile strength has severe consequences for whole-body physiology and bioenergetic integration. Thus, studying SCs that are essential cells for muscle self-repair, is of critical importance for both muscle pathobiology and regenerative medicine. To date, most comparative proteomic analyses in the field of neuromuscular disorders have focused on muscle tissue specimens from patients or animal models. However, it is imperative to study specialized myofibers and their complex cellular and acellular environment in isolation to fully comprehend the complexity of regulatory processes, cellular signaling pathways and pathobiochemical mechanisms. This would include the detailed analysis of the triggering factors causing progressive myonecrosis, the role of the inflammatory response and the cellular components that are involved in reactive myofibrosis.

The SCP-based identification and comprehensive characterization of distinct cells within the heterogenous skeletal muscle niche have the potential to become a key method for advanced studies in systems protein biochemistry. If current technical limitations in sample preparation and proteoform coverage are overcome, this approach could progress into biomedical and clinical practice using improved biomarker signatures. Crucial aspects of increasing the bioanalytical power of MS-based proteomics are the continuous improvement of research strategies in basic and applied myology, streamlined proteomic workflows, more sensitive MS instrumentation and comprehensive systems bioinformatic methodology. A combination of established LC-MS/MS technologies and rapidly advancing affinity-based methods could be the most promising strategy for the large-scale identification of novel biomarker candidates of myofiber regeneration and muscle self-repair.

Advanced SCP using an optimized LFQ-based analysis pipeline can be utilized to study individual stem cells and identify large numbers of protein groups using data dependent acquisition (DDA). A major challenge of SC research is sufficient access to patient specimens for developing new extraction methods for biomarker discovery of human muscle diseases. However, technological advances in single muscle cell preparation promise to enable highly improved proteomic biomarker research, which will be complemented by verification studies using routine immunochemical, biochemical, physiological, molecular biological, histological, histochemical and immunofluorescence microscopical methodology. It is important to stress that LFQ-based approaches are time-consuming and require a large number of individual runs for comparative studies. Alternative methods albeit more expensive, such as high-throughput and TMT-based workflows, can quantitate thousands of protein groups and much larger numbers of multiplexed samples can be analyzed per day.

These types of SCP approaches would be ideal to further elucidate the dysfunction of SCs in DMD, which seems to be based on reduced levels of asymmetric cell divisions due to deficiency in dystrophin. The systematic and differential analysis

of quiescent SCs, activated myogenic progenitors, myoblasts, and myocytes during the process of myofiber regeneration by SCP could shed new light on the pathogenesis of DMD. A more detailed cataloging of the proteomic profile of Dp427-deficient SCs would be extremely helpful to determine why these stem cells display an abnormal Dp427-MARK2-PARD3 signaling axis leading to the loss of SCs polarization and a greatly diminished capacity for muscular regeneration.

Thus, future SCP-based investigations will facilitate deep proteomics projects in the field of applied myology and have the potential to generate novel insights into pathobiology and enhanced concepts of how to treat muscle wasting diseases. The flexible usage of different SCP analysis modes will decisively advance biochemical and proteomic approaches to study the role of dynamic proteoforms and their PTMs in skeletal muscle pathobiology.

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Declaration of interest

The authors have no relevant affiliations or financial involvement with any organization or entity with a financial interest in or financial conflict with the subject matter or materials discussed in the manuscript. This includes employment, consultancies, honoraria, stock ownership or options, expert testimony, grants or patents received or pending, or royalties.

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Author contributions

All authors made a significant contribution to the work reported. Draft manuscript preparation was carried out by PD and KO. All authors reviewed and edited the drafts and approved the final version of the manuscript.

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