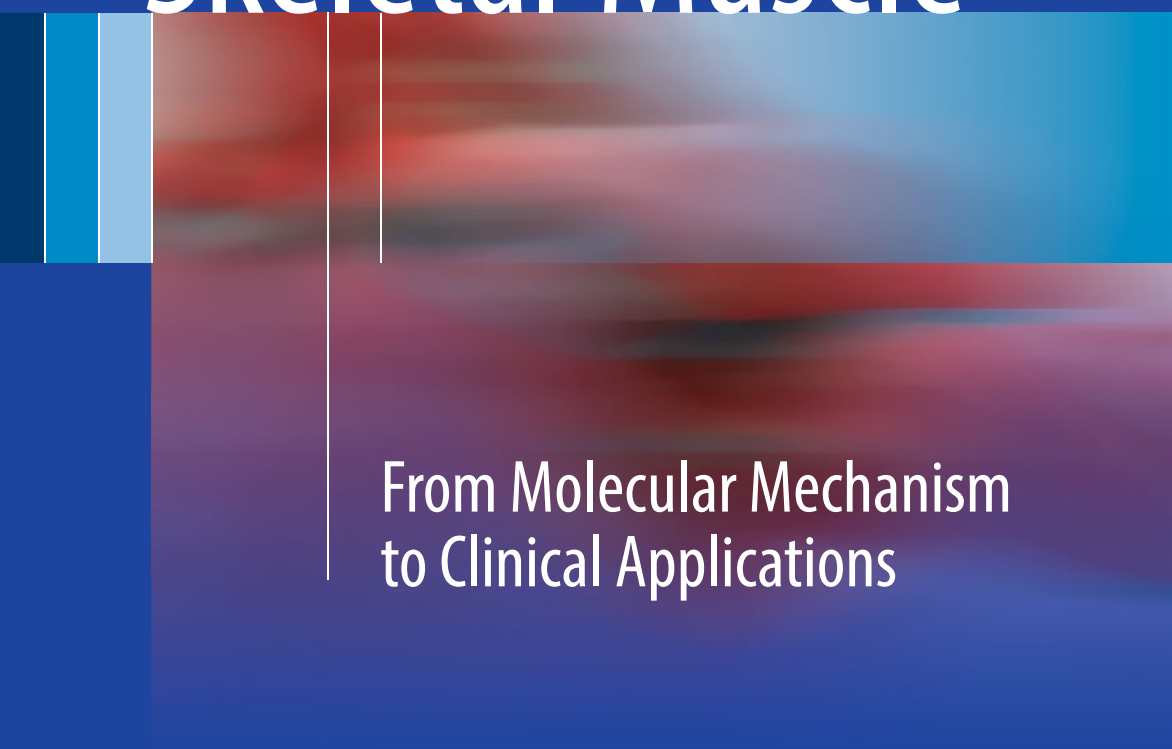
Kunihiro Sakuma *Editor*

The Plasticity of Skeletal Muscle



From Molecular Mechanism to Clinical Applications

Springer

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Preface

In humans, skeletal muscle is the most abundant tissue in the body, comprising 40–50% of body mass and playing vital roles in locomotion, heat production during periods of cold stress, and the overall metabolism. It is essential for our quality of life to have healthy muscles. Skeletal muscle possesses a high plasticity for environmental stimulation such as neuronal, mechanical, hormonal, and/or immune factors. For example, the increase of mechanical stress induces muscle hypertrophy probably due to the upregulation of protein synthesis and of transcription in muscle-specific structural components. This book is about skeletal muscles, molecular mechanism of muscle hypertrophy [AMP-activated protein kinase (AMPK) and ribosome biogenesis], and atrophy [ubiquitin-proteasome system, autophagy, cytokine, redox regulation (nitric oxide), and transient receptor potential cation channels (TRPC)]. In particular, it is a very intriguing and current topic that changes in ribosome biogenesis and translational capacity correlate finely with changes in muscle mass in both growth and wasting conditions.

Muscle loss occurs as a consequence of normal aging (sarcopenia) and several chronic diseases (cachexia). Muscle loss is also common in muscular dystrophy, in which markedly loss of various membranous structural proteins occurs around muscle fibers. This book includes various interventions such as therapeutic approach using muscle and pluripotent stem cells or nutritional and pharmacological approach for muscle wasting such as muscular dystrophy, sarcopenia, etc. In addition, this book also highlights the myokine [interleukin, brain-derived neurotrophic factor (BDNF), or secreted protein acidic and rich in cysteine (SPARC)] that is produced and released by muscle cells in response to muscular contractions and conducts various functional roles (e.g., prevention of several cancers). Furthermore, this book introduces versatile role of peroxisome proliferator-activated receptor gamma coactivator 1-alpha (PGC-1α) for the mitochondrial biogenesis, formation of neuromuscular junction, and so on. At present, no other book covering similar topics is available as a resource book. The majority of this book discusses which factors modulate the muscle mass of skeletal muscle and which interventions are effective for various muscular disorders by referencing current literatures.

For the completion of this book, I want express my personal thanks to all the chapter contributors who spent substantial effort and their valuable time to make this publication possible. I am also thankful to Ms. Hemalatha Gunasekaran who helped me with her excellent editorial assistance. This book can be interesting for graduate students, postdocs, teachers, physicians, and executives in biotech and pharmaceutical companies, as well as researchers in the fields of molecular biology and regenerative medicine in skeletal muscle.

Tokyo, Japan Kunihiro Sakuma

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About the Editor

Professor Kunihiro Sakuma Ph.D., currently works at the Department for Liberal Arts in Tokyo Institute of Technology. He is a physiologist working in the field of skeletal muscle. He was awarded a sports science diploma in 1995 by the University of Tsukuba and started scientific work at the Department of Physiology, Aichi Human Science Center, focusing on the molecular mechanism of congenital muscular dystrophy and normal muscle regeneration. His interest later was turned to the molecular mechanism and the attenuating strategy of sarcopenia (age-related muscle atrophy). Preventing sarcopenia is important for maintaining a high quality of life in the aged population. His opinion is to attenuate sarcopenia by improving autophagic defect using nutrient- and pharmaceutical-based treatments.

Chapter 1
Pluripotent Stem Cells and Skeletal Muscle Differentiation: Challenges and Immediate Applications

Elena Garreta, Andrés Marco, Cristina Eguizábal, Carolina Tarantino, Mireia Samitier, Maider Badiola, Joaquín Gutiérrez, Josep Samitier, and Nuria Montserrat

Abstract Recent advances in the generation of skeletal muscle derivatives from pluripotent stem cells (PSCs) provide innovative tools for muscle development, disease modeling, and cell replacement therapies. Here, we revise major relevant findings that have contributed to these advances in the field, by the revision of how early findings using mouse embryonic stem cells (ESCs) set the bases for the derivation of skeletal muscle cells from human pluripotent stem cells (hPSCs) and patient-derived human-induced pluripotent stem cells (hiPSCs) to the use of genome editing platforms allowing for disease modeling in the petri dish.

Keywords Pluripotent stem cells • Differentiation • Genome editing • Disease modeling

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1.1 Introduction

Regenerative medicine comprises multiple research areas and therefore can be defined as "an interdisciplinary field of research and clinical applications focused on the repair, replacement or regeneration of cells, tissues or organs to restore impaired function resulting from any cause, including congenital defects, disease or trauma" [1]. There are wide ranges of technologies that can contribute to achieve this goal. Some methodologies consist on cell therapy, tissue engineering, gene therapy, and biomedical engineering techniques, as well as more traditional treatments involving pharmaceuticals and devices. Overall, regenerative medicine is the key new discipline with true potential to change the field of current health care from traditional reactive to preventative and restorative health-care style.

Degenerative diseases in humans are characterized by loss or malfunction of specific cell types. Often, replacement of the whole organ is the unique treatment in clinics (i.e., muscular dystrophies). Unfortunately, for most of the pathologies involving organs, transplant is the unique possibility of treatment. Nevertheless, donor supplies are still scarce for clinical demand. In this regard, cell therapy is one of the most important approaches for tissue regeneration and in some cases a promising and feasible alternative to whole organ transplantation.

In this chapter we are going to revise the potential use of pluripotent stem cells (PSCs) for skeletal muscle disease modeling and differentiation. Our goal is to provide an extensive overview by revising from the early findings using mouse embryonic stem cells (ESCs) for the derivation of skeletal muscle cells to the latest findings making use of in vitro genome editing platforms for the study of skeletal muscle disorders in humans.

1.1.1 Human Pluripotent Stem Cells (hPSCs)

1.1.1.1 Human Embryonic Stem Cells (hESCs)

Totipotency is defined as the ability of a single cell to divide and produce all of the differentiated cells in an organism. This feature persists in human embryos until four- to eight-cell stage [2, 3]. Afterward genome activation initiates differentiation, with certain blastomeres forming the outer, polar trophectoderm, while others retain their pluripotent potential and generate the nonpolar inner cell mass (ICM) that will give rise to the future organism. Human embryonic stem cells (hESCs) are typically derived from the pluripotent ICM of the blastocyst [4]. After their first derivation by Thompson and colleagues [4], other groups reported the possibility to derive hESCs following this same method [5–7]. Interestingly within the last decade, hESC lines have also been derived from earlier stages of embryonic development, including single blastomeres of four- or eight-cell stage embryos [8–11] and 16-cell morulae [12, 13] (Fig. 1.1). Blastomere-derived hESCs could circumvent ethical

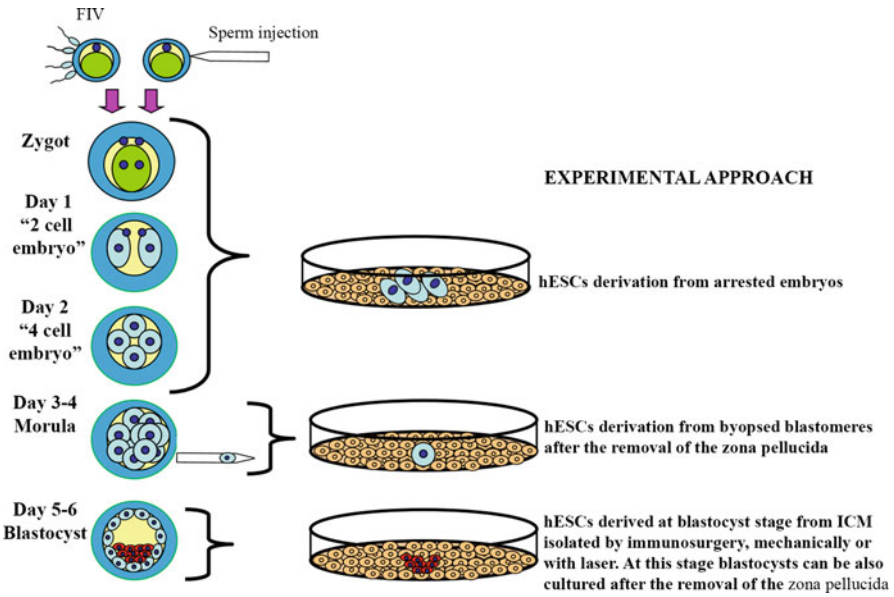

Fig. 1.1 Methods for derivation of human embryonic stem cells (hESCs) from samples coming from anonymous donations of processes such as in vitro fertilization or the injection of sperm into the egg. The diagram shows the various moments in the embryonic development in vitro in which different groups have derived hESCs lines (Schematic adapted from Ref. [213])

issues surrounding the use of hESCs in biomedical research, since the removal of single blastomeres from early-stage embryos would not hamper the ability of the remaining blastomeres to develop into a normal embryo. In this regard, Giritharan and colleagues have demonstrated that both blastomere-derived and ICM-derived hESC exhibit similar transcriptional profiles independent of the developmental stage of the embryo from which they were derived, highlighting their potential use for future applications such as cell therapy and drug screening [14].

After the discovery that hESCs could be easily isolated from human blastocysts, the scientific community pointed out that one of the main hurdles of blastomere- and ICM-derived hESC for clinical application is that transplantation of their differentiated derivatives might lead to allograft rejection. At that time several strategies were proposed to overcome such impediment, as the establishment of hESC bank containing cell lines covering the majority of human leukocyte antigen (HLA) genotypes. In this regard, tissues differentiated from homozygous hESCs express only one set of histocompatibility antigens, thus, being more readily matched to patients [15]. In addition, homozygous hESCs are routinely derived by parthenogenesis by the artificial activation of unfertilized metaphase II (MII) human oocytes into parthenogenetic ESCs (pESCs) [16–22]. Since all genetic material in parthenotes originates from the maternal genome, the resulting pESCs possess only maternal patterns of gene imprinting, becoming an instrumental platform for the

study of the mechanisms regulating maternal epigenetic regulation, as well as to explore disease-related mechanisms. Lately, parthenogenesis has been used to create a pESC line for the common deletion associated with spinal muscular atrophy type 1 [23], paving the way for the generation of pESCs for disease modeling. Also very recently, Sagi and colleagues have generated a collection of hESCs with a normal haploid karyotype from pESC lines originating from haploid oocytes, opening the door to the development of genetic screenings [24].

Somatic cell nuclear transfer (SCNT) consists in the transplantation of the nucleus of a somatic cell into an enucleated oocyte. In this regard, SCNT-derived ESCs (NT-ESCs) are genetically autologous to the nuclear donor somatic cell, offering great potential in regenerative medicine, including disease modeling and cell replacement therapy. NT-ESCs were reported first in mice [25] and later in primates [26]. Recently, Mitalipov group has shown that it is possible to generate hESCs via SCNT. In their work, differentiated fetal and infant fibroblasts were used as nuclear donors [27]. More recently others have demonstrated that age-associated changes in the nucleus donor cell do not hamper NT-hESC derivation [28], and that is also possible to generate insulin-producing beta cells from NT-ESCs from a patient with long-standing diabetes [29]. These last findings pinpoint SCNT as a suitable platform for the generation of autologous cells for clinical purposes.

1.1.1.2 Induced Pluripotent Stem Cells (iPSCs)

The "reprogramming history" started in 1958, when Gurdon et al. [30] by using the technique of SCNT, originally described by Briggs and King [31], showed that the nuclei of intestinal epithelial cells from *Xenopus laevis*, after transplantation into enucleated eggs, could develop into normal and healthy tadpoles, thus demonstrating successful nuclear reprogramming. Taken together, these first advances pointed out that the process of cell differentiation could be reversible and did not require irreversible nuclear changes. One of the most important advances in this field of research was the publication by Wilmut et al. in 1998 of the birth of a cloned sheep (Dolly) by transplanting the nucleus of an adult somatic mammary gland cell into an enucleated oocyte [32]. In the last 15 years, progress has been made producing "clones" for reproductive purposes in several species—cattle, goats, mice, and pigs [33–39]—culminating this period with the creation of the first cloned human embryo in 2013 by Mitalipov and colleagues [27].

In 1987 Schneuwly group found that in *Drosophila melanogaster*, the overexpression of certain transcription factors in somatic cells could activate the expression of genes arising from another cell type [40]. This group together with others also found similar results in mammals [41]. Pursuing the idea of changing cell fate and inducing dedifferentiation, Takahashi and Yamanaka in 2006 discovered that the pluripotent state could be artificially induced in somatic cell types through the overexpression of just four transcription factors (OCT4, SOX2, c-Myc, and KLF4-OSKM) [42–45]. The produced cells were called induced pluripotent stem cells (iPSCs), and they exhibited all the molecular and functional features of hESCs.

While, at first, somatic reprogramming was described using mouse embryonic fibroblasts, the Japanese team could show that also a reduced formula of the original "Yamanaka cocktail" could be used to reprogram human somatic cells toward human iPSCs (hiPSCs) [46]. Since 2007 several aspects have been considered when identifying the best cell source to be reprogrammed for regenerative medicine approaches. This also has conditioned the number of Yamanaka transcription factors used in each specific case (i.e., progenitor cells expressing endogenously any of the Yamanaka factors can be reprogrammed in the absence of it, as neural stem cells in the absence of SOX2), the strategy for Yamanaka factor delivery (i.e., proliferating cells can be easily transduced with retroviral vectors for reprogramming, as fibroblasts, keratinocytes, among others), and the cell type used (i.e., cell amenability has sometimes limited reprogramming applications, as for neural stem cells or intestinal cells, among others). Besides all these factors, up to day a huge variety of somatic cell types, which included fibroblasts, blood, keratinocytes, liver and gastrointestinal cells, as well as cancer cells, can be used to derive iPSCs [42, 46–56].

Interestingly, during the last years, the generation of protocols avoiding the use of lentiviral or retroviral vectors for the expression of Yamanaka factors has involved the definition of novel strategies for hiPSC generation, including the use of recombinant proteins [57, 58], episomal vectors [59], or mRNAs [60, 61], among others [62]. Thus, the generation of hiPSCs, especially the generation of patient-derived iPSCs suitable for disease modeling in vitro, opens the door for the potential translation of patient-derived iPSCs into the clinic. Successful replacement or augmentation of the function of damaged cells by patient-derived differentiated stem cells would provide a novel cell-based therapy for skeletal muscle-related diseases.

Integrative Methods for Cell Reprogramming

The first generation of iPSCs was accomplished by retroviral-mediated ectopic expression of "OSKM or Yamanaka factors" into mouse fibroblasts [42]. This method has been successfully used for several cell types, such as mouse and human fibroblasts, neural stem cells, keratinocytes, adipose cells, liver cells, and blood cells, with efficiencies of reprogramming between 0.01 and 0.02% [46]. An alternative approach to transduce OSKM factors to derive iPSCs is the use of a lentiviral system which yields a higher efficiency (0.1–2%) than retroviral transduction [62]. Both platforms have been intensively used during the first years of the reprogramming decade; however, the disadvantage of viral integration into the host genome, together with the use of oncogenic factors as KLF4 or c-Myc, bound the application of iPSCs for clinical purposes [62–64].

Since viral integration can cause insertional mutagenesis, interference with gene transcription, and genome instability and induce malignant transformation [65–68], several non-integrating virus-mediated iPSC reprogramming methods have been currently established [62, 64, 69]. One example is the use of doxycycline (dox)-inducible lentiviral vector harboring OSKM factors flanked by LoxP sites that can be subsequently excised by the use of Cre recombinase [70]. Also, replication-defective adenoviral vectors expressing OSKM factors have proven useful for derivation of

iPSCs because they do not integrate into chromosomal DNA [71, 72]. Adenoviral vectors have been mainly used to generate iPSCs from liver cells and fibroblasts without viral integration [69, 73]. While the non-integrating aspect of the adenoviral method is appealing, to be of significant use in translational medicine, optimization improving reprogramming efficiency is necessary [74].

Non-integrative Methods for Reprogramming

Lately, different laboratories have made use of episomal plasmids as another method for integration-free reprogramming of somatic cells into iPSCs [75, 76]. This procedure has also been used to derive iPSCs from cord blood and peripheral blood cells [77]. This technique yields a very low efficiency, but several modifications by different groups provide promising results for future use [75, 76, 78]. Interesting minicircle DNA vectors containing Lin28, Nanog, SOX2, and OCT4 factors have been described as a procedure to derive human iPSCs from human adipocytes with an efficiency of 0.005% [79].

Other approaches relay in the use of the single-stranded RNA Sendai virus (SV); this method allows for the generation of iPSCs with an efficiency around 0.1%, comparable to the lentiviral approach while avoiding transgene integration [80]. Similarly mRNA transfection has been proved as another appealing system for the generation of iPSCs avoiding transgene integration [81]. RNA-induced pluripotent stem cell procedures offer a safe and effective method to generate "safe iPSCs" providing a reduced immunogenic response. Using this method, Warren et al. [60] derived iPSCs from human keratinocytes, human neonatal fibroblasts, human fetal lung fibroblasts, and cystic fibrosis patient fibroblasts with conversion efficiencies and kinetics substantially superior to established viral protocols (around 2%).

Other strategies such as the use of bioactive OSKM proteins have also been tested for iPSC generation [57]. In this regard, Kim et al. demonstrated the successful generation of stable iPSCs from human fibroblasts by direct delivery of four reprogramming protein factors (OSKM) yielding an efficiency of 0.001% [58]. A major challenge of this procedure, however, stands in the efficient delivery of OSKM proteins [82]. In this regard others have shown the possibility to fuse OSKM proteins with a short basic segment with a high proportion of amino acids, namely, cell-penetrating peptide (CPP) [83, 84]. CPP-OSKM proteins, when delivered into somatic cells, can directly reprogram them successfully without genetic manipulation and/or chemical treatments [57, 58]. Nevertheless, bioactive reprogramming proteins are difficult to synthesize in large quantities, and reprogramming efficiencies by this method vary between 0.001 and 4%.

Cellular reprogramming using small molecules offers many advantages such as temporally and spatially manageable, reversible, cell permeability, and cost-effectiveness. Small molecules used to generate iPSCs are comprised of epigenetic modifiers, WNT signal modulators, cell senescence attenuators, metabolism modulators, and regulators of cell apoptosis/senescence pathways. Small molecules inducing iPSCs can be classified into three types: (1) small molecules that improve reprogramming efficiency [85], (2) compounds replacing one or more repro-

gramming factors [86–88], and (3) combinations of compounds that suffice for reprogramming [89, 90]. Small molecule methods have been successfully applied to reprogram mouse and human fibroblasts directly into iPSCs [62, 89–93].

1.2 General Approaches to Induce In Vitro Differentiation of Pluripotent Stem Cells (PSCs)

Both mouse and human PSCs are routinely cultivated in the presence of feeder layers (Fig. 1.2a). Initial studies made use of mouse embryonic fibroblasts mitotically inactivated as feeder cells in the presence of embryonic stem cell media for preserving hPSCs undifferentiated in culture. For mouse PSCs, LIF can substitute for feeder layers. However, since LIF is not needed for human PSC culture, in the last years, different chemically defined media have been produced in order to sustain human PSC culture and expansion in feeder-free substrates. PSCs grow on the feeder layers as colonies (Fig. 1.2b). Generally, human and mouse PSCs are enzymatically dissociated with different reagents as trypsin,

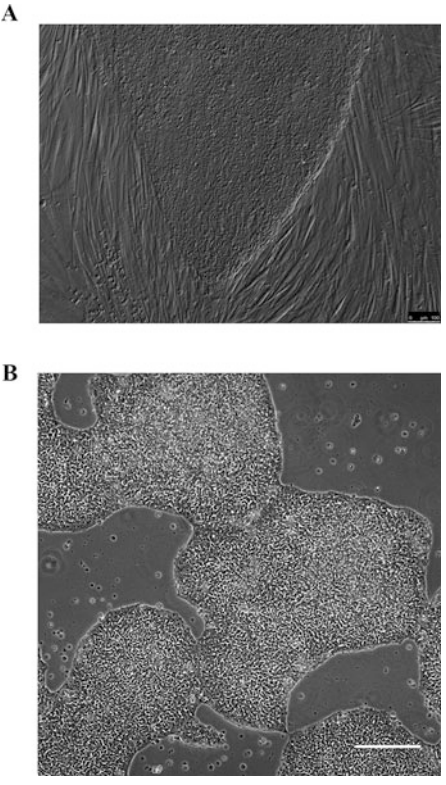

Fig. 1.2 Culture and propagation of human pluripotent stem cells (PSCs). (**a**) hPSCs can be cultured on top of irradiated human fibroblasts and grow as tight colonies that are manually expanded. (**b**) Lately, the culture of hPSCs is easily performed using defined matrices and medium sustaining pluripotency. hPSCs are routinely expanded by enzymatic methods allowing for the standardization of differentiation protocols worldwide. Scale bars 100 μm

acutase, or dispase; the obtained suspension of single cells is then transferred for subculture and expansion for differentiation purposes as guided differentiation, among others.

As an option for culturing human PSCs without feeder cells, Matrigel™ has proven to be a useful alternative enabling the stable culture of human PSCs. Moreover, others and we have also shown that Matrigel™ allows the generation of hiPSCs for disease modeling purposes without animal-derived feeder cells [94]. Since Matrigel™ was derived from Engelbreth–Holm–Swarm mouse sarcoma cells [95], other types of matrices which do not contain animal-derived agents have been tested and used as feeder cell substitutes for the successful maintenance and generation of human PSCs, such as CellStart [96, 97], recombinant proteins [98–100], and synthetic polymers [101, 102].

The culture media used in the early generation of hESCs contained fetal bovine serum [4]. In order to remove unspecific agents that might cause spontaneous differentiation of hESCs, knockout serum replacement (KSR) has now been established as a defined material for maintaining hESCs [103] and is also traditionally used for hiPSC generation [46, 104–106]. In this regard, mTeSR1 medium was developed as a chemically defined medium for maintaining human PSCs [107]. Importantly, in the last years, several authors have reported the generation of commercially developed *xeno*-free media for maintaining hiPSCs, and such media have already been used successfully for iPSC generation. These media include TeSR2 [108], NutriStem [109], Essential E8 [99], and StemFit [110].

When factors that sustain PSC stemness are deprived from the media, PSCs spontaneously differentiate into derivatives of the three embryonic germ layers. This capacity has been profited for more than 30 years in order to direct PSCs to the desired cell product. In this regard, up to day, an infinite number of protocols have been established to promote the development of the cell type of interest.

The following are basic strategies to induce in vitro differentiation of PSCs:

(a) **Embryoid bodies' (EBs) formation**: EBs are spherical structures that allow PSC culture in suspension when using nonadherent culture substrates (Fig. 1.3a). EBs can be induced from PSCs grown as monolayers by mechanical or enzymatic procedures. Interestingly, within the first 3 days of differentiation, PSCs propagated as EBs form three germ layers. The three-dimensional structure, including the establishment of complex cell adhesions and paracrine signaling within the EB microenvironment, enables differentiation and morphogenesis. The presence of ectoderm is manifested by the expression of fibroblast growth factor 5 (FGF5), endoderm by GATA-4, and mesoderm by Brachyury [111]. For all these reasons, the first protocols for muscle differentiation took advantage of EB induction, including those describing derivation of the first myogenic cells from mESCs and iPSCs [112, 113] and hESCs [114]. Although those first assays proved the feasibility of mouse and human PSCs to give rise to myogenic-like cells, lately, different works have proved the possibility to avoid the use of fetal bovine and/or horse serum in

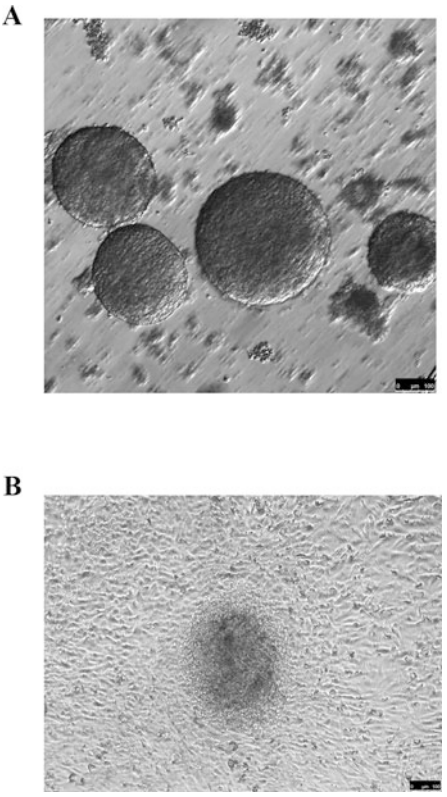

Fig. 1.3 hPSC differentiation following embryoid body formation (EB). hPSCs are able to differentiate toward the three germ layers of the embryo. (**a**) The generation of EBs from hPSCs has been widely use in order to generate cells with myogenic potential (**b**) After several days grown in suspension, EBs are then transferred onto supporting cells (feeder cells) sustaining for myogenic differentiation. Scale bars 100 μm

order to reduce potential contaminations of animal components by including serum-free-based media during the time course of differentiation.

Besides all the advances when using EB methodology for the derivation of muscle-like cells, their culture is laborious and time-consuming, limiting the control of growth factors/cytokines in a 3D setting. These limitations preclude the use of EB-based methods for the generation of PSC-derived muscle cells in a therapeutic setting, where large amounts of patient-derived muscle cells would need to be derived. Still EB-based methods may offer advantages when used as an intermediate step for the generation of myogenic cells from PSCs. In this regard, Hwang and coworkers have recently shown that cells differentiated as EBs and sorted for PDGF-αR expression could be successfully cultured in monolayer retaining the ability to undergo terminal myogenic differentiation despite culture pressure [115].

(b) **Guiding muscle differentiation modifying medium composition**: Traditionally monolayers of PSCs and/or EBs have been used as starting cellular populations to differentiate into specific lineages by mimicking developmental programs guiding tissue specification. Majorly, PSCs (grown as monolayers or EBs) have been subjected to changes in medium composition in order to induce

their differentiation toward the desired cell type. With respect to myogenic differentiation, PSCs from mouse and human have been differentiated toward different stages of myogenic differentiation, i.e., paraxial mesodermal cells, muscle progenitor cells, satellite cells, myoblasts, and myotubes. In this regard myogenic cells at initial stages of differentiation (those expressing Pax3 and/or Pax7) were shown to be characterized by higher regenerative potential than cells that reached more advanced stages of differentiation and expressed myogenic transcription factors [115–119]. Although these works used serum and cell culture media with animal-derived components, they set the basis for the definition of serum-free protocols for myogenic differentiation. In general, the use of such specific cell media together with the control of the expression of myogenic transcription factors crucial for muscle determination and differentiation has demonstrated promising results when differentiating mouse or human PSCs toward myogenic cells (i.e., control of MyoD1 expression under the control of promoters responsive for tamoxifen/puromycin treatment).

(c) **Genetic manipulation of PSCs**: For a long time, PSCs have represented an unprecedented platform for controlling the expression of transcription factors aiming to direct the differentiation of PSCs toward the lineage of choice. PSCs can be kept in culture in the absence of feeders and expanded as single cells, favoring different manipulations such as electroporation and nucleofection, methods generally used when performing PSC genetic manipulation. Whereas the first studies for the generation of myogenic-like cells from mouse or human PSCs took advantage of integrative gene expression systems (i.e., lentivirus or retrovirus), nowadays the use of these tools is limited, since they incur uncertain risks for potential cell-based therapeutic applications [120]. In this regard, the use of excisable vectors (i.e., transposons [121, 122] or mRNAs [60]) offers an unprecedented opportunity for the derivation of differentiated PSCs suitable for regenerative medicine. Moreover, the recent discovery of DNA meganucleases, TAL effector nucleases, or clustered regularly interspaced short palindromic repeats (CRISPR) will offer the possibility to target specific loci determinant for muscle differentiation with fluorescent reporters leading to the definition of robust protocols of PSC differentiation.

(d) **Coculture with supportive cells (feeder cells)**: Generally the coculture of mouse and human PSCs (either as monolayers or EBs) together with feeder cells has been used to induce PSC differentiation [123] (Fig. 1.3b). Different feeders have proven to commit PSC differentiation toward different lineages. In the context of muscle differentiation, Baghavati and coworkers showed that the coculture of EBs derived from mESCs together with primary muscle cells suffice for myogenic differentiation, since donor-derived myofibers could be occasionally found on the surface of the host muscle [124].

(e) **Extracellular matrix (ECM) as an instructive scaffold for PSC differentiation:** Extracellular matrix (ECM) is a dynamic and complex environment characterized by tissue-specific biophysical, mechanical, and biochemical properties. Different works have shown that muscle microenvironment (niche) enables freshly isolated muscle stem cells (MSCs) to contribute extensively to

skeletal muscle regeneration when transplanted in dystrophic mice (i.e., *mdx* model, among others). On the contrary when MSCs are grown on standard conditions (i.e., plastic substrate) for several passages, they lose their "stemness" leading to progenitors with diminished regenerative potential [125, 126]. It has been also described that muscle regeneration in higher vertebrates depends on the capacity of the injured tissue for retaining ECM scaffolding, which serves as a template for the de novo formation of muscle fibers [127]. In this regard, the interaction between PSCs and ECM via integrins determines the expression of signaling molecules that affect PSC differentiation [123]. Of note, myogenesis (i.e., proliferation of myoblasts and further fusion into myotubes) has been positively induced when mouse iPSCs have been cultured in the presence of Matrigel™ [128]. Similar results have been observed when using collagen-based matrix for the differentiation of human iPSCs expressing a dox-inducible expression cassette of MyoD1 [129]. In order to control the organization and alignment of muscle fibers, both the composition of the ECM and its anisotropic architecture are essential. Self-organized myotubes have been generated by using topography-based approaches based on nanofibers [130], microabrated surfaces [131], and microcontact printing of ECM proteins [132, 133]. In a complementary approach, biochemical cues have also been introduced in order to promote cell alignment and differentiation. By using inkjet bioprinting, spatially defined patterns of myogenic and osteogenic cells were derived from primary MSCs as a response to growth factor patterning [134]. In order to mimic native tissue organization, topographical and biochemical signaling has also been explored [135]. The vast majority of these works present cells to static microenvironments. Latest trends point out the relevance of presenting cells to spatially and temporally dynamic microenvironments [136]. Surfaces with gradient concentrations of growth factors (BMP-2 and BMP-7) have shown to successfully drive cell differentiation [137, 138]. Overall, these strategies appear a promising way to direct the differentiation of PSCs [139].

Tissue engineering strategies are intended to provide synthetic and natural 3D scaffold materials to mimic the structural, biochemical, and mechanical properties of the stem cell niche [140, 141]. Natural scaffolds based on ECM proteins have been used to form hydrogels for musculoskeletal tissue engineering [142–144]. Commercially available ECM substitutes such as Matrigel™ hydrogels are also showing promising results in the differentiation of PSCs toward cardiomyocytes [145]. Lately, technologies such as electrospinning, which allows organizing the polymers into thin sheets of fibrous meshes, are promising in this field [146, 147]. The use of acellular tissue scaffolds is also being explored in muscle regeneration since they offer a native ECM with the optimal biochemical and mechanical properties for MSC culture preserving the architectural features of the tissue.

(f) **The use of microfluidics for PSC differentiation:** PSC differentiation is affected by chemical, topographic, and mechanical effects and conventional culture methods. Microfluidic culture platforms have shown to accurately mimic physiological conditions for stem cell growth [148].This emerging technology

offers the possibility to (1) manipulate the environment controlling oxygen supply, pH, temperature, flow shear stress [149], material shear, topography, and stiffness [150, 151] and surface properties [149]; (2) identify, separate, and position desired cell types [152]; (3) stimulate cells through mechanical stretching [153] or electrically [154]; (4) develop screening of several parameters [155]; (5) apply gradients of chemical and soluble factors [156, 157]; (6) control fluid mixing through compartmentalized devices [158]; and (7) include sensors [159, 160]. Recently, Uzel and coworkers have shown that mechanical or electrical stimuli facilitate the differentiation of stem cells into myocytes [161]. Biochemical stimuli include the presence of several factors on the cell culture, as skeletal muscle differentiation factors promoting differentiation [162]. Mechanical stimuli are needed to induce desired interactions with cells or matrix, especially for muscle fibers. Passive mechanical stimuli, such as mechano-topographical cues [163], scaffold structure orientation [163, 164], and substrate stiffness or elasticity [165], have proved effect on myogenic differentiation. Active mechanical stimuli include stretching or forcing cells or the entire microfluidic chip [153, 166]. This stretch can be uniaxial or equiaxial, having different effects on stem cell differentiation, as reviewed by Watt and Huck [167].

Besides all these findings, differentiation toward myocytes is not enough to achieve physiologically relevant 3D models with fascicle-like sarcomere structure capable of contraction with uniform distribution of oxygen and nutrients or cell alignment. Several approaches have been developed on these regard, mostly trying to promote cell alignment, that include among others (1) the use of parallel linear microgroves [168, 169] or ECM molecule micropatterns on the surface [170, 171] in order to facilitate cell alignment; (2) the employment of microchannels for chemical delivery [172] or for 3D constructs of the skeletal muscle filled with hydrogels [173]; (3) the use of anchoring points for the ECM with Velcro anchors [174], tendon-like anchors [171], or steel mesh to induce cell alignment through a stretching freestanding construct; (4) and the use of capped pillar-based constructs to encourage freestanding muscle alignment and maturation through a controlled stress, enabling measurement of forces [175–178]. Despite all these improvements, self-aggregation of myoblasts happens frequently. Some studies developed by the group of Professor Asada [179, 180] have reported 3D fascicle-like muscle-on-a-chip devices without self-aggregation of cells, creating sarcomeric structures capable of contraction, with uniform distribution of oxygen and nutrients, spontaneous aligning stress, cell alignment along transmission axis encouraged by uniform tension, fibers with high length to diameter ratio, high cell density, and overall good mimic of motor units. Very recently, an integrated tissue–organ bioprinting procedure has been reported, which can fabricate stable, human-scale tissue constructs of any shape, such as the skeletal muscle [181]. In order to study the skeletal muscle in a biological and physiological context, it is necessary to include its interaction with motor neurons. Neuromuscular junction on a chip includes, mainly, the following three approaches: 3D coculture of neurospheres and

muscle fibers [178], 3D coculture of motor neurons and muscle fibers [182], and the use of compartmentalized microfluidic chips with chambers and microchannels [183–185].

1.3 Generating Myogenic Cells from Mouse and Human PSCs

Skeletal muscles in higher organisms originate from different areas of the embryonic mesoderm [186]. Head muscles derive from the unsegmented cranial paraxial mesoderm. In turn, muscles of the trunk and limbs arise in two subsequent stages from the dorsal part of the segmented paraxial mesoderm, commonly referred to as dermomyotome. In a first stage, postmitotic myogenic precursors delaminate from the borders of the dermomyotome and migrate ventrally to form the primary myotome [187]. This primary myotome serves as a scaffold for the second stage of myogenesis but also secretes factors that trigger an epithelial-to-mesenchymal transition (EMT) among muscle progenitors in the central dermomyotome that eventually migrate into the myotome [187]. This secondary migration of EMT-derived precursors from the dermomyotome also generates the satellite cells (SCs), the adult stem cell pool in the skeletal muscle, which are responsible for postnatal muscle maintenance, repair, and growth.

Over the last decades, the understanding of the transcription factors and intrinsic and extrinsic signals that govern SCs or terminally differentiated myogenic cells has represented a good starting point for the definition of protocols for the generation of myogenic cells from PSCs (both from mouse and human ESCs/iPSCs). In the same manner, the generation of patient-derived cell platforms can help us to develop experimental strategies toward generating muscle stem cells, either by differentiating patient-specific iPSCs or by converting patient's somatic cells toward myogenic cells (transdifferentiation). Overall, the possibility to generate disease-free patient iPSCs can help us to identify which are the mechanisms driving muscle disease and, more importantly, to develop new compounds for treating MDs.

1.3.1 Exogenous Expression of Muscle-Related Transcription Factors in PSCs: How to Generate Myogenic Precursors and/or Terminally Differentiated Multinucleated Myogenic Cells

The use of autologous derived muscle stem cells for restoring muscle function has been envisioned as a powerful therapeutic strategy for muscle degenerative diseases. Successful generation of myogenic precursors from mouse and human iPSCs has been achieved by exogenous expression of transcription factors crucial for myogenic

differentiation. Since PSCs are an expandable source amenable for genome editing (i.e., they can undergo extensive tissue culture manipulations, such as drug selection and clonal expansion, while still maintaining, e.g., their pluripotency signature and genome stability), latest advances in this field will increase our knowledge in PSC differentiation toward skeletal muscle lineage. Early studies in the field have relayed in the use of viral vectors for the generation of stable PSC lines expressing the myogenic transcription factor of interest under the control of specific drugs (i.e., Pax7 or MyoD1, Magic F-1, among others). Transduced PSCs are then subsequently exposed to culture media conditions promoting muscle differentiation. Other methods involve the use of non-integrative vectors such as adenovirus, transposons, or excisable lentiviral vectors in order to avoid undesirable effects when working with integrative systems (i.e., retrovirus or lentivirus). Following these different approaches, several studies have shown that PSC monolayers or PSC-derived EBs could be converted with different efficiencies into myogenic-like cells (see below).

Early Studies of Myogenic Differentiation from mESCs

Dekel and colleagues described the first protocol describing the generation of skeletal muscle cells from mESCs early in 1992. In their hands when mESCs were electroporated with MyoD1 cDNA driven by the β-actin promoter, some cells could be converted to skeletal muscle cells [188]. Although myogenesis was associated with the activation of MRF4 and Myf5 genes, the transient expression of MyoD1 did not lead to the efficient conversion of mESCs toward skeletal muscle cells. However, authors showed that contracting skeletal muscle fibers could be generated when the transfected cells were allowed to differentiate in vitro after EB formation in the presence of low-mitogen-containing medium. After that first work, other authors provided fine-tuned systems aiming to control the expression of the myogenic factor of choice at a precise moment during the onset of myogenic differentiation. Alongside this line, Ozasa and colleagues [189] established a mESC line by introducing a MyoD transgene controlled by a Tet-Off system (ZHTc6-MyoD). Under those conditions and only after 7 days, primed cells started to fuse into myotubes, and occasionally light muscle contractions were recorded. Intramuscular injections of MyoD–mESC-derived cells into *mdx* resulted in the generation of clusters of dystrophin-positive myofibers in the injected area.

Myogenic Differentiation from Human PSCs

Within the last years, different research groups have demonstrated the possibility to generate myocytes and even multinuclear myotubes from both hESCs and patient-derived hiPSCs. Already in 2012 two different reports indicated that after MyoD overexpression, mesodermal [190] or mesenchymal cells [191] could be generated from iPSCs. Similarly, Rao and colleagues (2012) generated a transgenic Tet-inducible MyoD cassette in which all the transgenic elements were inserted in hESCs making use of lentiviral vectors. Later on, Yasuno and colleagues [122] generated terminal multinucleated cells from iPSCs derived from patients affected with carnitine palmitoyltransferase II (CPT II) by the transduction of a self-contained Tet-inducible MyoD1 expressing piggyBac vector (Tet–MyoD1 vector) and transposase

into hiPSCs by lipofection. This system allowed the indirect monitoring of MyoD cells in response to doxycycline by co-expression of a red fluorescent protein (mCherry). Moreover, authors increased the purity of the generated myocytes by culturing the cells in low glucose conditions [192]. Also Abujarour and colleagues [129] found that it is possible to derive myotubes from control iPSC and iPSC lines from patients with either Duchenne or Becker muscular dystrophies using a lentiviral system expressing MyoD under the control of a Tet-inducible promoter.

Other factors apart from MyoD1 have been used to promote myogenic differentiation from hPSCs. In this regard, Iacovino and colleagues [193] integrated one single copy of Myf5 into mESCs and hESCs by means of a system that authors called inducible cassette exchange (ICE). Overall, Iacovino and colleagues showed that Myf5 expression is sufficient to promote the myogenic commitment of nascent mesoderm, thereby establishing a novel and rapid method of differentiating mESCs and hESCs into skeletal muscle tissue. Interestingly, Darabi and colleagues generated an improved version of ICE system in order to generate mESCs in which Pax7 expression was controlled under the control of doxycycline [194, 195]. Later on, the same group generated inducible Pax7 hPSCs by means of a doxycycline-inducible lentiviral vector encoding Pax7 incorporating an IRES–GFP reporter allowing for the monitoring of transplanted Pax7-derived myogenic progenitors into dystrophin-deficient mice (*mdx*). Interestingly, authors could show that after transplantation the differentiated cells led to long-term muscle regeneration [196].

1.3.2 Generation of Myogenic Precursors and/or Terminally Differentiated Multinucleated Myogenic Cells by Soluble Factors

The exogenous expression of muscle-specific transcription factors in PSCs by the methodologies described above has proved to be successful strategies to direct muscle differentiation. Although valuable, those strategies could not be applied in the context of clinics to treat compromised skeletal muscle tissues. For this reason, in the past years, many efforts have been also directed to the definition of specific culture media and conditions to produce myogenic precursor cells. Several groups have investigated the possibility to expose EBs or monolayers of mouse and human PSCs to stage-specific differentiation protocols based on the addition of soluble factors known to be crucial during embryonic myogenesis. Following such protocols authors have been able to derive different cell populations with myogenic potential (i.e., paraxial mesoderm) that could be further isolated using FACS-based selection strategies. In this manner, authors could evaluate the myogenic differentiation yield by quantifying the percentage of cells expressing specific myogenic markers. In the same manner, these works have characterized the myogenic differentiation process by analyzing the expression of myogenic-related markers by common techniques such as polymerase chain reaction or immunohistochemistry

Early Studies in Myogenic Differentiation from Mouse PSCs by Soluble Factors

mPSCs propagated as EBs are known to form the three germ layers within the first 3 days of in vitro differentiation in undefined culture media. However, transplantation of EBs without any induction to direct development along a specific pathway leads to a failure of integration into recipient tissues and often forms teratomas. Thus, successful derivation of myogenic cells from PSCs requires selective induction of the myogenic lineage in PSCs. In a pioneering study by Rohwedel and coworkers, the expression of myogenic-related factors (i.e., Myf5, MyoD, and myogenin) was identified in 7-day-old outgrowths obtained from EBs formed by differentiating mouse ESCs [112]. The EB system was also used in one of the first studies that addressed the myogenic differentiation potential of miPSC [197], in which Pax3 and Pax7 expression was followed by the expression of myogenic markers such as Myf5, MyoD, and myogenin, similarly as is observed during embryonic myogenesis. In an attempt to enhance the myogenic conversion of PSCs, Bhagavati and Xu [124] described the coculture of EBs with freshly isolated muscle cells as a novel method for myogenic differentiation. Although authors showed that differentiated cells generated by this method developed vascularized and muscle tissue when transplanted in dystrophic mice (mdx mice), still the number of engrafted cells was too low [124]. Others described that the temporarily supplementation of culture medium with retinoic acid [198] or ascorbic acid and activin A [199] could improve myogenic differentiation from mESC. Although these initial studies involving EBs and coculture methodologies yielded important information, they resulted to be rather inefficient and often used serum-containing medium hampering the experimental reproducibility and their further translation into the clinics, due to the presence of undefined factors in the medium. In this regard, many efforts have been directed to the development of defined culture conditions. Sakurai and colleagues [200] differentiated a mESC line toward paraxial mesodermal progenitors. Specifically, authors selected paraxial mesodermal progenitors based on the expression of platelet-derived growth factor receptor-α (PDGFR-α) and the absence of Flk-1—a lateral mesodermal marker. Later on, the same authors demonstrated that mESCs could be directed toward the paraxial mesodermal lineage by a combination of bone morphogenetic protein (BMP) and Wnt signaling under chemically defined conditions [201].

Generation of Myogenic Cells from Human PSCs by Soluble Factors

Myogenic differentiation from hPSCs forming EBs was also achieved by allowing the differentiation of cell outgrowths from human EBs exposed to medium supplemented with ITS (i.e., insulin, transferrin, selenium), dexamethasone, and epidermal growth factor (EGF) or to medium supplemented with horse serum [114]. In this manner, myogenic markers could be detected 2 and 4 weeks after EB plating. Interestingly, the treatment with the hemimethylating agent, 5-azacytidine for 24 h, led to significant increase in the number of cells expressing myogenic markers [114]. However, in vitro formation of myotubes could not be seen under none of these culture conditions. In contrast, when those hESC-derived myogenic precursors were

transplanted in NOD-SCID mice, they could incorporate into the host muscle and became part of regenerating muscle fibers [114].

Given that the EB culture system is a laborious and time-consuming method that does not allow for generation of large quantities of differentiated cells for therapeutic purposes, researchers have developed alternative myogenic differentiation protocols by omitting the EB formation step. Myogenic differentiation of hPSCs in monolayer cultures has been also proved to be feasible [202–204]. Following feeder-free monolayer culture of hESCs, Barberi and colleagues derived multipotent mesenchymal precursors (MMPs) that could be further differentiated into myogenin-expressing cells [202, 203]. Their monolayer differentiation method involved a serial of cell culture steps in specific culture media and two purification steps based on FACS sorting of CD73-positive mesodermal precursors that after 2–4 days of subculturing were subsequently sorted for NCAM-bright expression, a marker of the embryonic skeletal muscle. Forty-six percent of NCAM-positive cells revealed expression of myogenin, and importantly they were able to fuse and form MyHC-expressing contracting myotubes [202, 203]. First, MMPs were maintained in inactivated fetal serum and in the presence of the mouse skeletal myoblast line C2C12 [202]. Later, Barberi and colleagues could avoid the use of C2C12 cells by using serum-free N2 medium, allowing for the expansion of hESC-derived myoblasts in a serum-free N2 medium in the presence of insulin [203].

Following a similar strategy, Sakurai and colleagues [200] [201] developed a defined protocol for the production of paraxial mesodermal progenitors from mESCs and miPSCs that they could apply to differentiate hiPSCs toward PDGFR-α+/KDR- cells. Those progenitors could be further differentiated into osteocytes, chondrocytes, and skeletal muscle cells, demonstrating the suitability of their procedures for the generation of myogenic cells for regenerative purposes.

Other authors have also shown the possibility to generate PDGFR-α+ from hESCs, although low engraftment was observed after transplantation of such hESC-derived myogenic cells into injured skeletal muscle [205]. Interestingly, the same authors have recently demonstrated that addition of Wnt3a in the culture medium promoted a rapid myogenic commitment of hESCs and, more significantly, that those hESC-derived myogenic cells could contribute to muscle regeneration in a NOD/SCID mice model of the cardiotoxin-injured skeletal muscle [206]. In the same line, other works have demonstrated that inhibition of GSK3β and treatment with FGF2 could specifically induce skeletal muscle differentiation. In particular, Xu and colleagues [207] have demonstrated that simultaneous inhibition of GSK3B, activation of adenyl cyclase, and stimulation with FGF2 during EB formation could promote the generation of myogenic precursors that terminally differentiated in vitro and showed some functional characteristics typical of satellite cells upon transplantation. Similarly, Borchin and colleagues [208] have described that hPSCs could be differentiated toward Pax3/Pax7 double-positive cells after GSK3β and FGF2 treatment.

Moreover, Xu and colleagues have developed a massive platform for the identification of soluble factors promoting muscle differentiation making use of

zebra fish embryos [209]. Their system took advantage of zebra fish embryo culture system with reporters of early and late skeletal muscle differentiation, enabling for the examination of 2400 chemicals on myogenesis. Interestingly, authors identified six compounds expanding muscle progenitors, including three GSK3β inhibitors, two calpain inhibitors, and one adenylyl cyclase activator named forskolin. Of note, when bFGF, forskolin, and GSK3β inhibitor BIO were used in hiPSCs, they induced skeletal muscle differentiation and produced engraftable myogenic progenitors that contributed to muscle repair in vivo [209]. Taking advantage of these findings, the same group has recently demonstrated that the same protocol promoted the generation of myotubes from hiPSCs derived from patients affected from Donohue syndrome, offering the first model of human skeletal muscle insulin resistance [210].

1.4 How to Model Muscle Disease in the Petri Dish

Nowadays, the development of protocols to direct cell differentiation from human PSCs in a high range of cell types has set the basis to generate massive platforms for the study of differentiation procedures and disease progression. Furthermore, the correction of the genetic disorders in these cells with classical genetic engineering or emerged genome editing technologies not only allows molecular studies of MDs but also development of future strategies for gene and cellular therapies.

So far, different groups have demonstrated the suitability of patient iPSC approaches in order to model MDs. Abujarour and colleagues [129] have obtained myotubes by direct MyoD-mediated differentiation of hiPSCs from Duchenne muscular dystrophy (DMD) and Becker muscular dystrophy (BMD) patients. Authors validated the differentiated myotubes by a global expression profile that showed how they adopted the skeletal muscle program and the functional response to protein factors investigated as potential treatments for MD, in a similar manner to primary myotubes. These results prove that iPSC derived from DMD and BMD patients has no intrinsic barriers preventing from myogenesis. Although the delivery of MyoD by a lentiviral vector precludes the use of these iPSCs in a clinical setting, they still represented a scalable source of normal and dystrophic myoblast for immediate application in disease modeling and drug discovery.

Recently, Tedesco and colleagues [190] developed the first protocol for the differentiation of mesoangioblast-like cells from iPSCs generated from fibroblasts and myoblasts of limb–girdle muscular dystrophy 2D (LGMD2D) patients. After obtaining mesoangioblast-like cells, authors expanded and genetically corrected them by means of a lentiviral vector for the specific expression of human α-sarcoglycan in differentiated striated muscle cells. A tamoxifen-inducible lentiviral vector of MyoD–ER was also used to induce differentiation of the corrected cells into myotubes before its transplantation into α-sarcoglycan-null immunodeficient mice. Authors showed the engraftment of these cells in the dystrophic skeletal muscle and the related production of myofibers clusters expressing α-sarcoglycan. The amelioration of the dystrophic phenotype in terms of motor capacity was increased when the same experiments were conducted using mouse-derived mesoangioblasts. Overall, Tedesco and colleagues showed how to avoid the limited availability of

adult tissue-specific muscle progenitor cells by deriving patient-specific iPSCs and expanding their differentiated progeny. Together with the in vitro genetically correction and later transplantation, this approach could be useful for gene and cell therapies.

In the same line, Tanaka and colleagues [121] developed a myogenic induction system to differentiate iPSCs from patients affected by Miyoshi myopathy (MM), a congenital distal myopathy caused by mutations in dysferlin (DYSF). Authors obtained myotubes that showed MM associated phenotype with impaired expression of DYSF and defective membrane repair. These features were rescued by the expression of full-length DYS by a piggyBac (PB)-based vector. A similar work was performed by Yasuno and colleagues [122], where authors generated iPSCs from patients affected by carnitine palmitoyltransferase II (CPT II) deficiency, an inherited disorder involving B oxidation of long-chain fatty acids (FAO). Differentiated myocytes recapitulated the increase accumulation of C16 (palmitoyl-carnitine) that could be restored by bezafibrate, mimicking some clinical aspects of CPT II deficiency. All these data show how the patient-specific iPSCs and later differentiation result in the generation of validated in vitro models of both diseases.

Recently, Li and colleagues [211] have demonstrated the possibility to correct iPSCs derived from DMD patients by the use of genome editing technologies: TALEN and CRISPR/Cas9. Authors took advantage of the ability to expand iPSCs limitlessly to develop three different strategies: exon skipping, frameshifting, and exon knock-in, in order to correct the pathological mutation. The exon knock-in was the most effective approach to restore the full-length dystrophin protein in the iPSC-differentiated myocytes. In this context Turan and colleagues [212] have corrected limb–girdle muscular dystrophy 2B (LGMD2B) and 2D (LGMD2D) by DICE or TALEN-mediated integration of wild-type DYSF cDNA into the H11 safe harbor locus and single-stranded oligonucleotide-mediated gene editing by CRISPR/Cas9, respectively. These approaches resulted in the adequate protein expression for DYS and relocation of corrected α-sarcoglycan protein to the cell membrane in muscle progenitor cells differentiated from iPSC. These works demonstrate the capability of iPSC technology to provide in vitro muscle models and in combination with genome editing autologous corrected cells for ex vivo gene therapy approaches.

Very recently Salvatore Iovino and colleagues [210] derived iPSC from patients of Donohue syndrome related with insulin receptor mutations (IR-Mut). These cells were differentiated in myotubes that exhibited insulin resistance-like (IR) responses in vitro. IR-Mut myotubes fail to increase glucose uptake, glycogen synthase activity, or glycogen stores in response to insulin stimulation. Transcriptional regulation was also perturbed in IR-myotubes with reduced insulin-stimulated expression of insulin receptor protein and reduced insulin-stimulated phosphorylation of the receptor and downstream effectors. This work indicates an impairment of the insulin signaling to induce the expression of metabolic and early growth response genes. This data validated this model of skeletal muscle insulin resistance not only to dissect its genetic features related with Donohue syndrome but also to study epigenetic acquired features related with other insulin resistance states such type 2 diabetes. All these advances are summarized in Table 1.1.

Table 1.1 Summary of current approaches for modeling and correcting muscle diseases using patient-specific-induced pluripotent stem cells (iPSCs)

			Differentiation			Disease correction	
Author	Disease	Gene affected	Exogenous Expresion	Cullture	Differenciated cells obtained	Molecular strategy	Rescued phenotype
Abujarour et al.	Duchenne Muscular Distrophy (DMD) and Becker Muscular Distrophy (BMD)	Dystrophin (DMD)	Doxycycline induction of MYOD1 by lentiviral vector	· DAY -1: iPSCs culture media w/o bFGF+ROCKi+ Collagen or Matrigel	Myotubes	Only Modeled	
				· DAY 0-4: Dox+DMEM+15 % FBS			
				DAY 4-7: low-glucose DMEM and 5% HS			
Tedesco et al.	Limb-girdle muscular dystrophy 2D (LGMD2D)	α-sarcoglycan (SGCA)	—	· Week 1-2: Single cell, low O2+ROCKi+ αMEM+matrigel. Split at week 1	Mesoangioblast-like stem/progenitors (MABs)	· Lentiviral vector carrying wt SGCA under the muscle-specific myosin light chain 1F promoter and enhancer.	DMD expresion and muscle colonization of corrected cells in SGCA-null mice
				Week 3: Post trypsin, seed at 80% confuency, low O2+MegaCell or IMDM+matrigel		· Or human artificial chromosome containing the entire DMD locus.	
				Week 3 to 4: Post trypsin, after 100% seed at 80% low O2+MegaCell or IMDM w/o matrigel		Transduced at MABs state and then myogenic differenciated to multinucleated myotubes by tamoxifen inducible lentiviral vector of MyoD-ER	

Tanaka A et al.	Miyoshi myopathy (MM)	Dysferlin (DYSF)	Doxycycline induction of MYOD1 by piggyBac (PB) based vector	· DAY 0-2: w/o bFGF+Dox (at D1) DAY 2-7: 5% KSR αMEM+Dox DAY 7-9: 5% HS +DMEM+IGF-1	Myocytes	EF1a promoter-driven constitutive expression of DYSF by a piggyBac (PB) based vector	Expression of DYSF in differenciated myocytes
Yasuno et al.	Carnitine palmi-toyltransferase II (CPT II) deficiency	Carnitine palmitoyl-transferase II (CPT2)	Doxycycline induction of MYOD1 by piggyBac (PB) based vector	· DAY 0-2: w/o bFGF+Dox (at D1) DAY 2-7: 5% KSR+αMEM+Dox DAY 7-9: 5% HS +DMEM+IGF-1· DAY 10-11: Low-glucose +MEM+0.4% BSA+L-carnitine+palmitic acid+P/S	Myocytes	Only Modeled	
Li et al.	Duchenne Muscular Distrophy (DMD)	Dystrophin (DMD)	Doxycycline induction of MYOD1 by piggyBac (PB) based vector	· DAY 0-2: w/o bFGF+Dox (at 1)· DAY 2-7: 5% KSR+αMEM+Dox· DAY 7-9: 5% HS+DMEM+IGF-1	Myocytes	· TALENs and CRISPRs for Exon skipping, Frameshift or knock-In	Expresion of DMD in differenciated myocytes
	Limb-girdle muscular dystrophy 2B (LGMD2B)	Dysferlin (DYSF)	—	· DAY -7-0: Embryoid bodies (EB)+APEL media+bFGF +BIO+forskolin·	Muscle Progenitors	· Integration of wt DYSF cDNA into the H11 safe harbor locus by DICE or TALEN.	Expresion of DMD or SGCA in differentiated muscle progenitor cells

(continued)

Table 1.1 (continued)

Author	Disease	Gene affected	Differentiation			Disease correction	
			Exogenous Expresion	Cullture	Differenciated cells obtained	Molecular strategy	Rescued phenotype
Turan S. et al.	Limb-girdle muscular dystrophy 2D (LGMD2D)	α-sarcoglycan (SGCA)		DAY 0-21: matrigel+ DMEM+2%HS		· Singlestranded oligonucleotide-mediated gene editing by CRIPR/Cas9 (HDR).	
Iovino S. et al	Donohue syndrome	Insulin Receptor (INSR)		· DAY 0-7: STEM Diff Apel medium+bFGF+ Bio+Fordkolin	Myotubes	Only Modeled	
				DAY 7-9: Matrigel+Media Serum Free			
				DAY 9-36: 2% HS +DMEM			

The different approaches in terms of the differentiation protocol used and genotype/phenotype correction are listed in the table

Overall, the generation and differentiation of iPSCs constitute an innovative tool for modeling MDs. This next generation of in vitro models will speed up the understating of molecular basis involved into muscle development and muscle pathology. This knowledge will set the basis for the quicker development of new therapeutic compounds and approaches in muscle disease.

1.5 Concluding Remarks

Together with the use of emerging techniques as TALEN and CRISPR, nowadays hPSCs have become an unprecedented platform for the development of functional screens targeting specific genes related to disease gestation and progression, thus opening new venues in cell replacement therapies. Such advances, linked to the latest advances in the field of hPSC differentiation, have led to the generation of in vitro human disease models with a potential impact in drug discovery. Concerning skeletal muscle-related pathologies, primary myoblasts directly obtained from postnatal muscle tissues still represent an accessible cell source in the clinics compared with hPSC-derived myocytes; however, in some cases the possibility to obtain patient myoblasts remains a challenge due to the specific pathology or patient intrinsic characteristics as aging. In this regard, common efforts in the differentiation of human skeletal myogenic cells from hPSCs will soon provide clinical grade protocols ensuring the safety and efficacy of the generated cell products increasing our understanding in the definition of novel culture conditions for the expansion of undifferentiated primary myoblasts from patients. We believe that latest advances in the development of microfluidic systems will benefit the proper maturation of skeletal myogenic cells from hPSCs, allowing for the study of skeletal muscle interactions with other cell types as motor neurons or immune cells, also providing physiological environments mimicking skeletal muscle niche and disease. Next years are going to be determinant for the development of such platforms paving the way to the generation of novel treatments for MDs.

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Chapter 2
Role of the Ubiquitin-Proteasome Pathway in Skeletal Muscle

Yasuo Kitajima and Naoki Suzuki

Abstract Skeletal muscle is a plastic organ that is maintained by multiple pathways regulating cell and protein turnover. The ubiquitin-proteasome system (UPS) is now recognized as a major intracellular degradation system, and its proper function is critical to health and muscle homeostasis. Alterations in muscle proteasomes have been linked to several pathological phenotypes. Indeed, excessive or defective activity of UPS leads to detrimental effects on muscle homeostasis. Emerging evidence suggests that UPS can specifically target proteins that govern pathological signaling pathways for degradation, thus altering downstream effectors and disease outcomes. An increasing number of studies link abnormalities in the regulation of UPS to myofiber degeneration and muscle weakness. Therefore, an understanding of the pathogenic role of the proteostatic system in each inherited muscle disorder

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may provide novel therapeutic targets to counteract muscle wasting. In this section, we focus on the function of the proteasome system with respect to several diseases with altered proteostasis.

Keywords Muscle homeostasis • Proteasome • Sporadic inclusion body myositis • Aging • Muscle disease

2.1 Introduction

Skeletal muscle mass represents approximately 40% of human body weight, making it the largest tissue mass in the body. Muscle homeostasis is essential to the body's integrity and maintenance, and the muscle impairment associated with several associated diseases leads to a poor quality of life. Maintaining proteolysis is a critical factor in preventing cellular dysfunction and propagation of many disease states. Proteolysis requires both protein synthesis and degradation, although the latter is more often associated with pathological states than with normal cellular functioning.

Perhaps the most well-known cellular proteolytic system is the ubiquitin-proteasome system (UPS), which is responsible for degrading the majority of cellular proteins [1]. This is a system whereby proteins meant for destruction are enzymatically tagged with the polypeptide ubiquitin via E3 ubiquitin ligases. These tagged proteins are recognized by the 26S proteasome, which comprises a central barrel-shaped 20S core particle associated with two 19S regulatory subunits [2, 3]. The latter subunits recognize and bind the ubiquitinated proteins and begin their adenosine triphosphate (ATP)-dependent destruction within the catalytic core [2]. In the skeletal muscle, protein degradation via the proteasome is often associated with muscle atrophy. A number of recent studies have highlighted the importance of proteolysis by the UPS in the pathogenesis of muscle wasting in different inherited muscle disorders. The UPS has an important role in the protein quality control. Protein degradation by the UPS needs to be carefully regulated in muscles. Indeed, excessive or defective activity of the UPS leads to detrimental effects on muscle homeostasis [4]. A growing number of studies link abnormalities in the regulation of these two pathways to myofiber degeneration and muscle weakness. Therefore, an understanding of the pathogenic role of these proteostatic systems in each inherited muscle disorder may provide novel therapeutic targets to counteract muscle wasting. In this section, we focus on the function of the proteasome system with respect to several diseases with altered proteostasis.

2.1.1 Structure of the 26S Proteasome

The rapid degradation of ubiquitinated proteins is catalyzed by the 26S proteasome. This structure is found in the nucleus and cytosol of all cells and constitutes

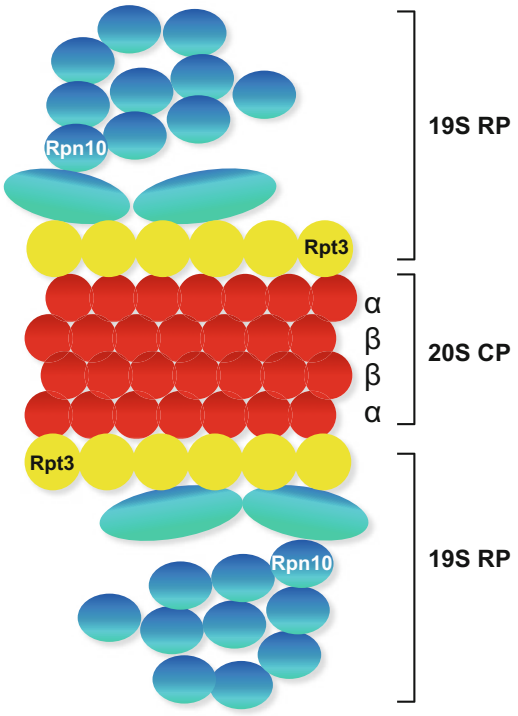

Fig. 2.1 Schematic diagram of the 26S proteasome. The 26S proteasome consists of the 20S core particle (CP) and the 19S regulatory particle (RP). The CP is made up of four heptameric rings: two *outer* identical α-rings and two identical *inner* β-rings. Each ring is made of seven distinct homologous subunits. Certain β-subunits contain the protease active sites facing inward into the sequestered proteolytic chamber. The 19S RP is divided into two subcomplexes: the *lid* and the *base*. The subunits forming the *lid* and the *base* are shown. Rpn10 can interact with either the *lid* or the *base* and stabilizes the interaction between the two

approximately 1–2% of cell mass [2]. The 26S particle comprises approximately 60 subunits (Fig. 2.1). Therefore, it is approximately 50–100 times larger than and differs in critical ways from the typical proteases that function in the extracellular environment. The most fundamental difference is that it is a proteolytic machine in which protein degradation is associated with ATP hydrolysis. The 26S complex comprises a central barrel-shaped 20S proteasome with a 19S regulatory particle at either one or both ends [2, 3]. The 20S proteasome is a hollow cylinder that contains the mechanisms for protein digestion. It is composed of four stacked, hollow rings, each containing seven distinct but related subunits [2]. The two outer α-rings are identical and so are the two inner β-rings. Three of the subunits in the β-rings contain the proteolytic active sites that are positioned on the interior face of the cylinder. The outer α subunits of the 20S particle surround a narrow, central, and gated pore through which substrates enter and products exit [5]. Substrate entry is a complex process that is catalyzed by the 19S particle. This complex architecture evolved to isolate proteolysis within a nano-sized compartment and prevents the nonspecific destruction of cell proteins. One can view protein ubiquitination and the functioning of the 19S particle as mechanisms that maintain proteolysis as a highly selective process; only certain molecules are degraded within the 20S proteasome [6]. The 19S regulatory particles on the ends of the 20S proteasome are composed of at least 18 subunits [2, 3]. Its base contains six homologous ATPases in a ring and adjoins

the outer ring of the 20S particle. These ATPases bind the proteins to be degraded and use ATP hydrolysis to unfold and translocate the protein into the 20S particle [7]. The outer lid of the 19S particle contains subunits that bind the polyubiquitin chains plus two deubiquitinating enzymes that disassemble the ubiquitin chain such that the ubiquitin can be reused in the degradation of other proteins [3]. There is growing evidence that additional factors associate with the 19S particle and actually help to deliver ubiquitinated proteins into the particle [8].

2.1.2 The Ubiquitin-Proteasome System

In the ubiquitin-proteasome system (UPS), proteins are targeted for degradation by the 26S proteasome through covalent attachment of a chain of ubiquitin molecules. The ubiquitin ligase enzyme, or E3, binds the protein substrate and catalyzes the movement of the ubiquitin from the E2 enzyme to the substrate. This is the rate-limiting step of the ubiquitination process, which affects the subsequent proteasome-dependent degradation. Once the protein is ubiquitinated, it is docked to the proteasome for degradation, unless the polyubiquitin chain is removed by the deubiquitinating enzymes. Among the different E3s, only a few have been found to regulate the atrophic process and to be transcriptionally induced in atrophying muscle.

The regulation of skeletal muscle mass largely depends on protein synthesis and degradation processes. Two muscle-specific E3 ubiquitin ligases, muscle RING finger 1 (MuRF1) and muscle atrophy F-box (atrogin-1/MAFbx), are believed to be key regulators of proteasomal proteolysis in skeletal muscle, particularly under atrophy-inducing conditions [9–11]. These two E3s are specifically expressed in muscle atrophy [12, 13]. Atrogin-1/MAFbx and MuRF1 knockout mice are resistant to muscle atrophy induced by denervation [12]. Also, knockdown of atrogin-1 spares muscle mass in fasted animals [14]. Thus far, MuRF1 ubiquitinates several muscle structural proteins, including troponin I [15], myosin heavy chains [16], actin [17], myosin-binding protein C, and myosin light chains 1 and 2 [18], whereas the substrates of atrogin-1 that have been identified appear to be involved in growth-related processes or survival pathways. Atrogin-1 promotes degradation of MyoD, a key muscle transcription factor, and of eIF3-f, an important activator of protein synthesis [19, 20].

Another E3 ubiquitin ligase found to play a critical role in atrophy is TNF receptor-associated factor 6 (TRAF6) [21], which mediates the conjugation of Lys63-linked polyubiquitin chains to target proteins. Lys48-linked polyubiquitin chains are a signal for proteasome-dependent degradation, but Lys63-linked polyubiquitin chains play other roles, such as regulating autophagy-dependent cargo recognition by interacting with the scaffold protein p62 (also known as SQSTM1) [22–24]. Notably, muscle-specific TRAF6 knockout mice have a decreased amount of polyubiquitinated proteins, with almost no Lys63-polyubiquitinated proteins in

starved muscles [25], and are resistant to muscle loss induced by denervation, cancer, or starvation [21, 25, 26]. The mechanism of this protection involves both direct and indirect effects of TRAF6 on protein breakdown. Inhibition of TRAF6 reduces the induction of atrogin-1 and MuRF1, thereby preserving muscle mass under catabolic conditions. Moreover, TRAF6-mediated ubiquitination may have an additional function on modulating intracellular signaling. In fact, TRAF6 is required for the optimal activation of c-Jun N-terminal kinase (JNK), AMP-activated protein kinase (AMPK), and forkhead box O3 transcription factor (FoxO3) pathways [25]. The effects on FoxO3 and nuclear factor κB (NF-κB) may explain why atrogin-1 and MuRF1 are less induced in TRFAF6 knockout mice.

In skeletal muscle, E3 ligases have important regulatory functions in signaling pathways. For example, it was recently found that anabolic signals were regulated by the ubiquitin ligase F-box protein 40 (Fbxo40) [27]. Fbxo40 ubiquitinates and affects the degradation of insulin receptor substrate 1 (IRS1), a downstream effector of insulin receptor-mediated signaling. Inhibition of Fbxo40 by RNA interference induces hypertrophy in myotubes, and Fbxo40 knockout mice display bigger muscle fibers [27].

2.1.3 Muscle-Specific Proteasome Dysfunction Mice

Proteasomal degradation is mediated by an ATP-dependent protease complex, the 26S proteasome, which is present in both the cytoplasm and nucleus. The 26S proteasome consists of the 20S proteasome and the 19S regulatory particles. The 19S regulatory particles unfold ubiquitin-conjugated proteins to enable their entry into the 20S proteasome. The 19S regulatory particles contain several putative ATPases, such as PSMC1–PSMC6 (proteasome 26S subunit, ATPase, 1–6). These subunits form a large family with a highly conserved ATPase domain [28]. PSMC4, also known as Rpt3 (proteasomal regulatory particle AAA-ATPase-3; Fig. 2.1), is an essential subunit of the 26S proteasome and is required for the degradation of most proteasomal substrates. In particular, Rpt3-deficient mice die before implantation because of a defect in blastocyst development [28]. Interestingly, an insertion/deletion variant in intron 5 of the Rpt3 gene was frequently found in a cohort of patients with Parkinson's disease [29]. Recently, Tashiro et al. reported that the conditional knockout of the proteasome subunit Rpt3 in motor neurons caused locomotor dysfunction that was accompanied by progressive motor neuron loss and gliosis in mice [30].

We also generated conditional Rpt3-knockout mice to specifically block proteasomal activity in skeletal muscle to clarify the role of the proteasomal system in skeletal muscle tissue (Fig. 2.2). Accordingly, we reported that muscle-specific deletion of a crucial proteasomal gene, Rpt3, resulted in profound muscle growth defects and a decrease in force production in mice with the accumulation of abnormal proteins [31]. In contrast to previous studies using proteasome inhibitors

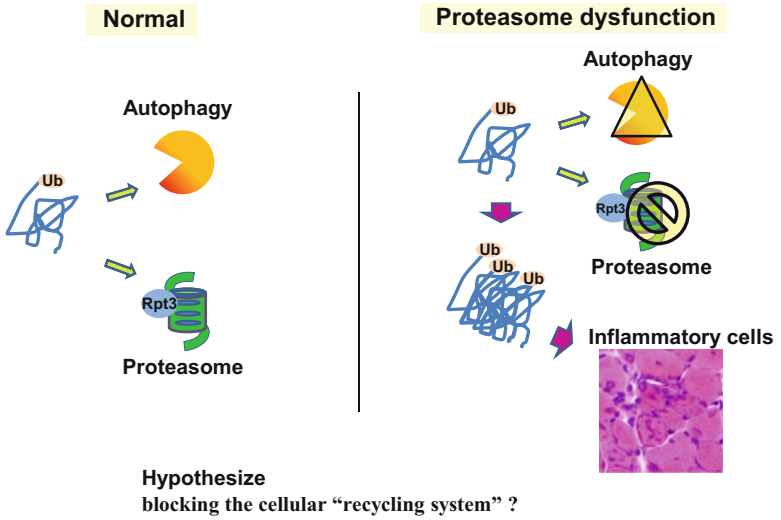

Fig. 2.2 A schematic of the pathophysiology of muscle-specific proteasome dysfunction mice. Usually, the ubiquitination of misfolded proteins are degraded by both the UPS and autophagy. In Rpt3 deficiency mice, the UPS is impaired, and the autophagy system is upregulated. The dysfunction of the UPS leads to the accumulation of ubiquitinated proteins. Finally, basophilic inclusions and inflammatory cells are found in the myofibers. Our results suggest that appropriate proteasomal activity is important for muscle growth and for maintaining myofiber integrity in collaboration with autophagy pathways [31]. The protein and organelle degradation by both UPS and autophagy may provide resources, such as oligopeptides and amino acids, for maintaining cellular integrity of the skeletal muscle tissue [33]. Therefore, the proteasome dysfunction mice may have resulted in a far more serious condition that deprives the cells of two major pathways responsible for pooling resources for cellular maintenance. We hypothesize that the skeletal muscle proteasome dysfunction may have led to blocking the cellular "recycling system" that is essential to the maintenance of skeletal muscle fibers, and this question needs to be further examined

on dystrophic or myostatin-defective mice, in which muscle hypertrophy was demonstrated [32], the specific deletion of the proteasome component Rpt3 resulted in a significant loss of muscle mass with premature death and significantly reduced physical activity. Furthermore, our study suggests that both the UPS and autophagy systems are required to maintain myocellular homeostasis and integrity. The protein and organelle degradation by both autophagy and UPS may provide resources, such as oligopeptides and amino acids, for maintaining cellular integrity in skeletal muscle tissue [33]. Therefore, Rpt3 deficiency may result in a far more serious condition that deprives the cells of two major pathways responsible for pooling resources for cellular maintenance. We hypothesize that the skeletal muscle Rpt3 deficiency may have resulted in blocking the cellular "recycling system" that is essential for maintaining skeletal muscle fibers; this question needs to be further examined.

2.2 Muscle Disease and the Proteasome System

2.2.1 Sporadic Inclusion Body Myositis

Sporadic inclusion body myositis (sIBM) is the most common form of inflammatory myopathy in those older than 50 years of age in Western countries [34–36]. Muscle weakness and atrophy in the quadriceps, wrist flexors, and finger flexors are the typical neurological findings of sIBM. The muscle biopsy typically reveals endomysial inflammation, the invasion of mononuclear cells into non-necrotic fibers, and rimmed vacuoles; these findings suggest that inflammation and degeneration are co-existent in the pathological mechanisms. An abnormal accumulation of intracellular multiprotein inclusions is a characteristic feature of the sIBM phenotype, and, as such, sIBM is considered to be caused by protein unfolding/misfolding combined with the formation of inclusion bodies [35]. The 26S proteasome system is involved in the elimination of various proteins, including ubiquitinated misfolded/unfolded proteins, and its inhibition results in the cellular accumulation of protein aggregates. Ubiquitinated protein aggregates in muscle fibers are characteristic of sIBM muscle fibers.

Two major types of aggregates exist, containing either amyloid beta (Abeta) or phosphorylated tau (ptau). Based on these findings, using cultured human muscle fibers, researchers examined the effect of Abeta precursor protein (AbetaPP) overexpression on proteasome function and the influence of proteasome inhibition on aggresome formation [37]. They reported that in sIBM muscle biopsies, the 26S proteasome subunits were immune detected in the gamma tubulin-associated aggresomes, which also contained Abeta, ptau, ubiquitin, and heat shock protein 70 (HSP70). Additionally, proteasome subunit expression was greatly increased, 20S proteasome subunit was co-immunoprecipitated with AbetaPP/Abeta, and three major proteasomal proteolytic activities were reduced. In the cultured muscle fibers, those that overexpressed AbetaPP displayed diminished proteasomal proteolytic activities, and the addition of a proteasome inhibitor strikingly increased aggresome formation. Accordingly, proteasome dysfunction in sIBM muscle fibers may play a role in the accumulation of misfolded, potentially cytotoxic proteins and may be induced by increased intracellular AbetaPP/Abeta levels. Abnormal intracellular accumulation of unfolded proteins may lead to their aggregation and the formation of inclusion bodies [38].

2.2.2 Cachexic State

Sarcopenia and cachexia are muscle wasting syndromes associated with aging and with many chronic diseases, such as congestive heart failure (CHF), diabetes, cancer, chronic obstructive pulmonary disease, and chronic kidney disease (CKD)

[39]. While their mechanisms are complex, these conditions are often accompanied by elevated angiotensin II (Ang II) levels. Patients with advanced CHF or CKD often have increased Ang II levels and cachexia, and angiotensin-converting enzyme inhibitor treatment prevents weight loss [39]. Ang II-induced muscle wasting is caused by alterations in insulin-like growth factor 1 (IGF1) signaling and by enhanced muscle protein breakdown via the UPS. In another study, researchers examined diaphragm muscle biopsies from 22 critically ill patients who had received mechanical ventilation before surgery and compared these with biopsies that were obtained from patients during thoracic surgery for resection of a suspected early lung malignancy. The cross-sectional areas of both the slow and fast twitch diaphragm muscle fibers in the critically ill patients were approximately 25% smaller, and this reduced their contractile force by one-half or more. Markers of the UPS were significantly upregulated in the diaphragm of the critically ill patients. These findings showed that the diaphragm muscle fibers of the critically ill patients displayed atrophy and severe contractile weakness and that the UPS was activated in the diaphragms of critically ill patients [40].

2.2.3 Mutants in the Protein Degradation Systems

Protein aggregate myopathies (PAMs) comprise of muscle disorders characterized by protein accumulation in muscle fibers. Clinicians diagnose new cases of PAM in patients with proximal muscle weakness and hypertrophic cardiomyopathy; these muscle fibers contain inclusions containing myosin and myosin-associated proteins and have an aberrant distribution of microtubules [41]. These lesions can appear as intact A and M bands that lack thin filaments and Z discs. These features are similar to those in mice that are deficient for MuRF1 and MuRF3. Sanger sequencing excluded mutations in the myosin storage myopathy (MSM)-associated gene for β-cardiac myosin heavy chain (MYH7), but identified mutations in tripartite motif-containing 63 (TRIM63) and TRIM54, which encoded MuRF1 and MuRF3, respectively. Both MuRFs are microtubule-associated proteins that are localized to the sarcomeric M bands and Z discs. They are E3 ubiquitin ligases that play a role in the degradation of sarcomeric proteins, the stabilization of microtubules, and myogenesis. A lack of ubiquitin and the 20S proteasome subunit in the inclusions found in the patient suggested impaired turnover of the thick filament proteins. The disruption of microtubules in cultured myotubes was rescued by the transient expression of wild-type MuRF1. The unique features of this novel myopathy point to defects in the homeostasis of A-band proteins in combination with the instability of microtubules as the cause of this disease.

Mutations in the non-lysosomal cysteine protease, calpain 3, cause limb-girdle muscular dystrophy type 2A (LGMD2A) [42]. Calpain 3 may mediate remodeling by the cleavage and release of myofibrillar proteins, targeting them for ubiquitination and proteasomal degradation [43]. The rate of atrophy and particularly the rate of growth are decreased in calpain 3 knockout mouse muscles under conditions

that promote sarcomere remodeling by hind limb unloading. In wild-type mice, an elevated level of ubiquitinated proteins was observed during muscle reloading, which was presumably necessary to remove atrophy-specific and damaged proteins [44]. This increase in ubiquitination correlated with an increase in calpain 3 expression. Calpain 3 knockout mouse muscles did not show any increase in ubiquitination at the reloading stage, suggesting that calpain 3 was necessary for ubiquitination and that it acted upstream of the ubiquitination machinery. These studies suggested that the accumulation of aged and damaged proteins can lead to cellular toxicity and a cell stress response in calpain 3 knockout muscles and that these characteristics were pathological features of LGMD2A.

2.2.4 Proteasomal Abnormality in Other Mutants

Mutations in the phosphoinositide phosphatase myotubularin (MTM1) result in X-linked myotubular/centronuclear myopathy (XLMTM), which is characterized by a severe decrease in muscle mass and strength in both patients and murine models [45]. Upregulation of atrogenes has been observed in the presymptomatic phase of the myopathy, which supports over-activation of the UPS.

Oculopharyngeal muscular dystrophy (OPMD) is a late-onset progressive muscle disorder caused by a poly-alanine expansion mutation in the poly-A-binding protein nuclear 1 (PABPN1). UPS is the most consistently and significantly OPMD-deregulated pathway across species [46]. The manipulations of the proteasome and immunoproteasome activity can specifically affect the accumulation and aggregation of mutant PABPN1 in vitro.

Myotonic dystrophy type 1 (DM1) is a neuromuscular disease caused by the expansion of a CTG repeat in the dystrophia myotonica protein kinase (DMPK) gene and is characterized by progressive skeletal muscle weakness and wasting. Using a transgenic mouse model that carried the human DM1 region with 550 expanded CTG repeats [47], a significant increase in trypsin-like proteasome activity and Fbxo32 expression was also measured in the DM1 muscles and indicated that an atrophic process mediated by the ubiquitin-proteasome pathway may contribute to the progressive muscle wasting and weakness in DM1 mice.

2.2.5 Nakajo-Nishimura Syndrome and Myositis

The disease spectrum currently known as the proteasome-associated auto-inflammatory syndromes (PRAAS) was first described in 1939 in patients who presented with recurrent fevers beginning in infancy or early childhood, which were accompanied by nodular erythema, a pernio-like rash, and joint contractures [48]. Since then, several syndromes, such as the chronic atypical neutrophilic dermatosis with lipodystrophy and elevated (CANDLE) temperatures syndrome;

the Nakajo-Nishimura syndrome (NNS); the joint contractures, muscle atrophy, microcytic anemia, and panniculitis-induced lipodystrophy (JMP) syndrome; and the Japanese auto-inflammatory syndrome with lipodystrophy (JASL), have been used to categorize the patients that had diseases within the same spectrum, including myositis. Recently, independent studies have identified mutations in the human proteasome subunit β type 8 (PSMB8) gene, which results in a sustained inflammatory response in all syndromes [49, 50]. PSMB8 encodes a catalytic subunit of the 20S immunoproteasomes called β5i. Immunoproteasome-mediated proteolysis generates immunogenic epitopes presented by major histocompatibility complex class I molecules.

Myositis is characterized by severe muscle weakness. Stable isotope labeling with amino acids in cell culture (SILAC) has been used to identify alterations in the skeletal muscle proteome of myositic mice in vivo [51]. Using the SILAC technique, the authors identified significant alterations in the levels of proteins that belonged to the pathway including UPS [51]. There was a significant increase in the ubiquitination of muscle proteins as well as a specific increase in ubiquitin carboxyl-terminal hydrolase isozyme L1 (UCHL-1) in myositis, but there was no increase in the muscles affected by other dystrophies or in normal muscles.

2.2.6 Motor Neuron Disease

Amyotrophic lateral sclerosis (ALS) is a fatal neurodegenerative disorder characterized by the progressive loss of upper and motor neurons. In particular, the accumulation of ubiquitinated inclusions containing these gene products is a common feature in most familial ALS models; it is also a pathologic hallmark of sporadic ALS, indicating that the failure to eliminate detrimental proteins is linked to the pathogenesis of both types of ALS. The presence of ubiquitinated inclusions such as skein-like and round hyaline inclusions strongly suggested dysfunction in the UPS. Indeed, the continuous expression of mutant (superoxide dismutase 1) SOD1 was shown to decrease proteasome activity and that primary cultured embryonic motor neurons were vulnerable to proteasome inhibition by lactacystin [52]. Ubiquilin 2 and p62, which are disease-causing genes in ALS, are mainly related to the protein aggregation and degradation pathways; therefore, mutations of the ubiquilin2 and p62 genes can cause related disorders [53]. On the contrary, other reports have documented an increase in proteasome activity [54]. As described in the previous paragraph, conditional knockout of the proteasome subunit Rpt3 in a motor neuron-specific murine model showed locomotor dysfunction accompanied by progressive motor neuron loss and gliosis [30].

Spinal and bulbar muscular atrophy (SBMA) or Kennedy's disease is an X-linked disease associated with the expansion of the CAG triplet repeat that is present at exon 1 of the androgen receptor (AR) gene. This results in the production of a mutant AR that contains an elongated poly-glutamine tract (poly-Q) in its N-terminus. Interestingly, the AR poly-Q becomes toxic only after its activation by

natural androgenic ligands; this may be because of the aberrant androgen-induced conformational changes of the AR poly-Q, which generates the misfolded species. These misfolded AR poly-Q species must be cleared from the motor neurons and muscle cells, and this process is mediated by proteasomal proteolysis. Molecular chaperones and degradative pathways including the UPS, the autophagy-lysosome pathway, and the unfolded protein response, which activates endoplasmic reticulum-associated degradation, are differentially affected in SBMA [55].

The expansion of the GGGGCC hexanucleotide repeat in the noncoding region of the chromosome 9 open reading frame 72 (C9orf72) gene is the most common cause of frontotemporal dementia (FTD) and ALS (c9FTD/ALS). Recently, it has been reported that an unconventional mechanism of repeat-associated non-ATG (RAN) translation arose from C9orf72 expansion. Sense and antisense transcripts of the expanded C9orf72 repeat, such as the dipeptide repeat protein (DRP), were deposited in the brains of patients with c9FTD/ALS. The evaluation of cytotoxicity revealed that overexpressed poly-GA, poly-GP, and poly-GR increased the substrates in the UPS, including transactive response DNA-binding protein 43 (TDP-43), and enhanced the sensitivity to a proteasome inhibitor, indicating that these DRPs were cytotoxic, possibly via UPS dysfunction [56].

2.3 Therapeutic Strategy Using Intervention into the Proteasomal System

As reviewed above, some diseases present UPS dysfunction, while others represent excessive UPS activity. The suppression of excessive UPS activity is a promising method for some muscle diseases.

The 427 kDa protein dystrophin is expressed in striated muscle where it physically links the interior of the muscle fibers to the extracellular matrix. A range of mutations in the gene encoding dystrophin can lead to a severe muscular dystrophy known as Duchenne muscular dystrophy (DMD) or a typically milder form known as Becker muscular dystrophy. Dystrophin missense mutations, however, cause a wide range of phenotypic severities in patients. When stably expressed in mammalian muscle cells, the mutations caused steady-state decreases in dystrophin protein levels that are inversely proportional to the tertiary stability and are directly caused by proteasomal degradation. Proteasome inhibitors are able to increase the mutant dystrophin to wild-type levels, establishing the new cell lines as a platform to screen for potential therapeutics personalized for patients with destabilized dystrophin [57]. Using systemic treatment with an osmotic pump, the administration of the proteasomal inhibitor MG-132 effectively rescues the expression levels and plasma membrane localization of dystrophin in skeletal muscle fibers from dystrophin-deficient mdx mice. Proteasomal inhibitors reduced muscle membrane damage, as revealed by vital staining (with Evans blue dye) of the diaphragm and gastrocnemius muscle that was isolated from treated mdx mice; it also ameliorated the histopathological signs of muscular dystrophy, as judged by

hematoxylin and eosin staining of muscle biopsies taken from treated mdx mice [58]. Bortezomib was systemically administered in mdx mice over a 2-week period. In this system, bortezomib restored the membrane expression of dystrophin and dystrophin-glycoprotein complex members and improved the dystrophic phenotype [59]. Golden retriever muscular dystrophy (GRMD) is a genetic myopathy that corresponds to DMD in humans. Five GRMD dogs were examined; two were treated with the proteasome inhibitor bortezomib, and three were control dogs. The skeletal tissue from the treated dogs had lower levels of connective tissue deposition and inflammatory cell infiltration that in the control animals as determined by histology, collagen morphometry, and ultrastructural analysis. Significant inhibition of 20S proteasome activity was observed 1 h after bortezomib administration. Proteasome inhibitors may thus improve the appearance of GRMD muscle fibers, lessen connective tissue deposition, and reduce the infiltration of inflammatory cells. In addition, proteasome inhibitors may rescue some dystrophin-associated proteins in the muscle fiber membrane [60].

Congenital muscular dystrophy, caused by mutations in LAMA2 (the gene encoding the laminin α2 chain), is a severe and incapacitating disease for which no therapy is yet available. Proteasome activity is increased in laminin α2 chain-deficient muscle and treatment with the proteasome inhibitor MG-132 reduces the muscle pathology in laminin α2 chain-deficient dy(3 K)/dy(3 K) mice. The proteasome inhibitor bortezomib (currently used for the treatment of relapsed multiple myeloma and mantle cell lymphoma) in dy(3 K)/dy(3 K) mice and in congenital muscular dystrophy type 1A muscle cells can also be effective in the improvement of muscle pathology. Bortezomib improved several histological hallmarks of the disease, partially normalized microRNA expression (miR-1 and miR-133a), and enhanced body weight, locomotion, and survival of the dy(3 K)/dy(3 K) mice. In addition, bortezomib reduced proteasome activity in congenital muscular dystrophy type 1A myoblasts and myotubes [61]. The same group explored the use of bortezomib in dy2J/dy2J animals. However, bortezomib neither improved the histological hallmarks of disease nor increased muscle strength or locomotive activity in dy2J/dy2J mice. It is possible that proteasome inhibition could be useful as a supportive therapy in patients with a complete absence of the laminin α2 chain [62].

Dysferlin is a transmembrane protein implicated in the surface membrane repair of muscle cells. Mutations in dysferlin cause the progressive muscular dystrophies Miyoshi myopathy, LGMD2B, and distal anterior compartment myopathy. A marked reduction in dysferlin in patients harboring missense mutations implies that dysferlin is degraded by the cell's quality control machinery. One study revealed that dysferlin-deficient human myoblast cultures harbored the common R555W missense allele and a dysferlin-null allele and that control human myoblast cultures harbored either two wild-type or two null alleles. Dysferlin protein and messenger RNA levels, resealing kinetics of laser-induced plasmalemmal wounds, myotube formation, and cellular viability were measured after treatment of the human myoblast cultures with the proteasome inhibitors lactacystin or bortezomib

(Velcade). Inhibition of the proteasome increased the levels of R555W missense mutated dysferlin. This salvaged protein was functional as it restored the plasma membrane, resealing in the patient-derived myoblasts, and reversed their deficits in myotube formation. These results raised the possibility that inhibition of the degradation pathway of missense mutated dysferlin could be used as a therapeutic strategy for patients harboring certain dysferlin missense mutations [63]. In the other study, the authors described dysferlinopathy due to a homozygous Arg555Trp mutation in dysferlin in patients treated with the proteasome inhibitor bortezomib and monitored dysferlin expression in monocytes and in skeletal muscle by repeated percutaneous muscle biopsy. Expression of the missense mutated dysferlin in the skeletal muscle and monocytes of the three patients increased markedly, and dysferlin was correctly localized to the sarcolemma of the muscle fibers on histological sections. The salvaged missense mutated dysferlin was determined to be functional in a membrane resealing assay in patient-derived muscle cells treated with three different proteasome inhibitors [64].

2.4 Conclusion

Skeletal muscle is a plastic organ that is maintained by multiple pathways regulating cell and protein turnover. The UPS is now recognized as a major intracellular degradation system, and its proper function is critical to health and muscle homeostasis. Alterations in muscle proteasomes have been linked to several pathological phenotypes. Indeed, excessive or defective activity of UPS leads to detrimental effects on muscle homeostasis. Emerging evidence suggests that UPS can specifically target proteins that govern pathological signaling pathways for degradation, thus altering downstream effectors and disease outcomes. In this section, we focus on the function of the proteasome system with respect to several diseases with altered proteostasis.

Our results suggest that appropriate proteasomal activity is important for muscle growth and for maintaining myofiber integrity in collaboration with autophagy pathways [31]. The protein and organelle degradation by both UPS and autophagy may provide resources, such as oligopeptides and amino acids, for maintaining cellular integrity of the skeletal muscle tissue [33]. Therefore, the proteasome dysfunction mice may have resulted in a far more serious condition that deprives the cells of two major pathways responsible for pooling resources for cellular maintenance. We hypothesize that the skeletal muscle proteasome dysfunction may have led to blocking the cellular “recycling system” that is essential to the maintenance of skeletal muscle fibers, and this question needs to be further examined (Fig. 2.2). In addition, we examine muscle stem cell specifically proteasome dysfunction to elucidate the functional role of proteasome system in the maintenance of stem cells. In the future, muscle and stem cell studies will be valuable to find out the function role of UPS comprehensively and help to better understand the molecular mechanisms of muscle and stem cell homeostasis.

Overall, the proteasome is a highly regulated complex that requires intensive research to properly understand the roles of many components in modulating proteasome function. An understanding of the pathogenic role of UPS in each inherited muscle disorder may provide novel therapeutic targets to counteract muscle wasting.

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Conflict of Interest There are no conflicts for interest to declare.

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Chapter 3
Stem Cell Therapy in Muscle Degeneration

Robin Duelen, Domiziana Costamagna, and Maurilio Sampaolesi

Abstract Skeletal muscle is one of the largest tissues in humans, reaching around 38% of the body weight for men and 30% for women (Janssen I, et al, J Appl Physiol 89(1):81–88, 1985/2000). Muscle tissue finely regulates essential body movements, including respiration, equilibrium maintenance and ambulation. In addition, it controls body temperature and energy balance and restores blood glucose levels. Genetic or acquired alterations in these enzymes can lead to metabolic syndromes (McArdle's syndrome (Vorgerd M, Neurotherapeutics 5(4):579–582 2008) or diabetes (DeFronzo RA, Tripathy D, Diabetes Care 32(Suppl 2):S157–S163 2009). Muscle tissue is originated during embryo development, from the fusion of mesoderm progenitors. During the neonatal and early juvenile period, even if the myofibre number remains constant, new postnatal multipotent cells, called satellite cells (SCs), can fuse and increase the number of nuclei per fibre (Biressi S, et al, Dev Biol 379(2):195–207, 2013). Adult skeletal muscle is organised in long fibres generated from the fusion of single cells into a unique syncytium able to collect thousands of nuclei and of myofibrils. Each myofibril contains multiple sarcomeres, formed by actin and myosin filaments to generate contraction and develop force. High heterogeneity is present among single fibres, including slow-contracting fatigue-resisting type I fibres (also known as slow-twitch, tonic or simply slow fibres) and fast-contracting fatigue-unresisting type II fibres (fast-twitch, phasic or fast fibres). To exert a good performance, the expression of specific contractile proteins and metabolic enzymes is necessary together with a regular connection to a single motor neuron and an efficient vascularisation. Myotendinous junctions connect myofibres from one side and bones in the skeleton on the other side, allowing transmission of contractions to other muscles. Daily wear and tear

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or excessive load and damage of the fibre integrity activate SCs that undergo fusion. The highly orchestrated process has been thoroughly studied. Nevertheless, understanding the role of local and circulating cell types in adult myogenesis still remains a matter of discussion (Relaix F, Zammit PS, Development 139(16):2845–2856, 2012). In this chapter after introducing the biological events occurring in acute and chronic skeletal muscle damages, cell and gene therapy approaches are considered as novel tools to preserve muscle function.

Keywords Skeletal muscle regeneration • Chronic inflammation • Adult and pluripotent stem cells • Gene editing • Clinical trials

3.1 Introduction: Muscle Damage

Muscle damage can be local and limited, so that it is easily repaired without the activation of the immune compartment. Focal myofibre damage is a reversible process involving the plasma membrane, myofibrils and myonuclei, not supported by inflammatory response activation. This kind of damage involves subcellular structures that are rapidly repaired by fusion to the plasma membrane of subsarcolemmal vesicles [6]. For instance, eccentric contraction can induce myofibrillar alterations and leads to sarcomere remodelling. The same stressing effort induces also disruption of focal plasma membrane, with involvement of dysferlin, caveolin-1 and other proteins. *Dysferlin* gene mutations generate Limb-Girdle Muscular Dystrophies 2B (LGMD2B) in humans [7].

The lesions can become severe due to traumatic reasons, such as muscle strains in sport medicine (in case of resistance training or myotoxin exposure), or are irreversible due to genetic defects, such as multiple kinds of musculopathies or muscular dystrophies. In these cases, necrosis of the whole or segments of the myofibre takes place and activates the inflammatory response. Infiltration of activated macrophages and final fusion of SCs with a pre-existing damaged myofibre are events observed during a process called muscle regeneration. This regenerative process has been described to recapitulate the phases of embryonic myogenesis, starting with fibre necrosis, inflammatory system involvement, SC activation, proliferation and, finally, differentiation of SC-derived myoblasts [8].

3.1.1 Necrosis and Chemoattractant Release

Whenever a fracture or any other trauma activate the inflammatory system, a defined cascade of events takes place, in order to ensure the repair of damaged muscle tissue, the complete reconstruction of the muscle structure and, most important, the physiology.

After muscle fibre degeneration, fibre integrity disruption and permeability increase. All the cytosolic components are released in the circulation, together with other common enzymes (i.e. creatine kinase) [9] and microRNA

(i.e. miR-133a) [10], playing the role of chemoattractant for the innate leukocytes. Desmin, heat shock proteins, chromatin-associated proteins, High-Mobility Group Box 1 (HMGB1) and many other intracellular players are dispensed actively through non-canonic pathways. These have been demonstrated to be responsible for the immune system activation. Lately, studies in a mouse model lacking leukocyte HMGB1 in the haematopoietic compartment have revealed a new role for HMGB1 in injured muscle. This molecule responds to hypoxia controlling neoangiogenesis through angiopoietin-2 expression [11].

Experimentally, damaged fibres are visible through the uptake of small molecules, like Evans blue or Procion-orange dyes, indicators of damage after extensive exercise or sarcolemmal permeability related to genetic diseases [12]. Membrane changes and calcium (Ca^{2+}) influx increment together with sarcoplasmic reticulum Ca^{2+} release contribute to the activation of Ca^{2+}-dependent proteases, such as calpains. These proteases, starting from the degradation of big myofibrillar and cytoskeletal proteins (i.e. titin), prepare the substrates for other proteolytic systems (such as ubiquitin proteasome and caspases) that participate to the degradation in amino acids and ultimately to muscle atrophy [13].

3.1.2 Inflammatory Response Activation

An increase in the local myeloperoxidase activity is correspondent to the activation of neutrophils, the first inflammatory cells, that can modify the damaged tissue to allow phagocytosis of debris [14]. Moreover, the neutrophils are responsible for the peak of cytokine secretion till 24 h after injury, contributing to the modulation of the regenerative process [15]. Thanks to the secretion of specific cytokines, i.e. Interleukin 4 (IL-4), eosinophils regulate macrophage attraction. Beyond this chemoattractant role, IL-4 has been demonstrated to promote skeletal muscle differentiation [16].

Due to the proximity to capillaries, myogenic progenitors attract monocytes from the blood through chemotactic molecules, such as Monocyte Chemoattractant Protein-1, macrophage-derived chemokine, fractalkine and Vascular Endothelial Growth Factor (VEGF) [17]. In vivo studies have shown the activation of both circulating and resident macrophages after injury [18].

SC activation and proliferation are concomitant to M1 macrophage activation in vitro and in vivo. The M2 macrophage population is more prominent during the first phases of muscle differentiation. In coculture with SCs, they can induce better differentiation with higher levels of myogenin, a marker for complete myogenic differentiation [19]. Both resolution of inflammation and myofibre repair are driven by the transition from M1 to M2 macrophages, through dephosphorylation of the Mitogen-Activated Protein Kinase (MAPK) p38 and anti-inflammatory cytokine production [20]. A decrease in fibre growth has been reported in case of ablation of anti-inflammatory IL-10 release in vivo, impairing the transition from M1 to M2 macrophages and, more recently, disturbing skeletal muscle differentiation of other muscle precursors [21].

Besides macrophages, emerging evidences stress the role of the lymphocyte subsets, such as Th17 and regulatory T cells, able to activate neutrophils in case of prolonged muscle activity or endurance exercise [22]. These two main groups of T lymphocytes can exert distinct roles in inflammatory conditions, both regulating muscle regeneration and secreting molecules able to cross-react with macrophages and contribute to the activation of the humoral immunity. In particular, activation of cell-mediated immunity can be due to $CD4^-$ Th1 secretion of anti-fibrotic cytokines, such as IL-12, IL-2, Tumour Necrosis Factor-alpha (TNFα) and Interferon-gamma (IFNγ). On the other side, pro-fibrotic cytokines, secreted by $CD4^+$ Th2, such as IL-4, IL-5, IL-6 and IL-13, activate humoral immunity [23] and maintain the polarisation of macrophages in vivo [24].

Other soluble mediators can impact on the myogenic process influencing both macrophages and SC proliferation, thereby their differentiation. For instance, TNFα, usually present in the injured muscle microenvironment, facilitate the phagocytosis of cell debris. Nevertheless, high and prolonged doses of TNFα impair regeneration through Nuclear Factor-kappa beta (NF-κB) activation and MyoD inhibition, resulting in the maintenance of proliferative SCs, without stimulus to differentiate [25]. The same block in differentiation is also the result of TNFα stimulation inducing phosphorylation of Small Mothers Against Decapentaplegic family members 2 and 3 (SMAD2/3) and fusion inhibition [26]. In addition, M1 macrophages produce Activin-A that delays the conversion to M2 macrophages in vitro and consequently the release of anti-inflammatory cytokines, such as IL-10 [27]. Besides the effect of TNFα, IFNγ also seems to delay myoblast fusion and differentiation in vitro, causing both defects in proliferation and low expression of late markers of myotube differentiation [28].

Besides inflammatory cytokines, the skeletal muscle itself is able to secrete myokines with both a paracrine and endocrine effect [29]. With paracrine effectors, people mainly refer to cytokines, such as IL-15 and Leukaemia Inhibitory Factor (LIF) [30], while the term myokines is used to list molecules able to induce anti-inflammatory cytokines, such as IL-10 and IL-1 [31], usually with an endocrine range of action, such as myonectin, IL-6 and oncostatin M [32], irisin and calprotectin [33].

3.1.3 Skeletal Muscle Regeneration: Activation, Proliferation and Fusion of Muscle Precursors

After muscle degeneration, the process of regeneration both in physiological (extensive exercise) or pathological conditions (musculopathies) is a finely organised sequence of events, characterised at first by the proliferation of muscle precursors. At a morphological level, newly formed fibres are small centronucleated structures, actively synthetising proteins, thereby basophilic and positive for embryonic

Myosin Heavy Chain (MyHC) [34]. Architectural changes due to incomplete fusion of some of the SCs or the myotubes can be the origin of forked myofibres that are usually more present in old subjects or patients suffering from neuromuscular diseases, suggesting a possible alteration in regenerative ability [35]. Finally, increased scar tissue could avoid the continuity between the two sides of a damaged myofibre, leading to formation of new myotendinous tissue and to the decrease in elasticity of the entire tissue.

SCs are quiescent cells characterised by a subliminal position and by a G_0 phase. Pax7 expression is verified for SCs till the quiescent state is maintained. The disruption of Pax7 in SCs leads to cell cycle arrest and dysregulation of myogenic transcription factors [36].

SCs upon activation are extensively migrating to the site of injury stimulated by different cues. Once arrived, SCs start to express two of the myogenic regulatory factors, Myf5 and MyoD [37], with different function in adult myogenesis [4]. *Myf5*-null mice develop SCs with compromised proliferation and show myofibre hypertrophy [38]. Genetic ablation of MyoD reduces murine muscle mass and induces impairment of muscle regeneration with an accumulation of cells in the damaged area unable to complete the differentiation process. The importance of this gene has been as well demonstrated by ectopic expression of MyoD in NIH3T3 fibroblasts, as sufficient to complete the myogenic activation. Terminal differentiation starts when the myogenin expression takes place. Myogenin controls the fusion of myoblasts in both myotubes in vitro and myofibres in vivo [39]. At the end of the process of regeneration, the centronucleated myofibres grow in size (protein content) but also in number of myonuclei that, once pushed to the periphery of the fibre, will allow recovery of the morphological and physiological properties of the entire muscle.

3.2 Chronic Inflammation: When the Inflammatory Process Goes Too Far

Numerous papers are pointing at the effect that the immune system has on the differentiating environment in skeletal muscle during the onset of regenerating conditions. To this extent, physical exercise, and, in particular, long-term training, has been demonstrated to educate the immune system to survey the host microenvironment against muscle micro-injuries [40]. Eventually, this activation has a role against potential malignancies and acquired pathologic conditions. Indeed, whenever the inflammation acquires a chronic aspect, it can compromise wound healing and cause fibrosis, with the possibility to induce muscle wasting. In these cases, inflammatory cells acquire a deleterious effect with consequences on the myeloid population and on the lymphoid compartment of the immune system [41].

3.2.1 Immune Cell Involvement in Skeletal Muscle Injuries

To discriminate the role of different cells in fibrosis and other molecular hallmarks, the development of different mouse models has been essential to display deficiencies in the immune system associated to the absence in dystrophin expression (*mdx* mice, representing characteristics of Duchenne Muscular Dystrophy, DMD). *Scid/mdx* mice deficient in functional lymphocytes have shown a decrease in Transforming Growth Factor beta (TGFβ) levels and fibrotic tissue in the diaphragm, allowing the study of T cells in the development of fibrosis [42]. Same results were obtained for osteopontin-deficient *mdx* mice, in which a decrease in Natural Killer (NK) cells and neutrophils has been associated to reduced infiltration and fibrosis [43]. Whether this is a peculiarity of DMD pathologic condition or the normal terminal point reached when the immune system pushes peripheral tolerance to breakdown is still a matter of discussion. The involvement of myeloid cells in the *mdx* mouse model has been demonstrated reporting macrophage activation. Moreover, since the diaphragm of *mdx* mice exhibits high levels of C-C Chemokine Receptor type 2 (CCR2) and its ligand, together with inflammatory monocytes ($Ly6C^{high}$) and accumulation of $CD11b^{high}$ monocyte-derived macrophages, a new role for monocytes has been proposed. Indeed, CCR2 loss of function reduces the number of monocytes recruited from the bone marrow and restores macrophage polarisation preventing a pro-inflammatory phenotype. Similar results have been obtained when a mutated isoform of the ligand is released by implanted Mesenchymal Stem Cells (MSCs), showing a better histopathological condition for the diaphragm and increased skeletal muscle force [44].

Modulation of the inflammatory response, minimal off-targets and survival improvement have been obtained in case of injections of myeloid cells ($F4/80^{+}$ mature macrophages) together with GFP^{+} SCs, in situ, in *mdx* or injured muscles. Conflicting results on which type of macrophages could be more suitable for transplantation make the need of further studies more and more necessary. Several studies have identified an involvement for mast cells in normal skeletal muscle repair. Of interest for this section, mast cells have been shown in the proximity of vessels in *mdx* mice and in human clinical trials, some stabilisers of these cells (oxatomide and cromolyn) have been tested for the possibility to improve muscle repair [45, 46].

3.2.2 Fibrotic Component in Skeletal Muscle Regeneration

If the injury is not solved and physiologic conditions are not restored, fibrinogen or fibrin fragments accumulate and induce the production of connective tissue, exacerbating chronic dystrophic failure. It has been described that *mdx* mice in a *DBA/2* background (*D2-mdx*) exhibit loss of skeletal muscle weight, weakness, fibrosis and fat accumulation and, after multiple injuries, poor regeneration ability. Differently, when fibrinolytic treatment in skeletal muscle avoids fibrin and myeloid

cell accumulation, in particular M2 macrophages, muscle tissue permeability is reduced in *mdx* mice. Activated M2 macrophages express high levels of TGFβ and arginase 1, both important molecules able to drive fibrosis and impair muscle regeneration. Moreover, inducing M2 macrophages to express arginase 1 at progressive stages of the disease, in the skeletal muscle and heart, worsens fibrosis and contributes to lethality, while its ablation decreases the fibrotic process. The role of fibrinogen is to drive inflammation signalling through Mac-1 ($\alpha_M\beta_2$-integrin) on the surface of myeloid cells inducing the expression of pro-inflammatory cytokines and chemokines [47]. As a matter of fact, lower levels of fibrosis in the diaphragm, decreased infiltration and low levels of pro-inflammatory cytokines were reported in mutated Mac-1 mice [48].

3.2.3 Immunomodulatory Drugs

Since fibrosis development could negatively interfere with both gene and cell therapies, reducing the integrity of the tissue for cell safe engraftment, many strategies are focusing on the treatment of fibrotic cells and molecules [49]. In particular, halofuginone (also known as HT-100), a drug promoting regeneration with decreased degree of inflammation, was reported to improve histopathology in dysferlinopathies. Interestingly, the same molecule was described as specific inhibitor of Th17 cell differentiation, suggesting an immunomodulatory effect in dystrophic muscles [50]. A phase I–II clinical trial has been started on the safety and tolerability of this drug (NCT01847573). Thereby, immunomodulator administration in DMD patients has positive effects, reducing M1 macrophages and pro-inflammatory cues [51, 52]. On this line, cyclosporin-A (CyA) treatment has been demonstrated to be beneficial when administered to *collagen VI α1*-null mice, both in restoring the mitochondrial compartment and recovering muscle strength. CyA increased the SC pool and stimulated the differentiation into myofibres [53]. Furthermore, immunosuppressive drugs have also shown to induce myogenesis in myopathic patients, recognising the link between immune system and muscle regeneration, not strictly reduced to Muscular Dystrophies (MDs) but extended to different myopathies. Patients with Ullrich Congenital Muscular Dystrophy (UCMD) have received a treatment of CyA able to correct mitochondrial dysfunction, increase muscle regeneration and decrease the number of apoptotic nuclei. Long-term treatment with CyA has been confirmed to ameliorate performance in the limbs [54].

3.2.4 Detrimental Effects of Cytokines During Chronic Inflammation

SC proliferation is repressed in case of IFNγ secretion and M1 subtype activation [19] with increase in degradation of damaged dystrophin [55]. In case of high

concentration of IL-4, IL-10 and corticosteroid, M2 macrophages are activated and produce TGFβ, accumulating in fibrotic areas. M2 macrophages have been found in the fibrotic areas of *mdx* mice diaphragm [47]. TGFβ has been related to inhibition of SC proliferation, differentiation and degeneration into fibroblast-like cells able to produce collagen and increased reactive O_2 species in *mdx* mice. TGFβ or TNFα and HMGB1 inflammatory cytokines can all mediate the activation of p38. In particular, α and β isoforms, once activated, promote MyoD heterodimerisation [56] and Mef2 transcriptional activation [57], while γ isoform in SCs inhibits MyoD transcriptional activity by direct phosphorylation [58]. In contrast, p38α represses SC determination, avoiding the exit from cell cycle [59]. On this line, other inflammatory cytokines, like IL-6 and LIF, through Janus Kinase (JAK) activation and Signal Transducer and Activator of Transcription 3 (STAT3) phosphorylation, repress *MyoD* target gene favouring the expansion of $Pax7^+$ cells [60].

3.2.5 Chronic Inflammation in Muscle Degenerative Processes

An inflammatory environment is not only the result of continuous damage and regeneration, happening in case of myopathies and MDs, but can be as well the final step of many other acquired syndromes (i.e. cancer cachexia). On this line, the inflammatory cues usually established during cancer are responsible for proteolytic system hyperactivation in myofibres and increased proliferation in skeletal muscle precursors. It has been reported that SCs and other precursors, both in mice and human samples, are able to proliferate but do not achieve an adequate muscle differentiation [61, 62]. New insights to limit such an increment or to unravel the stimuli that induce muscle precursors differentiation could be a useful strategy to counteract muscle atrophy. In this perspective, the activation of the transcription factor NF-κB following pro-inflammatory cytokines sustains Pax7 expression and inhibits MyoD activation [62]. Moreover, mesenchymal progenitors expressing Platelet-Derived Growth Factor Receptor-alpha (PDGFRα) and Stem cell antigen-1 (Sca-1) are unable to complete their support in myogenic differentiation. Analogously, proliferation of these cells seems dependent on IL-4/IL-13 signalling and able to inhibit adipocyte differentiation [63]. In old animals, this population is unable to activate any myogenic differentiation, showing a different behaviour depending on their environment [64]. Transplantation of young mesenchymal progenitors into an old dystrophic environment has shown their plasticity, opening the possibility for novel applications in clinical trial [65].

Recently, in old animals, SC number might not critically influence muscle atrophy, although it could delay skeletal muscle regeneration and induce fibrosis [66]. Moreover, the possibility for SCs to switch from a quiescent to a senescent state in geriatric conditions has already been addressed [67]. A fine regulation of STAT3 in mouse and human SCs is important for the progression of differentiation [68]. Chronic degenerative stimuli could favour this pro-differentiative pathway leading to SC pool exhaustion in condition of sarcopenia, the natural process

of muscle mass and function decline with age. A pharmacologic inhibition of STAT3 could have a therapeutic relevance in chronic regenerative cases. As already proposed in the past, SCs during elderly could easily convert into fibrogenic fate mainly due to humoral factors that activate Wnt signalling pathway [69]. Finally, also in old patients, a regular and not extensive exercise could educate the immune system in order to repress pro-inflammatory monocytes and increase regulatory T cells [40]. Indeed, exercise remains the most physiological stimulus that can regulate both myogenesis and metabolic pathways able to counteract muscle wasting and educate the inflammatory system [70].

3.3 Stem Cell Therapies in Degenerating Muscles

Several stem cell types have been described in the skeletal muscle tissue, mainly located in the interstitium. The majority of these cells seem to be directly involved in muscle regeneration, although some of these exert supportive effects on muscle fibres. The ideal cell candidate contributing to muscle regeneration (Table 3.1) should respect the following criteria: (1) to exist in humans, (2) to have a myogenic differentiation potential, (3) to exert migration ability, (4) to express homing abilities into areas of muscle degeneration, (5) to be easily accessible and expand under in vitro conditions and (6) to be genetically editable and suitable in autologous context.

3.3.1 Muscle-Resident Stem Cells

3.3.1.1 Satellite Cells (SCs)

SCs, first identified in 1961 by Katz and Mauro in the muscles of frog and rat [71], represent the adult stem cell population of skeletal muscles. SCs are mononucleated cells located between the basal lamina and sarcolemma of the skeletal muscle fibres. In mature healthy muscles, SCs are in a quiescent unipotent state and enter the cell cycle as multipotent precursor cells (MPCs) to contribute to the formation of new muscle fibres in response to muscle injury, disease or during muscle physical activity (exercise or stretching) [72]. After activation, SCs proliferate extensively generating myoblasts that fuse rapidly to form multinucleated myofibres. When SCs are ablated, no other cell types can start skeletal muscle regeneration, indicating the need of resident SCs to recruit or guide any other possible contributor to muscle differentiation [73]. The localisation in specific anatomical structures and the exposure to the skeletal muscle niche signalling direct SC self-renewal. SCs undergo both symmetrical and asymmetrical cell division, mainly depending on the location of the daughter cells with respect to the myofibre. A small proportion of SCs do not undergo terminal differentiation, but repopulate the reserve pool of quiescent cells

Table 3.1 Adult stem cells involved in adult myogenesis

Cell type	Source	Anatomic localisation	Molecular markers	Myogenic differentiation	Model	Systemic delivery	References
SCs (myoblasts)	Skeletal muscle	Between basal lamina and sarcolemma of muscle fibres	Pax7, MyoD, M-cadherin, CD56, desmin	Spontaneous	*nude* mice, *scid(/mdx)* mice, *scid/beige* mice, *nod/scid* mice, *Rag2*$^{-/-}$(*γC*$^{-/-}$*C5*$^{-/-}$*/mdx)* mice, *Rag2*$^{-/-}$*il2rb*$^{-/-}$*(/mdx)* mice and GRMD dogs	No	[71, 72, 74]
Pericytes	All body tissues	Perivascular, surrounding capillaries and microvessels in all tissues	ALP, CD140b, desmin, NG2, αSMA	Spontaneous	*scid* mice, *scid/beige* mice, *nod/scid* mice, *Rag2*$^{-/-}$(*γC*$^{-/-}$*C5*$^{-/-}$*)* mice	Yes	[88, 89]
MABs	Skeletal muscle and heart	Perivascular	ALP, CD140b, Sca-1, NG2	Embryonic: spontaneous; Adult: induced by C2C12 coculture	scid(/mdx) mice, scid/beige mice, Rag2$^{-/-}$(γC$^{-/-}$C5$^{-/-}$) mice and GRMD dogs	Yes	[94, 96]
MyoECs	Skeletal muscle	Interstitium	CD34, CD56, CD144	Spontaneous	*scid* mice	Not tested	[79]
MDSCs ("MuStem cells")	Skeletal muscle	Endomysium	Sca-1, vimentin, desmin	Spontaneous	*scid(/mdx)* mice and GRMD dogs	Not tested	[108, 110]
PICs	Skeletal muscle	Interstitium	PW1, CD34, Sca-1	Spontaneous	*mdx* mice and *scid/mdx* mice	Yes	[112, 113]
SP cells	Skeletal muscle	Interstitium	Abcg2 transporter	Induced by C2C12 coculture	*mdx* mice	Yes	[114]

FAPs	Skeletal muscle	Interstitium	CD34, CD140a, Sca-1	No myogenic differentiation, paracrine effect	*mdx* mice	Not tested	[118, 119]
MSCs	All body tissues	Bone marrow	CD73, CD90, CD105	Sporadic myogenic differentiation	*nude* mice, *mdx* mice and $Rag2^{-/-}(\gamma C^{-/-}C5^{-/-})$ mice	Yes	[120, 121]
$CD133^{+}$ cells	Peripheral blood	Peripheral bloodstream	CD133, CD34, CD90, CD44, CD45, LFA-1, PSGL-1,VLA-1, L.-selectin, CXCR4	Induced by C2C12 coculture	*scid(/mdx)* mice and $Rag2^{-/-}(\gamma C^{-/-}C5^{-/-})$ mice	Yes	[129]
$CD133^{+}$ cells	Skeletal muscle	Interstitium	CD133, CD34, CXCR4	Spontaneous, but limited	*scid(/mdx)* mice and $Rag2^{-/-}(\gamma C^{-/-}C5^{-/-})$ mice	Yes	[129]

to mediate further rounds of muscle regeneration. The identification in different cell states (quiescence, activation or proliferation) for both murine and human SCs is based on the expression of numerous molecular markers [74].

In mice, paired box transcription factor Pax7 [75], adhesion molecule M-cadherin, sialomucin surface receptor CD34, integrin α7β1, transmembrane Heparan sulphate proteoglycan syndecans 3 and 4 (Hspg3 and Hspg4), C-X-C chemokine Receptor type 4 (CXCR4) [76] and cytoskeleton protein desmin [77] are used as markers. In human CD56 (also known as Neural Cell Adhesion Molecule, NCAM) [78], although not SC specific, and the nuclear transcription factor Pax7 [75] are usually combined with the nuclear laminin A/C to identify SCs in their in vivo niche [78].

In the human adult skeletal muscle, a subpopulation of SCs that co-express vascular system markers has been identified as myoendothelial cells (MyoECs). These cells can be expanded for long-term in vitro and support muscle regeneration superior to myoblasts. MyoECs, located between the muscle fibres, represent approximately 0.4% of the muscle-resident stem cells, sharing myogenic ($CD56^+$, $CD34^-$, $CD144^+$) and endothelial features ($CD56^-$, $CD34^+$, $CD144^+$) [79]. The strong myogenic commitment and feasible isolation and expansion of SC-derived myoblasts in vitro pointed out these cells as the first candidates for stem cell therapy in muscular diseases. However, pioneering studies in mice models showed poor survival and migration abilities for transplanted SCs [80].

Preclinical and Clinical Studies Using SCs

SC-derived myoblasts were the first cell type used for cellular therapy, seen their rapid exhaustion in DMD patients [81]. In 1989, the group of T. Partridge demonstrated that intramuscular injected myoblasts derived from neonatal mice could restore dystrophin expression in *mdx* mice [82]. Subsequently, multiple studies confirmed these results by using murine neonatal [83] or adult [84] myoblasts as well as myoblasts from human origin [85]. In the beginning of the 1990s, these results were translated to several human clinical trials. Although no adverse effects were observed, only in a few cases myoblast-mediated dystrophin expression was documented. Overall, transplantations failed to provide sustained clinical benefits to the patients due to immunological rejection by the host, poor survival and dispersion of the injected myoblasts that significantly decrease their engraftment. Therefore, in general, SCs are cultured on soft substrates to preserve their engraftment ability. Another strategy to increase SC engraftment is to pretreat cells with growth factors, leading to partial colonisation in pilot clinical studies [86]. Nowadays, intramuscular injections of SC-derived myoblasts are still tested as potential treatment for localised forms of muscle disorders, although several multiple injections would be required (NCT02196467, Table 3.2). A recent study, investigating the possibility of the systemic delivery of myoblasts via the intraarterial route, showed that myoblasts are unable to cross the endothelial blood vessel barrier [87]. Promising results

Table 3.2 Stem cell-based clinical trials for MDs

MD type	Phase	Trial status	Number	Eligibility	Cell type	Dose	Route	Principal investigator sponsors/collaborators
DMD	I–II	Recruiting	NCT02196467	16 years and older, M	Eterologous myoblasts	30×10^6 cells/cm^3 in extensor carpi radialis	i.m.	Craig Campbell and Jack Puymirat-Centre Hospitalier Universitaire de Québec, CHU de Québec, Canada
OPMD	II	Completed	NCT00773227	18–75 years, M/F	Autologous myoblasts	178×10^6 cells into constrictor muscles of pharynx	i.m.	Assistance Publique – Hôpitaux de Paris, France
FSHD	I	Recruiting	NCT02208713	18–50 years, M/F	Autologous MDSCs and adipose-derived MSCs	Unknown	i.m.	Leila Arab, MD-Royan Institute, Tehran, Iran
DMD	I–II	Completed	EudraCT-2011-000176-33	6–14 years, M	HLA-identical allogeneic MABs	50×10^6/kg (0.5×10^8/kg), distributed in 4 increasing doses	i.a.	Giulio Cossu, Fondazione Centro San Raffaele del Monte Tabor, Milan, Italy
Refractory idiopathic inflammatory myopathy diseases	I	Recruiting	NCT00278564	16–65 years, M/F	Autologous HSCs	Unknown	i.v.	Richard Burt, MD, Northwestern University, Chicago, USA
DMD	I–II	Recruiting	NCT01834066	6–25 years, M/F	Autologous BM-MSCs	100×10^6 each dose, 6 doses in 3 months	i.v.	Dr. Sachin Jamadar, C0-Investigator, Chaitanya Hospital, Pune, India
DMD	I–II	Recruiting	NCT02285673	7–20 years, M	UCMSCs	Unknown	i.v.	Ercument Ovali, Acibadem University, Istanbul, Turkey

i.m. intramuscular, *i.a.* intraarterial, *i.v.* intravenous

have been achieved in the case of Oculo Pharyngeal Muscular Dystrophy (OPMD), characterised by muscle wastage in eyelid and pharyngeal muscles. Autologous transplantation of unmodified myoblasts isolated from healthy muscles has shown beneficial effects in preclinical work and has entered clinical trial (NCT00773227, Table 3.2) for the treatment of dysphagia in OPMD. Another clinical study for Facio Scapulo Humeral muscular Dystrophy (FSHD), involving myoblast autotransplantation without immunosuppression or genetic correction, is currently ongoing (NCT02208713, Table 3.2).

Overall, SCs/myoblasts have two main disadvantages for cell therapy purposes: (1) these cells are often exhausted in dystrophic conditions (like DMD), and (2) they cannot be administered systemically but only intramuscularly [88]. Therefore, other stem cell types, distinct from SCs, have been studied and identified extensively for their myogenic potentials in animal models over the past years or in clinical trials, including vessel-associated cells, Muscle-Derived Stem Cells (MDSCs), side population (SP) and muscle interstitial cells. In a human setting, the correspondent of these stem cell types has not yet been found.

3.3.1.2 Pericytes/Mesoangioblasts (MABs)

Pericytes, the mural cells of the blood microvessels, have been discovered and described in 1871 as a contractile cell population surrounding the endothelial cells of small blood vessels. Pericytes were initially described as regulators of the blood flow, although confusion still exists about their identity, ontogeny and progeny. They differentiate towards adipocytes, bones, cartilage and muscles [89]. Several molecular markers for pericytes have been characterised, depending on their presence in different tissues [90, 91]. Notably, their expression pattern is dynamic in developmental stages, in vitro culturing and after pathological insults, so that no single pericyte-specific marker has been identified. Among the general accepted markers for murine pericytes are CD140b and Nerve/Glial antigen 2 (NG2), crucial for survival and development, alanyl membrane aminopeptidase CD13, desmin and alpha-Smooth Muscle Actin (αSMA). However, in mice, quiescent pericytes are not positive for αSMA while its expression level is upregulated in tumours and inflammatory environments. In human skeletal muscles, pericytes are highly positive for NG2, Alkaline Phosphatase (ALP), annexin V, desmin, αSMA, vimentin and CD140b and negative for M-cadherin (CD146), NCAM, cytokeratins and neurofilaments (except for nestin), endothelial markers (CD31, CD34, KDR) or haematopoietic markers (CD45) [88]. Myogenic differentiation capacity is not restricted to muscle-resident pericytes, since pericytes isolated from human adult adipose tissue or placenta can contribute to myotubes in vitro and to dystrophin-expressing fibres in vivo [92]. Recently, a new expression profile has been described for the simultaneous purification of pericytes, MSCs and blood vessel-derived stem cell subpopulations from human skeletal muscles [93].

Mesoangioblasts (MABs) are vessel-associated progenitors, located in the interstitial space between the muscle fibres. MABs have been first isolated from the dorsal aorta of E9.5 mouse embryos [94] and in adult muscles from mice [95, 96], dogs [97] and humans [88]. Embryonic MABs are positive for CD34, c-Kit and Flk-1 but negative for Oct4, Nkx2.5 and Myf5 [94]. MABs of embryonic origin can differentiate to multiple derivatives of the mesodermal lineages in vitro and in vivo [98]. In mice and dogs, adult MABs are isolated from adult skeletal muscles based on their expression for ALP, Sca-1, integral membrane NG2, CD140a and CD140b (also known as PDGFRα and β isoforms, respectively) [88, 95, 99]. Adult MABs have a multipotent differentiation potential towards myo-, osteo-, chondro- and adipogenic lineages [100]. Human MABs are well studied both in vitro and in vivo. They express the adhesion molecule CD146, CD140b and NG2 but do not express haematopoietic or SC markers, including CD45, CD34, CD56, CD144 and Pax7 [88]. The human counterpart for the murine Sca-1 marker is still missing, although members of the human Ly6 proteins have been suggested as correspondent to Sca-1 with homologous functions. Intriguingly, a comparison among human MABs, MSCs and pericytes has identified some similar markers (CD10, CD13, CD44, CD73, CD90), opening new questions regarding their origin [101]. Human MABs easily proliferate in vitro and spontaneously differentiate into MyHC-positive myotubes. Moreover, they express immunomediated cytokines and receptors, indicating immunomodulatory properties by inhibiting the T-cell proliferation in vitro [102]. The ability to regulate immune responses by MABs opens new clinical applications.

Preclinical and Clinical Studies Using MABs

MABs are used in systemic cell therapy for MD in experimental models by intraarterial injection of either wild-type or genetically modified MABs. Injected MABs cross the blood vessel barrier and migrate to the dystrophic muscle, where, subsequently fused to muscle fibres, they reconstitute dystrophin expression. Moreover, MABs localise with the SCs and ameliorate morphologically and functionally the dystrophic phenotype [95, 97, 103–105]. MABs, in vitro transduced with a lentiviral vector carrying human mini- or micro-*dystrophin* after intraarterial injection in *scid/mdx* mice or Golden Retriever Muscular Dystrophy (GRMD) dogs, are able to migrate and contribute to the host skeletal muscle regeneration [88, 97]. Based on the outcome of these mice and dog studies, in March 2011, a phase I–II clinical trial has been started, in which four consecutive escalating intraarterial infusions of HLA-matched donor-derived MABs were given (EudraCT no. 2011-000176-33, Table 3.2). Safety and feasibility of the study have been proven. Two months after the last infusion, the first muscle biopsy was performed revealing low levels of donor DNA in the majority of the patients and low amount of donor-derived dystrophin just in one patient. Unfortunately, no functional improvements were observed [106].

3.3.1.3 Muscle-Derived Stem Cells (MDSCs): Skeletal Muscle Aldehyde Dehydrogenase-Positive Cells ($ALDH^+$) and "MuStem Cells"

Another muscle-resident stem cell population, known as MDSCs, has been identified from mouse skeletal muscle and seems to be different from late-stage myogenic populations, like SCs and myoblasts, by their multipotent differentiation potential. Besides their myogenic capacities, they can differentiate in vitro and in vivo towards adipo-, osteo-, chondrogenic, haematopoietic, endothelial, smooth muscle, cardiac muscle and neural lineages [107]. Moreover, MDSCs secrete high levels of VEGF, promoting vascularisation and consequently facilitating tissue regeneration. MDSCs have a high expression of Sca-1, low levels of the fibroblast marker vimentin and low levels of desmin. These cells are negative for CD45, M-cadherin. Moreover, the low expression of the Major Histocompatibility Complex (MHC)-1 is a promising feature towards more successful stem cell-based therapy applications [108]. Intramuscular delivery of MDSCs, 4 days after induced injury, showed enhanced angiogenesis and reduced scar tissue formation. Interestingly, 1 week after injection, high levels of VEGF and high expression levels of antioxidant, glutathione (GSH) and superoxide dismutase were detected, increasing their survival after administration [109]. Aldehyde dehydrogenase 1A1 (ALDH) is a ubiquitously detoxifying enzyme involved in the metabolism of aldehydes and retinoic acid. $ALDH^+$ cells are found in the bone marrow, umbilical cord and peripheral blood, although a cell population has been characterised with a high ALDH activity in human skeletal muscles [110]. These cells represent a small amount of the total mononucleated cells (2–4%). MDSCs have a higher survival ability compared to SCs and myoblasts after transplantation, probably due to their resistance to oxidative stress and high proliferation activity in vivo.

Preclinical and Clinical Studies Using $ALDH^+$ MDSCs and "MuStem Cells"

Two distinct subpopulations of $ALDH^+$ cells with different phenotypic and functional properties can be purified from skeletal muscle. The $ALDH^+$ $CD34^+$ cells have a mesenchymal profile, while $ALDH^+$ $CD34^-$ cells rapidly upregulate CD56 in vitro and give rise to multinucleated myotubes. In vivo, $ALDH^+$ $CD34^-$ cells injected in damaged muscles of immunodeficient mice contribute to muscle formation and migrate into the SC position. Interestingly, these $CD34^-$ cells display high proliferation rates after in vivo administration into the muscle of immunodeficient mice [110].

In 2011, MDSCs isolated from GRMD dogs, called MuStem cells, were isolated based on their delayed adhesion properties. These cells have high proliferation rates and seem to be committed to the myogenic lineage. MuStem cells have been shown to contribute to muscle regeneration in the GRMD dog, allowing dystrophin expression and relocation into the SC niche. A partial remodelling of the skeletal muscle tissue of GRMD dogs has been observed after intraarterial injection of

MuStem cells [111]. These data may indicate a potential therapeutic application for MuStem cells, although further studies have to be done to investigate their myogenic potential in humans.

3.3.1.4 PW1-Expressing Interstitial Cells (PICs)

PW1-expressing interstitial cells (PICs; also known as Paternally expressed gene 3, Peg3) are characterised by the localisation in the interstitium among muscle fibres. These cells are not embryonically related to SCs, since they do not derive from $Pax3^+$ myogenic progenitor cells. PICs have a $Pax7^-$/Sca-1^+ and $CD34^+$ marker phenotype [112]. Recently, a high expression of PW1 has been shown in MABs of mice, dogs and humans, and the expression levels correlate with their myogenic and migratory capacities. Silencing *PW1* significantly inhibits their myogenic differentiation capacity and their ability to cross the vessel wall, so consequently, their engraftment into damaged muscle tissue [113]. Therefore, PW1 may be used in the future as a biomarker to identify optimal donor populations for cell therapy in pathologic conditions.

3.3.1.5 Muscle Side Population (SP) Cells

SP cells are a rare subpopulation source of precursors associated to skeletal muscles. SP cells are characterised by the complete exclusion of the DNA-binding dye Hoechst 33342, due to their abundant expression of the Abcg2 transporter. They show heterogeneity inside their population. 80% of the total SP cells are positive for the vascular endothelial marker CD31, while 2–10% are blood derived and show positiveness for the immune marker CD45. However, a small fraction has been characterised as highly positive for Abcg2, CD31 and CD45, especially during muscle damage and the early phase of regeneration. Recently, another SP cell subgroup has been identified, representing 5% of the total population, which are negative for both CD31 and CD45, and may express Pax7, Sca-1 and syndecan 4 [114, 115]. In coculture with myoblasts, SP cells are able to fuse in vitro to form myotubes. Interestingly, in vivo engraftment experiments show high myogenic differentiation capacity in regenerating muscles after acute tissue damage [116]. Moreover, several studies have shown their ability to restore dystrophin levels when injected intravenously into *mdx* mice [117].

3.3.1.6 Fibro-/Adipogenic Progenitors (FAPs)

In 2010, a new muscle stem cell population located in the interstitium has been discovered. Fibro-/Adipogenic Progenitors (FAPs) are non-myogenic, mesenchymal progenitors that differentiate towards both adipo- and fibrogenic cells, indicating a

potential contribution to adipose and fibrotic deposition in diseased muscles [118, 119]. These cells were isolated as $CD34^+/Sca\text{-}1^+/CD31^-/CD45^-/Lin^-$/integrin $\alpha7^-$ [118] or $Sca\text{-}1^+/CD140a^+$/integrin $\alpha7^-$ [119]. They exert beneficial paracrine effects on the muscle fibres. FAPs were used in preclinical models of MDs with histone deacetylase inhibitors (HDACi), able to promote in vitro and in vivo myogenic differentiation. Transplantation of FAPs in more advanced dystrophic muscles enhanced the regeneration by interacting with the muscle-resident SCs. Several studies have shown that FAPs from dystrophic muscles of *mdx* mice retain a bipotency in their phenotype and functionality, either by supporting regeneration at early stages of disease progression or by differentiating in fat and fibroblasts [64, 65]. The human FAP counterpart has been isolated as CD140a-positive cells in healthy and diseased muscles, although further studies are needed to investigate the molecular and functional characterisation to develop novel pharmacological treatments or to understand their ability as a cell therapy source for muscular diseases in human settings.

3.3.2 Non-resident Stem Cells for Skeletal Muscle Regeneration

3.3.2.1 Mesenchymal Stem Cells (MSCs)

MSCs are non-haematopoietic multipotent stem cells that can be found in several tissues, including bone marrow, umbilical cord, blood, placenta, liver, adipose tissue, muscle and synovial membrane. The bone marrow is the principal source [120, 121]. MSCs can renew and differentiate into multiple mesenchymal lineages, including adipo-, chondro- and osteocytes. MSCs are characterised by the expression of several markers (CD73, CD90 and CD105), although not specific for MSCs, and by the lack of haematopoietic markers (CD45, CD34 and CD14 or CD11b, CD19 and HLA-DR) [122].

Preclinical and Clinical Studies Using MSCs

The clinical relevance of MSCs is their modulating properties of immunological responses, which could be important for immunotolerance induction and to prevent rejection of allogenic transplantation. Moreover, they can secrete trophic factors and chemokines, which alters the local environment to facilitate and regulate endogenous tissue homeostasis [123]. Unfortunately, studies using MSCs isolated from human tissue, like adipocytes [124] or synovial membrane [125], have shown a weak beneficial effect in muscle regeneration. However, their accessibility and abundance from different tissues and their immunosuppressive and trophic characteristics make

MSCs an interesting cell candidate to be further investigated [126]. In 1998, the first report was published, showing the transplantation of genetically modified bone marrow cells into the injured muscle of immunodeficient mice [127]. Nowadays, two clinical trials (Table 3.2) started for autologous transplantation of bone marrow-derived stem cells (BM-MSCs; NCT01834066) and for Umbilical Cord-derived Mesenchymal Stem Cells (UCMSCs; NCT02285673) involving DMD patients. In addition, genetically modified MSCs for their Notch expression were injected intramuscularly in *mdx* mice and showed a better rescue of dystrophin expression [128]. Further studies are necessary to determine the immunomodulatory properties of MSCs and their interactions with the inflamed environment into damaged tissue.

3.3.2.2 CD133$^+$ Cells

Another cell population with myogenic potential has been identified expressing CD133 [129–131]. CD133$^+$ cells are a small subpopulation of the mononucleated cells present in the peripheral blood [129], expressing the stem cell marker CD133. Some cells have also been identified as positive for CD133 in human skeletal muscle [131]. The function of CD133 is still unclear. However, it could be a useful marker to purify haematopoietic and endothelial progenitors. A drawback of these cells is CD133 loss in vitro after expansion, hampering cell tracking after expansion or in vivo administration. CD133$^+$ cells cocultured with murine myoblasts have the capacity to form MyHC-expressing myotubes [129].

Preclinical and Clinical Studies Using CD133$^+$ Cells

Intraarterial or intramuscular injections of human peripheral blood-derived CD133$^+$ cells in *scid/mdx* mice produce dystrophin, by fusion with the host fibres, and colonise the SC niche, expressing typical SC markers [129]. Moreover, local injections in rat injured muscle accelerate regeneration, suggesting the ability of these cells to produce VEGF and promote vasculogenesis, by differentiating to both endothelial and myogenic lineages. In addition, the regenerative capacity of CD133$^+$ cells isolated from human skeletal muscle has been studied. After intramuscular delivery in immunodeficient mice, CD133$^+$ cells showed a higher regeneration capacity compared to *bona fide* human SC-derived myoblasts. A study performed in immunodeficient and dystrophic mice opens the horizon for the use of (blood- and muscle-derived) CD133$^+$ cells as possible candidates for (autologous) stem cell therapy applications for DMD patients [132]. Moreover, genetic modifications together with their ability to cross the blood vessel barrier allow systemic delivery and point out CD133$^+$ as suitable cell population for cellular therapy.

3.4 Human Embryonic Stem Cells (ESCs) and Induced Pluripotent Stem Cells (iPSCs): Future Challenges for Stem Cell Therapies

Human Embryonic Stem Cells (ESCs) [133] and induced Pluripotent Stem Cells (iPSCs) [134] hold great potential for regenerative medicine. These PSCs are able both to repair damaged muscles and regenerate healthy tissues (Table 3.3). However, in vitro PSC-derived muscle precursor differentiation, obtained by well-defined medium conditions or genetic modification, is still challenging and needs further improvements in order to reproduce the in vivo environment. To favour expansion and differentiation, tissue engineering methods could be necessary to mimic the muscle progenitor stem cell niche and to accelerate myocyte maturation. Tissue engineering in combination with gene and cell therapy approaches opens new perspectives towards a successful development of therapeutic treatments in several MDs [135].

3.4.1 Embryonic Stem Cells (ESCs)

ESCs have been isolated from the inner cell mass of the murine [136] and human [133] blastocyst. The therapeutic applications of ESCs have been strongly debated in the scientific community, mainly because of ethical concerns and immune rejection issues. Moreover, their use needs to be carefully evaluated, since ESCs can lead to teratoma formation [137]. Few studies have described the use of human ESCs in muscle transplantation. One study documented the transplantation of mesenchymal precursors derived from human ESCs in cardiotoxin-injured tibialis anterior (TA) muscles of *scid/beige* mice. Although only a small cell proportion could be tracked in the muscle, a possible future role for ESCs in muscle cell transplantation could be suggested, since no tumorigenic effect was observed [138]. Several groups have documented the differentiation of myogenic progenitors from ESCs. Darabi et al. used inducible Pax3 and Pax7 overexpressing murine ESCs, injected intramuscularly or systemically administered in *mdx* mice, resulting in engraftment with dystrophin-expressing myofibres and improvements in the muscle function [139]. Similar outcomes were observed in mice affected with FSHD [140]. Zheng et al. showed that human ESCs exposed to serum in the presence of EGF directed differentiation towards myogenic precursors. Subsequent addition of the DNA-demethylating agent 5-azacytidine increases even more their myogenic potential. in vitro terminal differentiation and fusion into myotubes was not observed. However, transplantation of human ESC-derived cells into injured TA reached terminal differentiation, as well as localisation in the SC compartment [141]. Barberi et al. differentiated human ESCs to obtain mesenchymal precursors for myogenic progenitor cells. After transplantation in the hindlimb muscle of immunodeficient mice, long-term survival and myofibre commitment were reported

Table 3.3 PSC derivatives to improve adult myogenesis

Cell type	Source	Pluripotency markers	Myogenic differentiation	Acquired molecular markers	Model	Systemic delivery	References
ESC derivatives	Blastocyst inner cell mass	Oct3/4, Nanog, Sox2, Lin28, SSEA4, TRA-1-60	Induced by culture conditions or gene transfer	CD56, KDR, CD73, CD90, CD140a, M-cadherin, α7 integrin, SM/C2.6	*mdx* mice, *scid/beige* mice and $Rag2^{-/-}(\gamma C^{-/-} C5^{-/-})$ mice	No	[142]
iPSC derivatives	Reprogrammed somatic cells	Oct3/4, Nanog, Sox2, Lin28, SSEA4, TRA-1-60	Induced by culture conditions or gene transfer	CD56, KDR, CD73, CD90, CD140a, M-cadherin, α7 integrin, SM/C2.6	*mdx* mice, *scid/beige* mice and $Rag2^{-/-}(\gamma C^{-/-} C5^{-/-})$ mice	No	[143, 144, 146, 147, 148]

[138]. Nowadays, several preliminary studies have been performed with PSC-derived myogenic cells, showing their capacity to form muscles in vivo. However, their long-term engraftment into adult muscle and their functional contribution still have to be elucidated, as well as their depleted muscle stem cell pool repopulation.

3.4.2 Induced Pluripotent Stem Cells (iPSCs)

Human iPSCs can be generated from adult somatic cells by introducing a defined set of transcription factors to reprogram them towards an embryonic-like pluripotent state [134, 142–145]. Human iPSCs have been derived from fibroblasts [146] and Peripheral Blood Mononuclear Cells (PBMCs) of DMD and Becher Muscular Dystrophy (BMD) patients [147]. Myogenic lineage induction from murine ESCs and iPSCs has been described [140, 148], although terminal differentiation of human ESCs, as well as human iPSCs, is still facing some difficulties. Murine and human iPSCs have been provided to counteract muscle wasting in MDs [116]. So far, in vitro and in vivo studies have shown that myogenic precursors derived from iPSCs could form chimeric myotubes in coculture with C2C12 myoblasts. In addition, iPSC-derived myogenic precursors transplanted in dystrophic muscles contribute to an improvement in the contractile properties. The iPSC technology has been used to allow the generation of genetically corrected human DMD iPSCs by using a human artificial chromosome able to carry the full-length dystrophin gene. This strategy is an alternative to induce truncated forms of *dystrophin* (quasi-, mini-, micro-*dystrophin* genes) in mouse and human iPSCs [149]. Recently, Clustered Regularly Interspaced Short Palindromic Repeat (CRISPR)/Cas9-modified DMD human fibroblasts reprogrammed to iPSCs have been tested for skeletal and cardiac muscle differentiation abilities both in vitro and in *mdx* mice [150].

3.5 Ex Vivo Cell Therapies

In case of genetic diseases, it becomes more and more possible to envision an autologous source of muscle progenitors extracted from the patient, genetically corrected in vitro and consequently transplanted into the same donor. Autologous strategies would be preferable to avoid immune rejection or immunosuppressive treatment to restore the expression of the mutated proteins. Still, further studies need to be developed, to increase both efficacy and safety of genetic modification and to avoid immune reaction due to the expression of transgene products. Since most of the studies have been addressing DMD, truncated versions of synthetic *dystrophin* have been tested for their efficiency, since just the actin-binding and the cysteine-rich domains are essential for function, while many other region of the gene are dispensable [151]. Instead of native *dystrophin* 14 kb cDNA, transcript of 4–5 kb can be better included into viral vectors [152]. Therefore, lentiviral vectors have

been used to infect micro-*dystrophin* gene in SP cells [153] or SM/C2.6-positive cells [154] transplanted into *mdx* mice. In addition, genetically corrected canine [97] and human MABs [88] have been transplanted into experimental dystrophic mice and dogs.

Viral vector choice has to be carefully considered, especially with respect to vector insertional mutagenesis. Activating vector insertions near proto-oncogenes might induce T-cell leukaemia, 3–5 years after gene therapy, therefore requiring careful monitoring against oncogenic development [155]. In the last years, the Adeno-Associated Viruses (AAVs) have been pointed out as the first choice viral vectors for gene and cell therapy in MDs, due to their low immunogenicity and ability to exist in the transduced cells as an episome [156].

3.5.1 Adeno-Associated Virus (AAV)

AAV has already been used in many clinical trials since its broad tropism and infectivity, long-term expression and site-specific integration (NCT01344798; Table 3.4) [157]. Non-selective infection and organ-restricted transduction have pushed researchers to look for better vectors, based on different serotypes able to transduce more efficiently muscle tissue or to offer the best compromise between skeletal muscle expression levels and ability to escape human immune response [158]. A mini-*dystrophin* gene (rAAV2.5-CMV-mini-*dystrophin* gene vector) has been tested for safety (phase I) in the treatment of progressive muscle weakness due to DMD (NCT00428935) [159]. Analogously, a double-blind randomised study (NCT00494195) reported the administration of intramuscular injection of rAAV.tMCK.haSG gene vector in LGMD2D patients as being safe. In addition, AAV8 has been used to repress chronic inflammation and muscle degeneration specifically into *mdx* skeletal muscle, overexpressing an endogenous inhibitor of NF-κB signalling pathway under the truncated Muscle Creatine Kinase (tMCK) promoter. Short hairpin RNA carrier AAV9 has been successfully developed to target the major subunit of NF-κB (NF-κB/p65) and ameliorate muscle pathologic phenotype in *mdx* mice [160]. Finally, AAV9 mini-*dystrophin* gene delivery combined to the addition of octalysine (8K)-NF-κB essential modulator (NEMO)-Binding Domain (8K-NBD) peptide promoted a higher mini-*dystrophin* expression in skeletal muscle compared to AAV9 alone [161].

3.5.2 Exon Skipping

Since DMD is mainly caused by frameshift mutations in *dystrophin*, exon-skipping strategies have been developed. Indeed, skipping exons carrying an out-of-frame DMD mutation result in a final in-frame transcript, able to be translated in a

Table 3.4 Gene therapy or small molecule-based clinical trials for MDs

MD type	Phase	Trial status	Number	Eligibility	Strategy	Viral vector	Molecule	Principal investigator sponsors/collaborators
DMD	I	Completed	NCT00428935	5–15 years, M	Mini-*dystrophin*	rAAV2.5-CMV	N/A	Jerry R. Mendell, Nationwide Children's Hospital, Columbus, Ohio, USA
LGMD2D	I	Completed	NCT00494195	5 years and older, M/F	*Alpha-sarcoglycan*	rAAV1.tMCK	N/A	Jerry R. Mendell, Nationwide Children's Hospital, Columbus, Ohio, USA
LGMD2C	I	Completed	NCT01344798	15 years and older, M/F	*Gamma-sarcoglycan*	AAV1	N/A	Frederic Revah, CEO, Genethon, Evry Cedex, France
LGMD2B	I	Recruiting	NCT02710500	18 years and older, M/F	*Dysferlin*	rAAVrh74.MHCK7	N/A	Jerry R. Mendell, Nationwide Children's Hospital, Columbus, Ohio, USA
DMD	II	Completed	NCT01462292	5 years and older, M	Exon skipping (exon 51)	N/A	Drisapersen (GSK-2402968)	GlaxoSmithKline, Brentford Middlesex, UK
DMD	III	Recruiting	NCT01803412	5 years and older, M	Exon skipping (exon 51)	N/A	Drisapersen	BioMarin Pharmaceutical, San Rafael, CA, USA

DMD	II	Completed	NCT01396239	7–13 years, M	Exon skipping (exon 51)	N/A	Eteplirsen (AVI-4658)	Sarepta Therapeutics, Cambridge, MA, US; Jerry R. Mendell, Nationwide Children's Hospital, Columbus, Ohio, USA
DMD	II	Ongoing	NCT01918384	4 years and older, M	Reading through	N/A	Arbekacin sulphate (NPC-14)	PTC Therapeutics, South Plainfield, NJ, USA; Yasuhiro Takeshima, Kobe University, Japan
DMD and BMD	II–III	Completed	NCT00592553	5 years and older, M	Reading through	N/A	Ataluren (PTC124)	PTC Therapeutics, South Plainfield, NJ, USA; Leone Atkinson, MD, PhD

N/A not applicable

truncated but still functional protein product [162], thereby leading to a less severe phenotype (similar to BMD). Similar genetic modification has been successfully used as well to correct other mutations such as exon 32 in *dysferlin* [163]. Morpholino oligomers have been directed against exon 51 and tested in proof-of-concept studies and clinical trials [164]. The same exon skipping has been extended to correct different cell types like $CD133^+$ myogenic progenitors from DMD patients transplanted afterwards into *scid/mdx* mice. The corrected cells were able to recover histologically and functionally dystrophin presence [132]. Lately, the greatest percentage of *mdx* muscle fibres rescued in dystrophin has been calculated by immunostaining around the 38%, underlying the importance of undefined factors in the variable success of exon skipping method [165].

It is worth noticing that two advanced clinical trials have been carried out describing two drugs promoting exon 51 skipping. Drisapersen is a 2′O-Methyl-Phosphorothioate oligonucleotide (2′OMePS) that has already been tried successfully in a phase II clinical trial on DMD patients (NCT01462292, Table 3.4). In a phase III randomised, double-blind, placebo-controlled trial, it did not show statistical difference between treated patients and placebo (NCT01803412, Table 3.4). Eteplirsen is a more promising phosphorodiamidate morpholino oligomer (PMO) that gives a gradual increase in dystrophin fibres (from already 23% at a first time point till 52% at lower or 43% at higher concentration, after 48 weeks of treatment). The presence of functional dystrophin in the sarcolemma has been confirmed both by detection of sarcoglycans and neuronal Nitric Oxide Synthase (nNOS) and resulted in ambulation benefit compared to placebo patients (NCT01396239, Table 3.4). Eteplirsen-treated patients undergoing a 6 min walking test (MWT) have reported a lower decline in ambulation compared to untreated matched healthy controls [166].

3.5.3 Reading Through

Two other clinical trials (phase II randomised, placebo-controlled, double-blind studies) have been started in patients with nonsense mutations in *dystrophin*, comparing the efficacy and the safety of two different drugs with a similar effect: arbekacin sulphate (NPC-14; NCT01918384, Table 3.4) and ataluren (NCT00592553, Table 3.4). These drugs are targeting the translation machinery and inducing the reading through premature stop codons. The study with ataluren has already been concluded reporting at lower doses an improvement in the 6 MWT at week 48, while at higher doses it did not differ from placebo effect. Moreover, time function test of skeletal muscle, from patients treated with lower doses, showed amelioration in both trends for climbing and descending four stairs and running/walking 10 m. These positive results have promoted the drug for a phase III clinical trial, in which the ability to slow the disease progression in DMD population will be hopefully reconfirmed [167].

3.5.4 Small Nuclear RNA (snRNA) Sequences

Small nuclear RNA (snRNA) sequences that favour sublocalisation for the RNA and ameliorate the inclusion of antisense oligonucleotides into the spliceosome are the next-generation molecules, able to be packaged in adenoviral vectors, allowing persistent repair of the DNA without continuous administration. Therapies using this approach have been developed and applied to *dystrophin* exon 51 skipping [168]. Recently, a study was performed in 18 juvenile GRMD dogs intravenously infused with different volumes of rAAV8-U7snRNA-E6/E8 [169]. rAAV8-U7snRNA-E6/E8 is an antisense nucleotide able to avoid the point mutation in the acceptor splice site of intron 6 of the canine *dystrophin* gene, causing skipping of exon 7, disruption of the reading frame and premature termination of translation. The injection was performed into one forelimb, aiming to model human treatment, to define the therapeutic dose, to set up the injection protocols and to assess both gene therapy and delivery safety. A dose-dependent exon skipping has been reported for the dogs, opening the possibility for a phase I–II clinical trial, since the highest dose resulted even in 80% of dystrophin-expressing fibres, without acute or delayed adverse effects [169].

3.5.5 Engineered Nucleases

Improvements in different kinds of nucleases have opened the possibility to replace in phase small frameshift and point mutations [170]. In the last years, genome editing at exon 51 by mean of engineered nucleases, able to correct the disrupted reading frame like Transcription Activator-Like Effector Nucleases (TALENs), has been demonstrated to efficiently correct and restore the expression of a functional dystrophin protein in vitro, in immortalised myoblasts [171]. A newer approach to correct specific genome sequences consists of RNA-guided nucleases and type II CRISPR/Cas9 system [172]. Single-guide RNA molecules drive Cas9 nuclease to a targeted locus in the proximity of protospacer adjacent motif and generate a double-strand break that is then repaired by either nonhomologous end-joining (with the possibility of insertion/deletion mutations) or by homology-directed repair. To favour this last foreseen repair mechanism and remove the defect, an exogenous template can be provided, in order to generate a perfect mutation of the desired locus [173].

In particular, as a proof of concept, this technique has been used to correct mutations in the germ line of *mdx* mice with a nonsense mutation in exon 23 of the *dystrophin* gene. The study has reported a high percentage (47–60%) of dystrophin-positive fibres associated to 17% of gene correction, and, in older animals, almost no negative dystrophin fibres have been observed selectively in skeletal muscle, meaning a progressive rescue of dystrophin protein, as well in the SC compartment [174]. Alternative strategies have been foreseen to correct

postnatal tissues, since germ line editing is not affordable in human settings and homology-directed repair does not occur in postmitotic cells [175]. AAV9-delivery of gene-editing components has been administered in different ways to postnatal *mdx* mice, including intraperitoneal at postnatal day 1 (P1), intramuscular at P12, and retro-orbital at P18. Dystrophin protein expression has been restored with different degrees, increasing from 3 to 12 weeks after injection, in cardiac and skeletal muscle, the latter proved by grip strength test [176]. These recent results are encouraging and in the range of therapeutic benefit, since it has been estimated that low-level expression of dystrophin (4–15%) could be enough to protect from myo-injury due to eccentric contraction and could effectively ameliorate cardiomyopathy [177]. Still, gene-editing techniques need improvements to avoid off-target effects if an eventual translation to human settings is foreseen.

3.6 Conclusion

So far many efforts have been made to increase our understanding of molecular cues of MDs, since the use of anti-inflammatory drugs administered alone, or in combination with other molecules, showed poor beneficial effects in treated patients. In the last two decades, stem cell therapies for MDs are gaining momentum. In particular, the rising on the knowledge about the biology of multipotent and PSCs allowed to identify novel muscle progenitor cells besides SCs. Those novel identified myogenic progenitors have been tested in several animal models, including mice and dogs. Promising results have been obtained in decreasing the inflammatory burden, improving dystrophin expression and tissue architecture, with a beneficial effect on muscle function. AAVs have been approached in preclinical and clinical studies to mediate the delivery of micro-dystrophin or mini-utrophin, while exon skipping strategies have been proposed to correct the mutation of the endogenous *dystrophin* and rescue protein expression. Unfortunately, so far, both gene and cell therapy strategies produced poor results in terms of dystrophin restoration and muscle function amelioration. Nevertheless, intraarterially transplanted MABs from donor cells have been proved to be feasible and relatively safe, recovering dystrophin expression, at least in one muscle biopsy from treated patients. An intriguing perspective is also to consider the non-genetic cues to improve the myogenic fates of local or transplanted progenitors, maybe in combination with miRNAs and gene-editing (CRISPR and TALEN) technologies (Fig. 3.1). However, several practical issues need to be urgently solved to improve stem cell efficacy in clinic. Low cell survival, cell motility after transplantation and immune rejection are still the main problems for cell-based therapies of MDs. In conclusion, more preclinical studies are expected on myogenic derivatives from PSCs and possibly in combination with exosomes, miRNAs and gene-editing technologies to improve the efficacy of stem cell treatment in MDs.

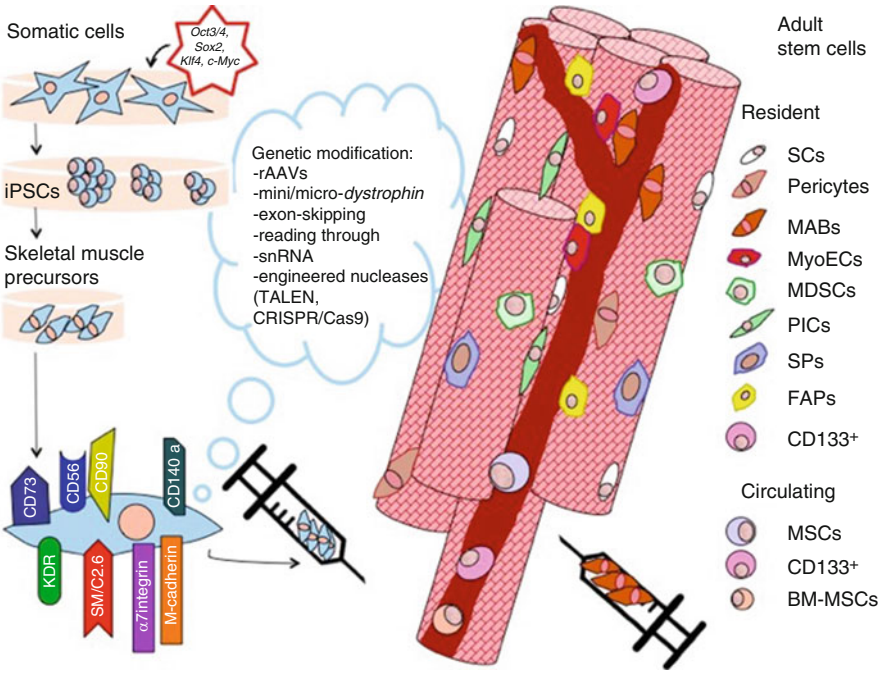

Fig. 3.1 Present and future of stem cell therapies in MDs. Schematic representation of skeletal muscle tissue focusing on the resident and circulating cells with myogenic potential (*right panel*). On the *left* side, somatic cells reprogrammed to a pluripotent state (iPSCs) and differentiated to myogenic precursors show early markers of muscle progenitors and represent the future challenge. Marker expression profile makes iPSC-derived muscle progenitors suitable for isolation and subsequent transplantation. Genetic modifications are necessary for autologous cell therapies and eventually to improve myogenic commitment and safety of iPSC-derived muscle progenitors

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Chapter 4
The Autophagy-Dependent Signaling in Skeletal Muscle

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Abstract Skeletal muscle provides a fundamental basis for human function, enabling locomotion and respiration. Autophagy occurs in all eukaryotic cells and is evolutionarily conserved from yeast to humans. The autophagy machinery is a critical pathway for cell homeostasis, but it has long been forgotten in skeletal muscle. Particular emphasis has been placed on the role played by atutophagic defects in disease pathogenesis, its involvement in atrophy, and the possible effects of exercise as a countermeasure. Recent studies have indicated the age-related defects of autophagy signaling in skeletal muscle, whereas the autophagic activation occurs in cancer cachectic muscle probably due to enhancing protein degradation. Endurance training possesses a positive effect for some disease model (ex. inclusion body myopathy) by activating autophagy system. This review provides a recent research advances dealing with autophagy-dependent signaling in various muscular adaptations.

Keywords Autophagy • Skeletal muscle • Exercise • Sarcopenia • Cachexia • Atrophy • Unloading

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Abbreviations

AMPK	AMP-activated protein kinase
Atg	Autophagy-related genes
Atrogin-1	Atrophy gene-1
BCL2	B-cell lymphoma 2
BNIP	BCL2/adenovirus E1B 19 kd-interacting protein
C26	Colon 26 (C26)
CHF	Chronic heart failure
COPD	Chronic obstructive pulmonary disease
DHM	Dihydromyricetin
DMD	Duchenne muscular dystrophy
FOXO	Forkhead box O
GSK	Glycogen synthase kinase
LC3	Microtuble-associated protein light chain 3
LLC	Lewis lung carcinoma
mTORC1	Mammalian target of rapamycin signaling complex1
MuRF-1	Muscle ring-finger-1
PGC-1α	Peroxisome proliferator-activated receptor γ coactivator 1α
ULK1	Unc51-like kinase-1
UPS	Ubiquitin-proteasome system
VCP	Valosin containing protein

4.1 Introduction

In humans, skeletal muscle is the most abundant tissue in the body, comprising 40–50% of body mass and playing vital roles in locomotion, heat production during periods of cold stress, and overall metabolism. That skeletal muscle consists of the largest pool of proteins in the whole organisms highlights why this specific tissue is highly sensitive under conditions that act to alter the balance between protein synthesis and degradation. Loss of muscle is a serious consequence of many chronic diseases and of aging itself because it leads to weakness, loss of independence, and increased risk of death.

Previous studies using preferential animal models have demonstrated that muscle atrophy receiving various catabolic stimuli exhibits similar activation of protein degradation by both ubiquitin-proteasome system (UPS) and autophagy. Most muscle proteins, particularly myofibrillar components, seem to be degraded by the UPS. Two muscle-specific ubiquitin ligases, muscle RING finger-1 (MuRF-1) and atrophy gene-1 (atrogin-1), are markedly induced in a wide range of in vivo models of skeletal muscle atrophy including diabetes, cancer, denervation, unweighting, and glucocorticoid treatment [1, 2]. The importance of these atrophy-regulated genes in muscle wasting was confirmed through studies in these knockout

mice attenuating denervation-, fasting-, and dexamethasone-induced muscle atrophy [3–5]. Interestingly, recent findings indicate that atrogin-1-knockout mice are short-lived and experience higher loss of muscle mass during aging than control mice [6], indicating that chronic inhibition of these atrogenes should not be considered a therapeutic target to counteract sarcopenia [7, 8].

Autophagy occurs in all eukaryotic cells and is evolutionarily conserved from yeast to humans [9]. Turnover of most long-lived proteins, macromolecules, biological membranes, and whole organelles, including mitodhoncria, ribosomes, the endoplasmic reticulum and peroxisomes, is mediated by autpophagy [10]. Three major mechanisms of autophagy have been described. Microautophagy, in which lysosomes directly take up cytosol, inclusion bodies and organelles for degradation. Chaperone-mediated autophagy, in which soluble proteins with a particular pentapeptide motif are recognized and transported across the lysosomal membrane for degradation. Macroautophagy (herein autophagy) is a ubiquitous catabolic process that involves the bulk degradation of cytoplasmic components by interacting lysosome [11, 12]. This process is characterized by the engulfment of part of the cytoplasm inside double-membrane vesicles (autophagosomes). Autophagosomes subsequently fuse with lysosomes to form autophagolysosomes in which the cytoplasmic cargo is degraded.

The autophagy machinery, a critical pathway for cell homeostasis that had long been forgotten in skeletal muscle, has been intensively studied in the past few years. Particular emphasis has been placed on the role played by autophagic defects in disease pathogenesis, its involvement in atrophy, and the possible effects of exercise as a countermeasure [13–15]. Indeed, sarcopenic muscle of human and rodent exhibits a marked autophagic defect [16, 17], in which can not degrade the accumulated denatured proteins, and abnormal mitochondria and sarcoplasmic reticulum. On the other hand, previous studies possess several discrepancies on autophagy activation in muscle according to the intermittent and/or continuous exercise [15]. This review aims to outline the functional role of autophagy-dependent system for various muscular adaptations.

4.2 Autophagy-Dependent Signaling

Autophagy represents an extremely refined collector of altered organelles, abnormal protein aggregates, and pathogens, similar to a selective recycling center [18]. The selectivity of the autophagy process is conferred by a growing number of specific cargo receptors such as p62/SQSTM1, Nbr1, and Nix [BCL2/adenovirus E1B 19 kd-interacting protein (BNIP) 3 L] [19]. These adaptor proteins are equipped with both a cargo-binding domain, with the capability to recognize and attach directly to molecular tags on organelles. At the same time, these adaptor proteins bind essential autophagosome membrane proteins. Three molecular complexes mainly regulate the formation of autophagosomes: the microtuble-associated protein light chain 3 (LC3) conjugation system and the regulatory complexes governed by unc51-like

kinase-1 (ULK1) and Beclin-1. The conjugation complex is composed of different proteins encoded by autophagy-related genes (Atg) [20]. The Atg12-Atg5-Atg16L1 complex, along with Atg7, plays an essential role in the conjugation of LC3 to phosphatidylethanolamine, which is required for the elongation and closure of the isolation membrane [20]. This system is under the regulation of at least two major cellular energy-sensing complexes. Under basal conditions, the ULK1 complex is inactivated by phosphorylation through mammalian target of rapamycin signaling complex1 (mTORC1), whereas during autophagy induction mTORC1 is inhibited, thus enhancing the formation of a complex between ULK1, Atg13, and FIP200. In addition, mTORC1 can also be negatively regulated independently of Akt by energy stress sensors such as AMP (Adenosine monophosphate)-activated protein kinase (AMPK) and, in a mechanical-activity-dependent manner, through tuberous sclerosis complex 1/2. Moreover, AMPK can also directly phosphorylate ULK1 and Beclin-1 [21]. During autophagy, the ULK1 complex is localized to the isolation membrane, where it facilitates the formation of autophagosomes through interaction with the Beclin-1 complex.

The UPS and the lysosomal-autophagy system in skeletal muscle are interconnected [22, 23]. Both these studies identified forkhead box O (FOXO)3 as a regulator of these two pathways in muscle wasting. FOXO3 is a transcriptional regulator of the atrogin-1 and MuRF-1. FOXO3 modulates the expression of autophagy-related genes in mammalian skeletal muscle and C2C12 myotubes [23]. Masiero et al. [24] found an intriguing characteristic using muscle-specific autophagy knockout mice, which exhibit fiber atrophy, weakness, and mitochondrial abnormalities. Autophagy-dependent protein degradation seems to be also modulated by tumor necrosis factor receptor-associated factor 6 and peroxisome proliferator-activated receptor γ coactivator 1α (PGC-1α) [25]. Wenz et al. [26] recognized age-related increase in the ratio of LC3-II to LC3-I in MCK-PGC-1α mice, Therefore, PGC-1α would attenuate the autophagic process probably through increased anti-oxidant defense and mitochondrial biogenesis.

4.3 Exercise and Autophagy

The merits of regular physical activity on lipid and glucose homeostasis and in muscle mass maintenance have been for decades. Energetic sensors, such as AMPK, sirtuin (SIRT)1, and p38-mitogen activated protein kinase, are well-established factors activated by muscle contraction. These molecules are possible modulators for autophagy signaling, which is important for maintaining cellular energy homeostasis acutely, as well as for efficient organelle and protein turnover following exercise. Currently the majority of research elucidating the molecular details of exercise-induced changes in autophagy pathways has been conducted in animal models. By using electron microscopy, Salminen and Vihko [27] observed autophagosome formation after exhaustive exercise more prominently in oxidative

fibers rich in mitochondria than in glycolytic fibers. Several autophagic markers such as LC3B, Atg4b, Atg12, and BNIP3 mRNA were upregulated immediately after a 24-h ultra-endurance exercise [28], as were the protein levels of some of these markers. Data characterizing the impact of more common exercise protocols have yielded mixed results. For example, acute endurace exercise in young mice results in rapid changes in ULK1, LC3-II, and p62 [29, 30] and the upstream signaling (AMPK and mTOR). In addition, an increase in LC3-II and a decrease in p62 expression were found from 120 min of exercise to time to exhaustion [30], consistent with an increase in autophagic activity in response to a common endurance exercise. Furthermore, Pagano et al. [30] observed that the autophagy initiator ULK1 was quickly phosphorylated on AMPK phosphorylation residues (Ser-317 and Ser-555) in oxidative muscles during a progressive endurance exercise. In contrast, mice subjected to shorter moderate to high intensity treadmill exercise showed a general decline or no change in Atg7, Beclin-1 and lysosome-associated membrane protein 2a [31]. Saleem et al. [32] also found no increase in several autophagic markers (LC3-II, p62, and Beclin-1) with exercise duration of about 90 min, even though the intensity was more elevated. Although it is not clearly determined whether acute exercise modulates the mRNA and/or protein levels of autophagic markers, exercise (muscle contraction) activate this atutophagy-dependent signaling. Indeed, He et al. [29] reported that mice with BCL2 knock-in mutations, displaying intact basal autophagy but defective stress-induced autophagy, show decreased phosphorylation and activation of AMPK and its downstream target acetyl-CoA carboxylase, as well as glucose gtransporter 4 (GLUT4). In addition, these mice fail to achieve exercise-mediated protection against high fat diet-induced glucose intolerance, supporting beneficial metabolic effects of stimulus-induced autophagy probably due to the disruption of the BCL2-Beclin-1 complex [29].

With regard to chronic endurance training, long-term voluntary running in young mice (4 weeks) increased autophagy flux (higher LC3-II and Beclin-1 and reduced p62/SQSTM1 proteins in the plantaris muscle [33]. These changes may be muscle-type specific as similar adaptations in basal autophagy were not detected following endurance training in the slow-oxidative soleus muscle. Nine weeks of resistance training prevented the loss of muscle mass and improved muscle strength in 18–20-month-old rats, accompanied by reduced LC3-II/LC3-I ratio, reduced p62 protein levels, and increased levels of autophagy regulatory proteins (Beclin-1, Atg5/12, and Atg7). Moreover, life-long exercise (regular exercise in past 30 tears) was found to attenuate the age-dependent autophagic disfunction (marked decrease in LC3-II and Atg7 proteins) in elderly sportsmen when compared with age-matched healthy sedentary [16].

Endurance training possesses a positive effect for some disease model by modulating autophagy. McMillan et al. [34] conducted 6 weeks of treadmill running for spontaneously hypersentive rats (SHR), which exhibits the elevation of Atg7 anv LC3-I proteins in white gastrocnemius muscle. Chronic exercise training increased Beclin-1, LC3, and p62 mRNA, but reduced these protein levels in muscle of

SHR rats. Intriguingly, exercise training had little effect on autophagy-related signaling factors (reduced AMPKa and LC3-II proteins and elevated p62 protein) in left ventricle in spite of increased proteasome activity and reduced apoptosis-dependent signaling. Exercise training-induced autophagic modulation is prone to skeletal muscle (exercise-recruited muscle) but not cardiac muscle. Mutations in the valosin containing protein (VCP) gene lead to Inclusion body myopathy associated with Paget's disease of bone and frontotemporal dementia and more recently affect 2% of amyotrophic lateral sclerosis-diagnosed cases. Nalbandian et al. [35] investigated the effect of downhill and uphill running training for experimental VCP mouse model carrying the R155H mutation. Progressive uphill exercise in VCP (R155H/+) mice revealed significant improvement in muscle strength and performance by grip strength and Rotarod analyses when compared to the sedentary mice. The uphill trained VCP (R155H/+) mice displayed an improvement in muscle atrophy, and decreased expression levels of several autophagic markers (ubiquitin, p62/SQSTM1, and LC3-I/II). There was also an improvement in the Paget-like phenotype. In contrast, mice trained to run downhill did not show any significant improvement. Thus, the manner of exercise-type may influence the effect of dystrophic phenotype. Collagen VI-null-mice also exhibits marked autophagic defect in skeletal muscle. These mice exhibit marked dystrophic phenotype in muscle showing an accumulation of defective mitochondria, as well as significant degeneration and exacerbated apoptosis [36]. In addition, these mice presented decreased endurance performance and altered glucose metabolism, including a decrease in the sarcolemma relocalization of GLUT4 during acute exercise. Intriguingly, neither long-term or shorter spurts of intense physical activity stimulated autophagy in Collagen VI-null-mice. Therefore, exercise therapy for muscular disease in order to improve autophagic defect should be considered with caution.

4.4 Unloading and Autophagy-Dependent System

Several studies investigated the changes in autophagy-linked molecules in the unloaded mammalian muscle. Recently, Smith et al. [37] demonstrated that hindlimb unweighting elicited a marked increase in mRNA expression of LC3B, Gabarapl1, and Atg4b mRNA in the quadriceps femoris muscle of mice (16–18 weeks of age) at earlier (2 days after operation) but not the later (7 days after this) periods. Using six-month-old male mice, hindlimb unweighting for 3 but not 7 days induced the siginificant up-regulation of p62 mRNA in the soleus msucle, in spite of no change in Beclin-1 mRNA at both periods. Intriguingly, Cannavino et al. [38] demonstrated that significant induction of p62 mRNA in unloaded muscle is prevented by PGC-1α overexpression but not Trolox (antioxidant treatment). Thus, the decrease in PGC-1α expression recognized in the unloaded muscle would be attributable to the increase in p62 expression. In contrast,

Table 4.1 The adaptive response in autophagy-linked molecules in the unloaded muscle

Author, year	Journal, volume, pages	Manner of analysis	Results	Species
Andrianjafiniony et al. [41]	Am J Physiol Cell Physiol 299: C307–C315	Western blot	Beclin-1 protein ⇔	Mouse
Liu et al. [42]	IUBMB Life 64: 393–402	Real-time PCR	Beclin-1mRNA ↓	Mouse
			p62/SQSTM1 mRNA ⇔	
		Western blot	LC3 protein ⇔	
			p62/SQSTM1 protein ↑	
Cannavino et al. [38]	J Physiol 592: 901–910	Real-time PCR	Beclin-1 mRNA ⇔	Mouse
			p62/SQSTM1 mRNA ↑	
Dupré-Aucouturier et al. [39]	J Appl Physiol 119: 342–351	Real time PCR	LC3B mRNA ⇔	Rats
			Gabalapl1 mRNA ⇔	
		Western blot	LC3II/I protein ratio ↑	
Smith et al. [37]	PLoS One 9: e94356	Real-time PCR	LC3B mRNA ↑	Mouse
			Gabarapll mRNA ↑	
			Atg4b mRNA ↑	
Baehr et al. [40]	Aging 8: 127–146	Western blot	Beclin-1 protein ⇔	Mouse
			p62/SQSTM1 protein ⇔	
			Atg7 protein ⇔	

Dupré-Aucouturier et al. [39] showed no significant change in mRNA levels of Gabarapl1, LC3B, and ULK1 in rat soleus muscle after 2 weeks of hindlimb suspension. Although some researcher reported the mRNA induction of autophagy-related molecules after hindlimb unweighting, many researchers demonstrated no siginificant change in protein level of autophagy-linked molecules. For example, the Western blot analysis of Baehr et al. [40] demonstrated that the levels in p62, Beclin-1, and Atg7 proteins did not significantly change in the unloaded (2 weeks) soleus and tibialis anterior muscles of male F344BN rats (9 month). In addition, hindlimb unloading elicits no significant change in the amount of Beclin-1 protein [41] and LC3 [42]. In contrast, some researchers indicated the elevatgion of p62 [42] and LC3-II/I [39] at the protein levels. Since the adaptive changes in autophagy-related molecules have been elucidated in the muscle of rodents only, the manner of adaptation should be investigated using human muscle sample by unloading model. Table 4.1 is summary of the adaptation in autophagy-linked molecules in muscle by hindlimb unloading.

4.5 Autophagic Adaptation in Sarcopenic Muscle

Sarcopenia, the age-related loss of skeletal muscle mass, is characterized by a deterioration of muscle quantity and quality leading to a gradual slowing of movement, a decline in strength and power, increased risk of fall-related injury, and

often, frailty [43]. von Haehling et al. [44] have estimated its prevalence at 513% for elderly people aged 60–70 years and 11–50% for those aged 80 years or above. Lean muscle mass generally contributes up to ∼50% of total body weight in young adults, but declines with aging to 25% at 75–80 years old [45].

A decline in autophagy during normal aging has been described for invertebrates and higher organisms [46]. Inefficient autophagy has been attributed a major role in the age-related accumulation of damaged cellular components, such as undergradable lysosome-bound lipofuscin, protein aggregates, and damaged mitochondria [46]. Demontis and Perrimon [47] showed that the function of autophagy/lysosome system of protein degradation declined during aging in the skeletal muscle of *Drosophila*. This results in the progressive accumulation of polyubiquitin protein aggregates in senescent *Drosophila* muscle. Intriguingly, overexpresssion of the FOXO increases the expression of many autophagy genes, preserves the function of the autophagy pathway, and prevents the accumulation of polyybiquitin protein aggregates in sarcopenic *Drosophila* muscle [48]. Several investigators reported the autophagic changes in aged mammalian skeletal muscle [17, 26, 49–51]. Compared with those in young male Fischer 344 rats, amounts of Beclin-1 were significantly increased in the plantaris muscles of senescent rats [51]. Using Western blot of fractionated homogenates and immunofluorescence microscopy, we recently demonstrated the selective induction of p62/SQSTM1 and Beclin-1 but not LC3 in the cytosol of sarcopenic muscle fibers in mice [17]. In addition, we observed a significant smaller p62/SQSTM1-positive muscle fibers in aged muscle compared to the surrounding p62/SQSTM1-negative fibers [17], (Fig. 4.1). In contrast, aging

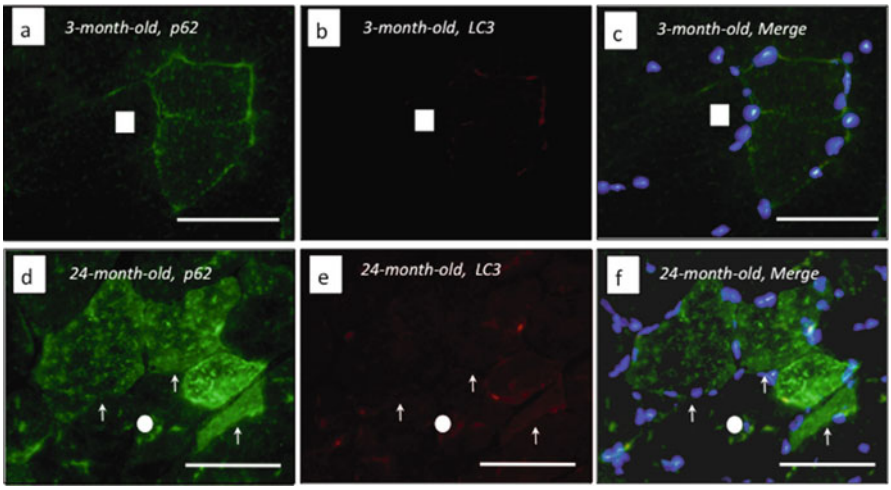

Fig. 4.1 Serial cryosections of the quadriceps muscle of 3- and 24-month-old mice. p62/SQSTM1 and LC3 immunoreactivity. In young quadriceps muscle, immunofluorescence labeling showed that p62/SQSTM1 was present in the membrane and at a low level in the cytosol of several muscle fibers (**a**). Marked increases of p62/SQSTM1 immunoreactivity were observed in the membrane and the cytosol of aged muscle fibers (**d**). No apparent difference in LC3 immunoreactivity was observed in the muscle between 3- and 24-month-old mice (**b** and **e**). White arrows denote the muscle fibers possessing p62/SQSTM1. Bar = 50 μm (Data from Sakuma et al. [17])

did not influence the amounts of Atg7 and Atg9 proteins in rat plantaris muscle [51]. Western blot analysis by Wohlgemuth et al. [51] clearly showed a marked increase in the amount of LC3 in muscle during aging. However, they could not demonstrate an aging-related increase of the ratio of LC3-II to LC3-I, a better biochemical marker to assess ongoing autophagy. In addition, we failed to detect marked increase in LC3-I and LC3-II (active form) proteins in aged quadriceps muscle [17]. In contrast, Wenz et al. [26] recognized a significant increase in the ratio of LC3-II to LC3-I during aging (3 vs. 22 months) in the biceps femoris muscle of wild-type mice. None of the studies determining the transcript level of autophagy-linked molecules found a significant increase with age [17, 49, 51]. Not all contributors to autophagy signaling seem to change similarly at both mRNA and protein levels in senescent skeletal muscle. Therefore, sarcopenia may include a partial defect of autophagy signaling, although more exhaustive investigation is needed in this field. Intriguingly, more recent study [16] using biopsy samples of young and aged human volunteers clearl showed tha age-dependent autophagic defect such as the decrease in the mount of Atg7 protein and in the ration of LC3-II/LC3-I protein.

Life-long caloric restriction alone, or combined with voluntary exercise, resulted in mild reduction of LC3 expression and lipidation coupled with increased lysosome-associated membrane protein 2 expression, suggesting a potential increase in autophagy flux. No significant age-related increase in autophagy-linked molecules was observed in MCK-PGC-1α mice. PGC-1α may also enhance autophagic flux. More recently, glycogen synthase kinase (GSK)-3α was proposed as a critical regulator of aging in various organs (skeletal muscle, heart, liver, bone, etc.) via modulating mTORC1 and autophagy [52]. Intriguingly, mice with null mutation of GSK-3α showed premature death and acceleration of age-related pathologies such as vacuolar degeneration, large tubular aggregates, sarcomere disruption, and striking sarcopenia in cardiac and skeletal muscle [52]. These GSK-3α knockout mice exhibited marked activation of mTORC1 and associated suppression of several autophagy molecules. Indeed, unrestrained activation of mTORC1 leads to profound inhibition of autophagy [53]. Therefore, it is expected that pharmacological inhibition (everolimus) of mTORC1 rescued the muscular disorder resembling sarcopenia in GSK-3α knockout mice [52]. Enhancement of autophagy flux (exercise, caloric restriction, etc.) would be a potential strategy attenuating sarcopenia as well as various type of muscular dystrophy with autophagy defect [36, 54]. Figure 4.2 summarizes a possible adaptation of autophagy-linked molecules (LC3 and p62/SQSTM1) in sarcopenic muscle.

4.6 A Marked Contribution of Autophagic Signaling to Cachexia

Cachexia is a complex metabolic syndrome characterized by a severe and involunrtary loss of muscle mass. Cachexia is associated not only with chronic diseases, most commonly cancer, but also with other inflammatory conditions such as chronic obstructive pulmonary disease (COPD), heart failure, chronic kidney disease, AIDS

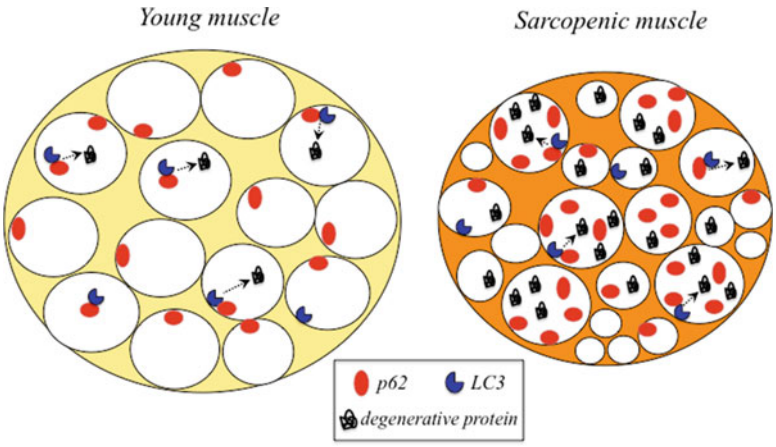

Fig. 4.2 The comparison of an autophagy-dependent system between young and sarcopenic muscle. In contrast to young muscle, sarcopenic muscle exhibits abundant p62/SQSTM1 proteins with no activation of LC3, showing apparent autophagy defects, which cannot destroy the degenerative (denaturing) proteins (Data from Sakuma et al. [55])

and sepsis [56]. The overall prevalence of cachexia is approximately 1% of the global patient population, which can increase to 50–80% in cancer patients [56, 57]. Indeed, almost 80% of cancer patients suffereing cachexia will be dead within 1 year of diagnosis.

As for cancer cachexia, earlier results obtained on muscles isolated from cachectic animals led us to rule out a substantial role for lysosomes in overall protein degradation [58]. In contrast, an elevation of total lysosome protease activity has been observed in the skeletal muscle and liver of tumor-bearing rats [59]. In addition, increased levels of cathepsin L. mRNA have been reported in the skeletal muscle of septic or tumor-bearing rats, whereas cathepsin B gene expression has been shown to be enhanced in muscle biopsy samples obtained from patients with lung cancer [60, 61]. Furthermore, a few general observations suggested that autophagy can be activated in the muscle of animals bearing Lewis lung carcinoma (LLC) or colon 26 (C26) tumor [62, 63]. More recently, Penna et al. [64] investigated whether autophagy signaling was elevated in muscle using three different models of cancer cachexia. They observed marked increases in the levels of Beclin-1, p62, and LC3-II (the lipidated form; a reliable marker of autophagosome formation) in muscle in C26-bearing mice. In addition, Penna et al. [64] evaluated autophagic markers in the gastrocnemius muscle of rats bearing Yoshida AH-130 hepatoma or of mice transplanted with LLC. Several autophagic markers were upregulated in the muscle of these two cancer cachexia rodent models, although there was some difference in the adaptive manner. Furthermore, OP den Kamp et al. [65] indicated that the levels of both LC3-I and -II proteins but not LC3B mRNA were significantly increased in the vastus lateralis muscle of patients with lung cancer cachexia. Esophageal cancer patients also appear to exhibit higher

Table 4.2 The adaptation of autophagy-related molecules in cancer cachectic muscle

Author, year	Journal, volume, pages	Manner of analysis	Results	Subjects
Penna et al. [63]	Am J Pathol 182:1367–1378	Western blot	Beclin-1 protein ↑	mouse (C26 tumor), rat (Yoshida AH-130 hepatoma & lewis lung carcinoma)
			p62/SQSTM1 protein ↑	
			LC3 II protein ↑	
Talbert et al. [67]	J Cachexia Sarcopenia Muscle 5:321–8	Real-time PCR	Beclin-1 mRNA ↑	mouse (C26 tumor)
			ATG5 mRNA ↑	
Tardif et al. [65]	Am J Clin Nutr 98: 1485–1492	Western blot	LC3 II protein ↑	human (esophageal cancer)
			p62/SQSTM1 protein ⇔	
			Cathepsin B & L protein ↑	
Op den Kamp et al. [64]	Am J Clin Nutr 98: 738–748	Real-time PCR	LC3 II protein ↑	human (lung cancer)
			LC3B mRNA ⇔	
		Western blot		
Johns et al. [68]	PLoS One 9: e83618	Western blot	Beclin-1 protein ↑	human (esophageal & pancreatic cancer)
			ATG5 protein ↑	

LC3-II/I ratios and levels of cathepsin B and L. expression in muscle [66]. Since they did not detect a significant change of proteasome, calpain, or caspase 3 activity in the muscle of these patients, they consider that the autophagic-lysosomal pathway is the main proteolytic system in the muscle in esophageal cancer cachexia. Table 4.2 summarizes the adaptive changes in several molecules in utophagy in cancer cachexia.

The functional importance of autophagy in the pathogenesis of lung disease in COPD patients has been demonstrated by Chen et al. [69] who described significant increases of autophagy in clinical lung samples taken from COPD patients. LC3B, Beclin-1, Atg7, and Atg5 were all upregulated, and autophagosome formation was visualized using electron microscopy.

In addition, Ryter et al. [70] have also described increased autophagy in clinical specimens of the lung from patients with COPD. They showed the increased expression and activation of autophagic regulator proteins (i.e., LC3B, Beclin-1, Atg5, Atg7) in lung. Similar evidence of increased autophagy was observed in mice subjected to chronic inhalation of cigarette smoke [69] and in lung epithelial cells exposed to aqueous cigarette smoke extracts [71]. Taking these findings together, autophagy seems to be activated in lung as a stress response. To date, little research has been completed on the contribution of the autophagy system to protein degradation and loss of skeletal muscle mass in COPD patients. Using muscle biopsy samples obtained from severe COPD patients with marked atrophy [forced expiratory volume in 1 s value of 35 ± 2% of predicted], Plant et al. [72] demonstrated that there was no difference in the levels of Beclin-1 and LC3

transcripts in the quadriceps muscle of patients with COPD compared with those in control individuals. On the basis of these results, Plant et al. [72] concluded that autophagy is not activated in muscles of COPD patients. However, they assessed the degree of autophagy by measuring mRNA levels only. More recently, Guo et al. [73] performed a pilot experiment using Western blot and real-time PCR mRNA measurements to evaluate autophagy-related gene expression of muscle biopsies obtained from cases of severe COPD. These experiments revealed significant increases in the intensity of LC3-II protein in muscle of COPD patients compared with that in control subjects. In addition, they also observed significant increases in Beclin-1 and p62 protein levels in muscle biopsies of COPD patients indicating the activation of autophagy. More complete elucidation of the functional role of autophagy in muscle of COPD patients remains to be determined, but some research in this field has been undertaken. It is probable that the activation of autophagy in the muscle of COPD patients is modulated by several factors, such as oxidative stress, inflammation, malnutrition, and therapeutic medication, as proposed in an excellent systematic review by Hussain and Sandri [74].

One original study investigated the relationship between chronic heart failure (CHF) and autophagy signaling in skeletal muscle [75]. It was suggested that there is a difference in the manner of autophagic adaptation between soleus (slow-type) and plantaris (fast-type) muscles by using rats with myocardial infarction. In fact, the transcription levels of GABARAPL1 and Atg7 were increased in the plantaris but not the soleus muscle. However, the expression levels of other autophagic markers (Beclin-1 and Atg12) did not change significantly. In addition, an autophagy-activating marker (LC3-II/I) also did not increase in both muscles. However, there have been no studies examining the autophagy in muscle in cases of CHF. It remains to be elucidated whether CHF includes autophagic activation in skeletal muscle similar to muscle in cancer cachexia and COPD.

4.7 Autophagic Adaptation in Muscular Dystrophy

A finely tuned system for protein degradation and organelle removal is required for the proper function and contractility of skeletal muscle [14]. Inhibition/alteration of autophagy contributes to myofiber degeneration leading to accumulation of abnormal (dysfunctional) organelles and of unfolded and aggregation-prone proteins [24, 76], which are typical features of several myopathies [36, 77]. Generation of Atg5 and Atg7 muscle-specific knockout mice confirmed the physiological importance of the autophagy system in muscle mass maintenance [24, 78]. The muscle-specific Atg7 knockout mice are characterized by the presence of abnormal mitochondria, oxidative stress, accumulation of polyubiquitinated proteins, and consequent sarcomere disorganization [24]. In addition, the central role of the autophagy-lysosome system in muscle homeostasis is highlighted by lysosomal storage diseases (Pompe disease, Danon disease, and X-linked myopathy), a group

of debilitating muscle disorders characterized by alterations in lysosomal proteins and autophagosome buildup [79]. Intriguingly, all of these myopathies exhibit the accumulation of autophagic vacuoles inside myofibers due to defects in their clearance.

Apparent defect of autophagy-dependent signaling is also observed in various muscular dystrophies. The first evidence of impaired autophagy in these models was provided by studies in mice and patients with mutations in collagen VI [76]. Mutations that inactivate Jumpy, a phosphatase that counteracts the activation of VPS34 for autophagosome formation and reduces autophagy, are associated with centronuclear myopathy [77]. De Palma et al. [54] have described marked defect of autophagy in dystrophin-deficient mdx mice and Duchenne muscular dystrophy (DMD) patients. This evidence included the electron microscopic evaluation of muscle tissue morphology as well as the decreased expression of autophagic regulator proteins (i.e., LC3-II, Atg12, Gabarapl1, BNIP3). In addition, starvation and treatment with chloroquine, potent inducers of autophagy, did not activate autophagy-dependent signaling in both tibialis anterior and diaphragm muscles of mdx mice [54]. Furthermore, mdx mice and DMD patients exhibited an unnecessary accumulation of p62 protein, which was lost after prolonged autophagy induction by a low-protein diet [54]. A similar block in autophagy progression was described in lamin A/C null mice [80]. LGMD2A muscles showed up-regulation of p62 (2.1-fold) and BNIP3 (3-fold) mRNA and slightly increased LC3-II/LC3-I protein ratio and p62 [81]. Conversely, laminin-mutated (dy/dy) animals displayed excessive levels of autophagy, which is equally detrimental [82]. These findings suggest that the defect of autophagy signaling has a central role in the degenerative symptoms in various types of muscular dystrophy. Figure 4.1 shows a schematic diagram of possible relationship between Akt-mTOR signaling and autophagy in muscular dystrophy.

4.8 The Autophagy and Glucose Metabolism

Skeletal muscle is the important tissue to maintain insulin sensitivity and optimal glucose concentration by glucose disposal. Glucose metabolism is the necessary process for our bodies to make energy and deterioration in this system causes diabetes mellitus. Diabetes population of the world continues to grow explosively. At present, type 2 diabetes mellitus is the most common endocrine disorder in the world. Type 2 diabetic patient exhibits high insulin resistance and decrease of glucose uptake. Autophagy-dependent signaling is expected to solve their problems by improving insulin resistance and glucose uptake in the skeletal muscle. Shi et al. [83] confirmed the decreased insulin sensitivity in autophagy-deficient L6 skeletal muscle cells by overexpressing an inactive mutant of Atg5. In addition, pharmacological inhibition of autophagy using bafilomycin or chloroquine led to the reduction of insulin-stimulated glucose uptake [84]. The autophagy-defective BCL2 AAA

mice, which contains knock-in mutations in BCL2 phosphorylation sites, showed an impaired insulin sensitivity and the plasma levels, and lower glucose uptake than in wild type mice [29]. In the pancreas, β-cells with autophagy deficiency induced an accumulation of amyloidgenic peptide oligomer that promotes type 2 diabetes [85]. Thus, autophagy is considered a necessary system for the maintenance of glucose metabolism.

The insulin secretion stimulates both AMPK and mTOR via the PI3K/Akt pathway. Increase of AMPK activates PGC-1α and ULK1 to induce autophagy. Autophagy improves PA or high fat diet induced-insulin resistance via the activated ULK1 or the AMPK – PGC-1α – SIRT3 signaling. DHM, an AMPK inducer, encourages autophagosome formation and enhances insulin sensitivity and glucose uptake.

Palmitic acid, a free fatty acid inhibits glucose uptake by enhancing the production of reactive oxygen species and insulin resistance. However, short-term administration of palmitic acid, is known to promote glucose uptake. Continuous administration with palmitic acid, for 30 min significantly increased the activity of several autophagy-linked mediators (LC3 and pULK1) in C2C12 myotubes [86]. Sixty percent high-fat diet feeding for 6 weeks significantly increased the levels of LC3-II, Beclin-1, fibroblast growth factor 21 compared with mice fed regular chow, but such insulin sensitivity of autophagy is impaired in adiponectin knockout mice [84]. In addition, treatment with adiponectin in L6 cells accelerates autophagosome formation and immune activity of LC3. Adiponectin seems to directly induce the activity of autophagy in vitro via AMPK-dependent pathway. Indeed, the inhibition of AMPK using compound C attenuates the adiponectin-induced expression of LC3-II and phosphorylation of ULK1. In other studies, short-term administration with palmitic acid increased the protein level of p-AMPK, whereas autophagy inhibitor 3-MA decreased expression of p-AMPK [87].

AMPK signaling is required for maintaining glucose homeostasis. Particularly, insulin and AMPK signaling pathways co-operates to regulate plasma glucose levels. Activation of both pathways induces the glucose uptake by increased translocation of GLUT4 to the plasma membrane in skeletal muscle [87]. Linying et al. indicated that Dihydromyricetin (DHM), a kind of flavonoid, improves the abnormalities in glucose metabolism via AMPK signaling [88]. In C2C12 myotubes treated with palmitic acid for 16 h, DHM increased protein levels of insulin signaling- (p-insulin receptor substrate-1 and p-AKT), autophagy- (LC3, Beclin-1, Atg5), and AMPK-related factors (p-AMPK, PGC-1α, SIRT3). At the same time, the glucose uptake is also improved with increasing the time and volume with DHM administration. siRNA transfection with PGC-1α or AMPK prevented DHM-induced autophagy activity. Therefore autophagy regulates the glucose metabolism through the AMPK in the skeletal muscle. Figure 4.3 summarizes the relationship between AMPK and autophagy in modulating glucose uptake in skeletal muscle.

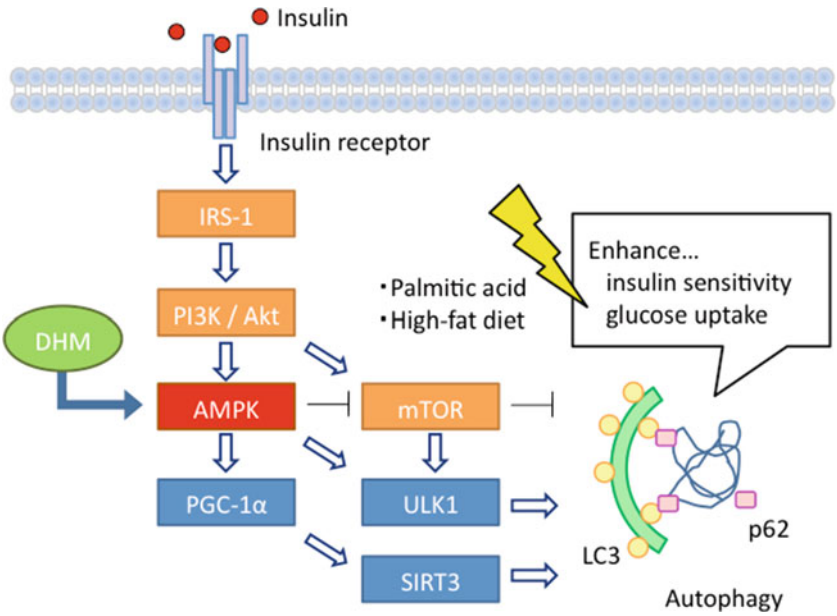

Fig. 4.3 The relationship between AMPK signaling and autophagy in skeletal muscle

4.9 Concluding Remarks

Particular emphasis has been placed on the role played by autophagic defects in disease pathogenesis, its involvement in atrophy, and the possible effects of exercise as a countermeasure. Indeed, endurance training possesses a positive effect for some disease model by modulating autophagy. In addition, autophagy regulates the glucose metabolism through the AMPK in the skeletal muscle. Over the past decade, studies using rodent muscles have indicated that atrogin-1 and MuRF-1 contribute to the protein degradation in various muscular wasting [89]. However, recent studies using human muscle do not necessarily support such a role for these atrogenes [90]. In addition, chronic inhibition of these atrogenes should not be considered a therapeutic target to counteract sarcopenia [6, 8]. The evidence described above supports that autophagy-dependent system regulates several atrophy model sucs as sarcopenia, cancer cachexia, and muscular dystrophy but not unloaded muscle. Intriguingly, the disorganization of the autophagy system seems to accerelate sarcopenic symptom in rodents and human because of no disposal of denaturing proteins and unfunctional mitochindria.

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Chapter 5
Cytokines in Skeletal Muscle Growth and Decay

Arkadiusz Orzechowski

Abstract By definition, cytokines are the first messengers of intercellular communications observed among leukocytes. Numerous cytokines control immune system and biological reactions thereof, but are functionally grouped into pro- and anti-inflammatory varieties (the latter are also involved in allergic reactions). The bulk of evidence points to substantial role played by cytokines in skeletal muscle growth and wasting. Cytokines and growth factors of immune origin affect skeletal muscle growth and organ formation, regeneration, and wasting but are also produced and secreted by muscle fibers as myokines. To orchestrate skeletal muscle growth, hepatocyte growth factor/scatter factor (HGF/SF) and insulin-like growth factors (IGF-I and IGF-II) play the primary physiological role being mediated through PI3-K/Akt signaling pathway. Skeletal muscle mass is in turn controlled negatively by myostatin, a member of transforming growth factor beta (TGF-β) superfamily. Following muscle injury, the immune-derived cytokines are most important in activation of muscle satellite cells: tumor necrosis factor alpha (TNF-α) and interleukin 6 (IL-6); with IL-8 to arrange growth/regeneration; and IL-15 to control muscle hypertrophy. Some life-threatening diseases are associated with muscle wasting featured by accelerated muscle protein breakdown. In these catabolic states, cytokines such as TNF-α was often reported as causal factor. Moreover, cross talk between myokines (IL-6, IL-15) and adipokines (leptin) is vital for correct metabolic interorgan relations. Thus, cytokines and growth factors exist as basic chemical signals that orchestrate skeletal muscle fate in normal and diseased states.

Keywords Cytokines • Growth factors • Skeletal muscle • Growth • Regeneration • Cachexia

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5.1 Cytokines in Skeletal Muscle Myogenesis and Somatic Growth

In vertebrates skeletal muscles develop during embryonic life from paraxial mesoderm (dermomyotome, myotome) specified to the myogenic lineage by paracrine signals from surrounding tissues. Wingless-related integration sites (Wnts), sonic hedgehog (SHH), and Noggin (NOG) stimulate, whereas bone morphogenetic protein 4 (BMP4) inhibits the commitment to the myogenic lineage. *Pax-3* and *Pax-7* are paired box genes targeted and co-expressed in the myotomal cells (epithelial spheres of paraxial mesoderm) [166]. Next, sequential activation of basic helix-loop-helix transcription regulators known as myogenic regulatory factors (MRFs) determines muscle progenitors termed myoblasts. Initially, *MyoD* and/or *Myf5* gene expressions are upregulated followed by *Myogenin* and/or *MRF4* gene activation. The latter are essential for induction of muscle-specific genes and terminal differentiation of mononuclear myoblasts [16]. Some of muscle-specific gene expressions such as myosin heavy chain (MyHC) or muscle creatine kinase (MCK) are valuable skeletal muscle cell markers. Dividing myogenic cells express *MyoD*/*Myf5* (myoblasts); subsequently they withdraw from the cell cycle to fuse upon *Myogenin* and/or *MRF4* control. Replication of activated satellite cells is possible by elevated levels of cyclin D, whereas terminal differentiation is upregulated by *Myogenin* gene expression [144]. Finally multinucleated cell syncytium (myotubes) is formed before establishing the fully mature contracting skeletal muscle fiber. Some myoblasts do not differentiate and remain dormant (satellite cells) embedded to the plasma membrane of contracting muscle fibers. After asymmetric cell division, they form a subpopulation apt to regenerate damaged muscle [46].

The major role in postnatal skeletal muscle growth is played by the growth hormone (GH)-IGF axis. Receptors for GH are present in sarcolemma, although direct binding of GH to muscle cells has never been demonstrated. In contrary to GH, IGF-I and IGF-II are able to alter MRF expression and promote muscle growth by increasing the proliferation and the differentiation of myoblasts [4, 38]. IGFs augment muscle mass through elevated DNA and protein synthesis [121, 158]. Increased number (hyperplasia) and fiber size (hypertrophy) are attributed to activation of satellite cells that in turn supply myonuclei to existing or newly formed muscle fibers [18, 19]. The hypertrophic effect of IGF-I differs from the general meaning of hypertrophy, as it is not barely increase in cytoplasmic-to-DNA volume ratio. IGFs are upregulated in skeletal muscle undergoing regeneration [48, 102], and these cytokines ameliorate muscle progenitor cells' (MPCs) viability and myogenic potential [95, 96]. Muscle growth and regeneration controlled by IGFs have distinct time windows, IGF-I being induced before IGF-II during muscle regeneration. In addition, IGFs' activity brought about in target cells is altered by local growth factor availability modulated by IGF-binding proteins (IGFBPs) [207, 208]. Furthermore, by suppressing the expression of muscle-specific atrophy-related ubiquitin ligases, muscle RING-finger protein 1 (MuRF1) and muscle

atrophy F-Box/atrogin 1 (MAFbx/atrogin 1), IGF-I inhibits protein degradation and protects skeletal muscle mass from loss. IGF-I differs from other growth factors as it stimulates both proliferation and differentiation of muscle cells [59]. Several existing isoforms of IGF-I (IGF-IEa, IGF-IEb, MGF) suggest distinct tasks played by each isoform and give possible explanation for apparently opposite roles played by IGF-I in muscle growth. Mechano growth factor (MGF) is believed to stimulate myogenic cell proliferation, while IGF-IEa seems to be decisive for terminal differentiation and muscle cell fusion [216]. It is less clear how IGF-II affects skeletal muscle growth, even though it is known that it acts on target cells through IGF-IR (no functional IGF-IIR was found) by autocrine and/or paracrine route. IGF-II was reported to accelerate terminal differentiation of C2C12 murine myoblasts in vitro and has some importance in prenatal skeletal muscle development [161]. Thus, IGF-I and IGF-II activate distinct signaling cascades, with IGF-II eliciting a stronger differentiation effect correlated with downregulation of $G_{\alpha i2}$ protein [160]. Insulin, the main anabolic hormone in postnatal life, has numerous actions on target cells with one that mimics IGF-I-dependent skeletal muscle growth-promoting effect. Similar to IGF-I, insulin encourages skeletal myogenesis through activation of membrane receptor-associated phosphatidylinositol 3-kinase PI3-K/Akt signaling cascade that in turn inhibits glycogen synthase kinase 3-beta GSK-3β and forkhead box protein O1 (FoxO1) [104].

5.2 Skeletal Muscle Repair and Regeneration

Regardless of injurious agent, any severe skeletal muscle damage starts the series of events that recapitulate myogenesis. Skeletal muscle regeneration is a highly coordinated process that absorbs adult muscle satellite cells to proliferate and differentiate. Following muscle injury, several biologically active substances are released, such as molecules from the injured fibers, soluble factors from connective tissue, and, finally, cytokines from infiltrating leukocytes. Some of them have been identified as trophic factors: fibroblast growth factor (FGF) and transforming growth factor beta (TGF-β) families, IGFs, hepatocyte growth factor/scatter factor (HGF/SF), tumor necrosis factor alpha (TNF-α), ciliary neurotrophic factor IL (interleukin)-6 (IL-6), and leukemia inhibitory factor (LIF). There are also neural-derived low molecular weight components including nitric oxide (NO) and ATP. Not only extracellularly released factors, but cell-cell and cell-extracellular matrix (ECM) communications are decisive to instigate muscle repair [49, 101, 182]. Disruption of muscle fiber integrity (necrosis) is critically important in the inflammatory response and recruitment of satellite cells to the site of injury (degeneration phase). Disturbed calcium homeostasis activates phospholipase A_2 with arachidonic acid being converted to prostaglandins and thromboxanes. Sarcoplasmic proteins released through discontinued sarcolemma, as well as inflammatory mediators, are believed to be chemoattractants for resident-activated mononucleated inflammatory and muscle satellite cells (regeneration phase).

Next, chemotactic signals attract circulating inflammatory cells. Reconstruction of injured muscle is a highly orchestrated process driven by chemical signals that grant the balance between pro- and anti-inflammatory factors. To fully resolve damage with newly formed or reconstituted muscle fibers, the kinetics of order of each phase (degeneration vs. regeneration) must be precisely synchronized. The magnitude of inflammatory reaction is highest within 1–4 days after muscle injury and lasts for another week or more [34]. Finally, after successive 3 weeks, skeletal muscle is fully restored morphologically and functionally. The exact timetable of regeneration is based on the observations obtained from laboratory animal experiments carried out after intramuscular myotoxin injections [42, 75, 76], crushing and/or freezing the muscle [98, 99], single muscle autologous transplantation, or after repeated bouts of eccentric exercise [15]. Alternatively, animal models of skeletal muscle dystrophies were in extensive use as they give opportunity to monitor the degeneration/regeneration process continuously. The *mdx* mouse line, i.e., the animal model of human Duchenne muscular dystrophy (DMD), is caused by deficiency of functional dystrophin protein, a fundamental component of dystrophin-glycoprotein complex (DGC). Truncated form of dystrophin renders skeletal muscle highly susceptible to contraction-induced injury as links between cytoskeleton and ECM are weak and during exercise sarcolemma is ruptured leading to myofiber necrosis [31]. From this and other mouse models established to study consequences of skeletal muscle damage, it is clear that, e.g., fibroblast growth factor (FGF) limits the efficiency of muscle regeneration [12]. Several FGFs have been shown as potent MPC mitogens and inhibitors of differentiation [4, 47, 70, 83, 98, 99, 112, 214]. Apparently, the main task of FGFs is to expand the MPC compartment. Among FGFs, the FGF-6 isoform is of particular interest, as this growth factor is specific for skeletal muscle and it is elevated during regeneration phase [43, 60, 83]. Similarly to FGF-6, the mitogenic effect was observed for FGF-2 and FGF-4 indicating possible redundancy among FGFs. Although FGFs stimulate satellite cell proliferation and muscle regeneration, the exact nature of FGF-induced skeletal muscle regeneration is still debated and some authors even point to improved revascularization as a cause, as FGFs are also highly angiogenic [100]. Physiological effects of FGFs are mediated through cognate transmembrane FGF receptors (FGFR 1–4) with the FGF-1R known to be the most prominent in skeletal muscle cells [162]. FGFRs possess intrinsic tyrosine kinase domain typical to mediate trophic actions of cytokines and growth factors. One has to admit that picture is more complex as ECM limits the access of several molecules to the plasma membrane receptors. Thus, it is obvious that availability of FGFs and other cytokines to regulate skeletal muscle growth and regeneration is altered by the composition of ECM. Heparan sulfate proteoglycans (HSPGs) are the most important for local modulation of signals from muscle and non-muscle cells. The synergy between FGFs and HGF/SF in MPC activation is believed to be dependent on HSPGs, as heparan sulfate assists in c-Met (tyrosine kinase domain) transmembrane receptor activation and amplifies downstream cellular signaling pathway [154]. For FGFRs

the most potent HSPGs are syndecans because syndecan-3 and syndecan-4 were identified on membrane surfaces of dormant and stimulated satellite cells [39]. Thus, MPCs are prepared by ECM for early activation by FGFs and/or HGF/SF, and changes in ECM composition (especially protein sulfation) may considerably impair MPC activation. With regard to HGF/SF, this growth factor is known to play substantial role in primary and secondary organogenesis in prenatal as well as organ regeneration in postnatal life [122, 123, 219]. Injured or crushed muscle is a rich source of HGF/SF, as both HGF transcript and the peptide are elevated at the onset of muscle regeneration [185]. It is not only a potent mitogen related to serine protease plasminogen (precursor peptide has a catalytic inactive serine protease homology domain which is cleaved through proteases like plasminogen activator), but it also stimulates morphogenesis and cell migration (scattering) [163]. HGF/SF is the ligand for proto-oncogene c-Met, plasma transmembrane receptor that contains the tyrosine kinase domain liable for autophosphorylation on tyrosine residues [118]. It plays dual role in myogenesis, as it encourages muscle satellite cells to divide while it mitigates their subsequent differentiation and fusion [64]. MPCs sense HGF/SF merely at the initial phase of muscle regeneration as exogenous injection of this peptide do not improve muscle repair at later stages [118]. Similarly, HGF/SF immunostaining is reduced at specimens obtained from damaged muscle with time after injury [185]. c-Met exposed to HGF/SF is able to activate MPCs through several signaling pathways (Gab1/Shp2/Ras/Raf/Erk, Grb2/Sos/Ras/Raf/Erk, Gab1/PI3K/Akt, Gab1/Crk/C3G/Rap1) which regulate cell proliferation/morphogenesis, survival, and actin reorganization, respectively. Pleiotropic effects of HGF/SF lead to increased MPC population and backing up satellite cell migration that in overall provide optimal myoblast density to the site of muscle injury. Besides HGF/SF secreted by MPCs in an autocrine route [165], certain pool is available from the ECM, especially when basal lamina is disrupted [186] or NO is released by nNOS at the motor end plates [13]. Other organs can also contribute to skeletal muscle regeneration, e.g., muscle damage stimulates spleen to secrete HGF/SF in rats [183]. Altogether, HGF/SF comes out to be a primary mitogen and the most important growth factor involved in skeletal muscle regeneration (Fig. 5.1).

5.3 Muscle Injury and Immune Cells

Even though molecular regulation of myogenesis and skeletal muscle regeneration are almost indistinguishable, the immune response is observed solely in harmed muscle. Immune cells are of myeloid origin and amount to high numbers ($10^5/mm^3$) in damaged muscle site. Immune cells actively secrete numerous cytokines and growth factors in order to modulate own inflammatory activity, and they affect the viability and transcriptional activities of regenerating muscle cells [190]. Most of the biological effects observed in the immune system are mediated by cytokines divided

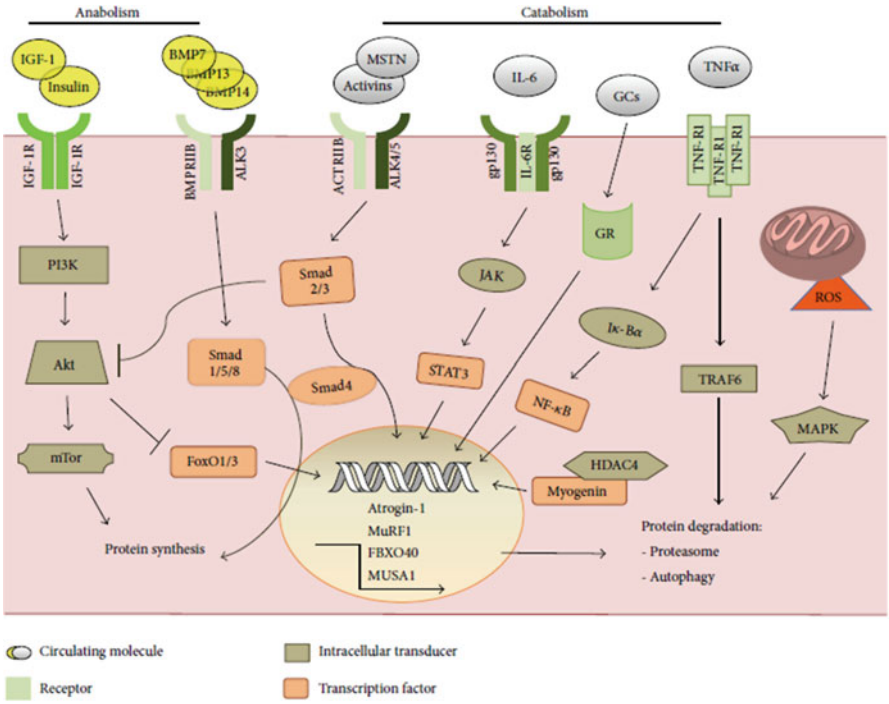

Fig. 5.1 Humoral mediators and associated pathways drive anabolic and catabolic responses in the skeletal muscle (Costamagna et al. [40])

into two groups: one represented by pro-inflammatory activity and second suited by anti-inflammatory action. Nowadays, there is a strong belief that the balance between pro- and anti-inflammatory cytokines is essential for accurate immune response to injury. Although expression of myogenic genes occurs in the absence of myeloid cells, the latter can control the extent and timing of expression. It is obvious that some cytokines endorse muscle satellite cell proliferation in vitro [29, 109].

T lymphocytes embody major source of cytokines with CD4-expressing T lymphocytes ($CD4^+$) also known as helper cells, the most useful cytokine producers. $CD4^+$ T lymphocytes could be further subdivided into Th1 and Th2, while the cytokines they secrete are termed Th1-type (pro-inflammatory such as interferon-γ, TNF-α, interleukin 1β) and Th2-type (anti-inflammatory such as interleukins 3, 5, 10, and 13), respectively. Inflammatory lesion in skeletal muscle begins the fast infiltration with $Ly6C^+/F4/80^-$ neutrophils during acute phase of inflammation (the second hour) peaking between 6 and 24 h postinjury. This phase ends up with phagocytic $CD68^+$ macrophages of M1 phenotype that rise in number between 24 and 48 h postinjury. M1 macrophages decline sharply 2 days postinjury, and this decline heralds the infiltration of non-phagocytic CD206-expressing macrophages of M2 phenotype (M2a, M2b, M2c) [170, 191]. The induction of MRF in skeletal

muscle progenitors is in striking coincidence with the release of inflammatory and vascular mediators by immune cells. First, the degree of inflammatory reaction affects regenerating muscle, with greater injury observed in wide-ranging infiltration by neutrophils and M1 macrophages. Free radicals (oxygen, nitrogen, and chlorate species) released by neutrophils and M1 macrophages lead to plasma membrane degradation (lipid peroxidation) and an extracellular debris for phagocytosis [27]. Thus, reductions in phagocyte-mediated damage facilitate and accelerate muscle regeneration [220].

One must bear in mind that innate immune response begins with TNF-α- and interferon-γ-driven activation of macrophages. These cells acquire M1 pro-inflammatory phenotype with vast generation of nitric oxide (NO) by inducible form of nitric oxide synthase (iNOS). By the way, other destructive nitrogen species such as peroxynitrite could come out [201]. Importantly, M1 macrophages express CD68, the receptor for oxidized LDLs which in turn stimulates phagocytosis and secretion of pro-inflammatory cytokines [153].

TNF-α plays a critical role in inflammation as this is the first cytokine released by neutrophils and M1 macrophages to trigger production of other Th1 cytokines. Besides, TNF-α was frequently reported to encourage skeletal muscle regeneration at early stage following acute injury as TNF-α null mutants and TNF-α receptor mutants had lower expression levels of MyoD and MEF-2 than wild-type controls [35, 204]. In differentiating C2C12 myotubes, TNF-α administration augmented cell growth [103], whereas it inhibited MyHC IIa protein expression in differentiated myotubes [145, 146]. Apparently, muscle cell fusion is inhibited, whereas myoblasts proliferation is stimulated by TNF-α that might trigger network of complex intracellular signaling pathways engaging NF-κB, signal transducer and activator of transcription (STAT-1α) and other transcription factors [120, 145, 146]. NF-κB activation by alternative pathway plays important function in skeletal muscle, as it promotes the expression and stability of cyclin D1 leading to increased cell proliferation and inhibition of differentiation [73]. Another explanation of NF-κB-dependent skeletal muscle growth retardation comes from the observation that MyoD and MyoD mRNA template are NF-κB targets for degradation [91]. Finally, NF-κB was shown to bind skeletal muscle transcriptional repressor, namely, YY1 which depresses activation of muscle-specific genes [203]. Thus, the exact role of TNF-α in muscle growth and regeneration varies on the degree of muscle damage, particular phase (transition from early to terminal differentiation), and cellular target such as NF-κB [218]. Although TNF-α levels peak at 24 h postinjury, this cytokine levels remain elevated up to 2 weeks following acute muscle injury which advocates principal role of TNF-α played in skeletal muscle repair [204]. It also points to TNF-α targets other than NF-κB muscle regulatory pathways, possibly p38 MAP kinase signaling [221]. Blocking the p38 kinase pathway resulted in diminished myogenesis and increased NF-κB activity in C2C12 myotubes [90]. TNF-α-dependent effects on muscle growth/regeneration are also associated with its chemoattractant attribute to stimulate directional migration of myoblasts and satellite cells [195]. Presumably, when TNF-α is released from polymorphonuclear

cells (PMCs) and M1 macrophages at the site of injury, it is chemical attractant for muscle stem and progenitor cells, by sensing this cytokine peel direction where the cell damage is to be compensated. Chemoattraction can be inhibited by deletion of TNF-α with specific antibodies or by the suppression of M1 macrophages [106, 115]. Elevated muscle protein loss is another important product of TNF-α activity regardless of circumstances. It is now well established that this effect of TNF-α is associated with induction of *FBXO32/Atrogin1* gene and its expression product atrogin-1 protein [24, 145, 146] mediated by p38 MAPK [103]. Apparently, TNF-α plays two opposite roles in muscle repair, one related to degenerative phase where it stimulates proliferation and migration of muscle stem and progenitor cells and the second where it holds back regenerative phase by impaired muscle cell fusion and accelerated protein decay [68, 145, 146].

Immune, type II, or γ-interferon (IFN-γ) is secreted by thymus-derived (T) cells under certain conditions of activation and by natural killer (NK) cells. IFN-γ controls several aspects of the immune response, comprising stimulation of bactericidal activity of phagocytes, stimulation of antigen presentation through class I and class II major histocompatibility complex (MHC) molecules, or by orchestration of leukocyte-endothelium interactions linked to cell proliferation and apoptosis [25].

IFN-γ is also another pro-inflammatory cytokine of Th1 series widely known from its pleiotropic activity taking in muscle growth and repair [36, 61]. It is worth noting, IFN-γ cooperates with TNF-α in many aspects of initial adaptation to skeletal muscle injury [10, 145, 146, 194, 207, 208]. Furthermore, IFN-γ and TNF-α relations are also evident during muscle cell differentiation when muscle fibers grow up and develop [145, 146, 168]. While early stages of skeletal myogenesis are apparently stimulated by both cytokines (augmented proliferation), later phases are markedly hampered, with noticed ATP-dependent proteolysis associated with E3 ubiquitin ligases atrogin-1/MAFbx and MuRF1 [145, 146]. The latter muscle-specific RING-finger protein 1 ligase and MAFbx are indicated as important IFN-γ and TNF-α targets in cancer cachexia [1]. Transcriptome analysis of differentiating C2C12 muscle cells revealed that IFN-γ affects the expression of several genes. The cytokine promoted cytokine/growth factor expression, cell proliferation, and migration but impaired muscle cell differentiation [72]. Presumably, the implementation of such a variety of effects by a single cytokine is achieved by complex patterns of skeletal muscle cell-specific gene regulation with IFN-γ response regulated by interaction with responses to other cytokines including TNF-α.

IFN-γ blocks the secretion of Th2 cytokines bulk which inhibits the inflammatory response. Thus, IFN-γ probably upholds inflammatory response of injured muscle in order to postpone tissue repair unless the population of muscle progenitor cells is sufficient for muscle regeneration. Th2 cytokines represented by IL-4, IL-10, and IL-13 stimulate M2 macrophages to take over M1 macrophages [69]. There are several subcategories of M2 macrophages indicated by small letters (M2a, M2b, M2c) (Fig. 5.2).

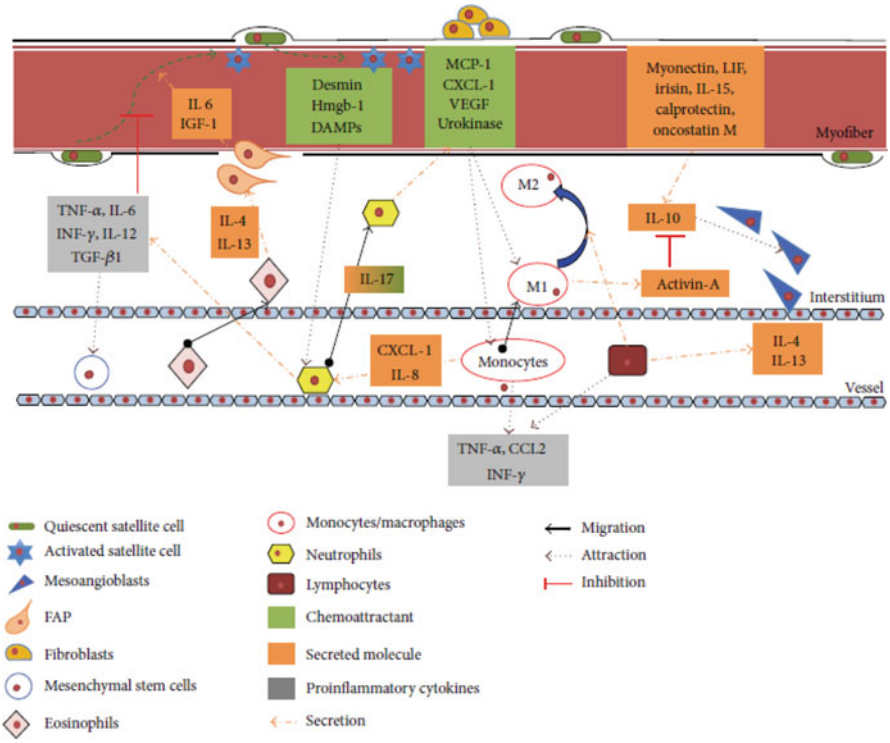

Fig. 5.2 Secreted molecules and paracrine effects from resident and circulating cells involved in skeletal muscle inflammation (Costamagna et al. [40])

5.4 Skeletal Muscle as an Important Source of IL-6

Myokines, a term used to describe a group of cytokines produced by skeletal muscle during and following exercise, point to a brand new role played by this organ [138]. Except MGF, remaining myokines are identical to previously described and identified leukocytes (IL-6, IL-1, interleukin 1 receptor antagonist (IL-1ra), IL-8, macrophage inflammatory protein α and β (MIP-1α and MIP-1β), TNF-α). Nowadays, it is widely recognized that strenuous prolonged exercise, e.g., marathon run, causes huge increase in the concentration of several pro- and anti-inflammatory cytokines in the peripheral blood [51, 52, 138]. Previously, it was well established that physical exercise stimulates the leukocytosis (increased number of leukocytes in peripheral blood). However, the origin of myokines is different from immune cells as both transcripts and proteins concentrate in skeletal muscles [66, 86, 176, 179]. The most prominent among myokines is IL-6 for the reason that following exercise this cytokine basal plasma levels may increase up to 100-fold

[58, 132, 135, 140–142]. The peak IL-6 plasma concentration is observed at the end of the exercise or soon after [56, 57, 58, 132]. For a long time, IL-6 has been considered as classical inflammatory cytokine suggesting that muscle fiber injuries promote almost exponential raise of the cytokine plasma level during a bout of heavy exercise. This assumption has no evidence as increase in IL-6 is observed in both non-damaging (concentric) [211] and damaging (eccentric) skeletal muscle contractions [107]. What is the role of IL-6 and why this myokine is that much upregulated in exercised skeletal muscle? Since IL-6 release by skeletal muscle is proportional to the muscle mass, exercise intensity, and duration (twofold increase in IL-6 level was observed after 6 min, tenfold after 2 h, and 100-fold after 6 h of running), there is presumption that this myokine is of paramount importance for homeostasis in contracting muscles, as resting muscles exposed to identical hormonal milieu do not release IL-6 [78]. Fluorescence-activated cell sorting (FACS) of peripheral monocytes isolated from blood samples of cycling or running athletes did not demonstrate any significant changes in IL-6 even during prolonged exercise [173–175]. These and other experiments show that contracting skeletal muscle is the major source of IL-6 in the peripheral blood in response to exercise and that muscle fibers but not myoblasts, endothelial cells, fibroblasts, or smooth muscle cells account for the systemic increase of plasma IL-6 [77, 86, 108, 143]. Interestingly, exercise-induced increase in IL-6 release from the exercised legs (based on arteriovenous difference) could be blocked completely by the supplementation of vitamins C and E for 4 weeks prior to the challenge [56, 57]. It appears that *IL-6* gene activation is redox mediated in skeletal muscles following exercise as antioxidants abolish this effect. Ablation of redox stimulus was also observed in differentiating myoblasts following vitamin C preincubation [131]. Given that IL-6 derived from skeletal muscle is the normal physiologic response to exercise, one may ask about its role and significance. IL-6 appears to be involved in glucose homeostasis [181, 66], and IL-6 response to exercise is dependent on pre-exercise skeletal muscle glycogen content [176]. Moreover, carbohydrate supplementation attenuates the increase of plasma IL-6 of skeletal muscle origin [174, 175]. In addition, IL-6 infusion is accompanied by extensive lipolysis and accelerated fat oxidation pointing to its metabolic rather than immunologic function [199]. Nowadays, the consistent findings indicating IL-6 secretion from exercised muscles in the absence of noticeable inflammatory markers designate this cytokine as a master switch that stimulates utilization of neutral fat when carbohydrates are in shortage. With respect to IL-6, skeletal muscle is prompted to transcriptional, translational, and secretory activities in reaction to rise in sarcoplasmic Ca^{2+} level during repetitive contractions. Importantly, the effect is calcineurin/nuclear factor of activated T-cell (NFAT) dependent (inhibited by cyclosporin A and potentiated by ionomycin). Sustained intracellular Ca^{2+} is a well-known calcineurin activator which is calcium- and calmodulin-dependent serine/threonine protein phosphatase upstream to NFAT [62]. Furthermore, *IL-6* gene expression at transcriptional and translational levels is controlled by calcineurin/NFAT in cultured muscle cells [86]. NFAT may be rephosphorylated by NFAT kinases like glycogen synthase

kinase (GSK-3β) which makes NFAT inactive and withdrawn from the nucleus [54]. We observed critical role played by GSK-3β in myogenesis, as both insulin and metabolic inhibitors of this kinase led to ameliorated muscle fiber formation [104]. Strict pro-inflammatory TNF-α is repressed in the same circumstances (exercised muscle) demonstrating that IL-6 and TNF-α are regulated differently in skeletal muscle cells by altered Ca^{2+} levels [84]. In addition, other possible signaling pathways explain IL-6 increase in exercised skeletal muscle. Abundantly expressed neuronal NO synthase isoform (nNOS) points toward NO production within contracting skeletal muscles with cGMP and/or nitrosative mechanisms leading to IL-6 production [93, 94, 156, 167]. Bulk of evidence collected from the experiments with NO donors showed increase in IL-6 mRNA content and IL-6 release from skeletal muscles challenged with NO, whereas opposite response was noted upon the use of nNOS inhibitors [178]. Nuclear factor kappa light chain enhancer of activated B cells (NF-κB) is another candidate as it is redox-sensitive transcription factor, while trained skeletal muscle is a rich source of reactive oxygen species (ROS) [167]. Antioxidants were mentioned as IL-6 repressors [189, 200]; thus it is very likely that ROS formation in skeletal muscle following exercise causes IL-6 release in a NF-κB-dependent manner. In order to position NF-κB in the redox-dependent cell signaling, nonsteroidal anti-inflammatory drugs (NSAIDs) were used as NF-κB inhibitors [88]. Actually, indomethacin diminishes the exercise-induced increase of IL-6 further supporting the notion of causal relationship between skeletal muscle contractions, NF-κB and IL-6 synthesis [155]. Moreover, basal IL-6 plasma concentrations are highly correlated with the skeletal muscle training, being lower in trained vs. non-trained individuals [32, 137]. Despite the marked rise in IL-6 mRNA during exercise, the long-term training (1 h, 5 times a week for 10 weeks) led to almost ten times lower IL-6 mRNA content in exercised skeletal muscles [56, 57]. Concomitantly IL-6R mRNA content was almost doubled in trained skeletal muscle [85]. It reveals the nature of feedback mechanism of IL-6 myokine; while plasma IL-6 is apparently downregulated by training, the muscular expression of the IL-6R is upregulated at the same time to augment IL-6 sensing by muscle fibers. The last but probably not the least, signaling pathway activated in skeletal muscle during contraction is p38 MAPK known as stress-activated protein kinase [21]. In fact, fatigued muscle with reduced glycogen content has been found to boost the p38 MAPK activity and its nuclear translocation [33]. Moreover, nuclear p38 MAPK phosphorylation through MAPK kinase (MKK-3 and MKK-6) is known to activate *IL-6* gene since inhibition of p38 MAPK results in loss of the IL-6 mRNA transcriptional control [33]. For years, p38 MAPK was shown to be essential for muscle fiber formation in myogenesis; thus its additional control of *IL-6* gene brings the role of the kinase updated [41, 90, 210]. It is very likely that the downstream targets of p38 MAPK, ATF-2, and Elk1 may participate in regulating the expression of *IL-6* gene, owing to the fact that ATF-2 is a subunit of the AP-1 heterodimer (Jun:ATF) [198], while Elk1 is a member of the Ets superfamily of transcription factors [217].

5.5 Cytokines Negatively Targeting Skeletal Muscle

Chronic systemic inflammation (CSI) is a cause of cardiometabolic syndrome (CMS) associated with higher risk of cardiovascular disease, type 2 diabetes, and muscle cachexia (muscle wasting) [44, 53, 141, 142, 149, 157, 192, 209]. Two- to fourfold rise in plasma concentrations of pro- and anti-inflammatory cytokines and acute-phase proteins are regarded as a common attribute of CSI [28]. Regular exercise prevents CMS almost certainly through the elevated levels of anti-inflammatory cytokines [IL-1ra, soluble TNF-α receptors (sTNF-R), and IL-10] which in turn override inflammatory response [134, 136, 139]. Throughout CSI several pro-inflammatory cytokines are elevated in blood plasma, TNF-α, IL-1β, and IL-6, even though TNF-α plays the central role in insulin desensitization of skeletal muscle [147, 148, 196]. Insulin resistance results in marked decline of its anabolic effect and accelerated loss of skeletal muscle mass typical for type 2 diabetes [147, 148]. A bulk of evidence confirms that TNF-α-induced insulin resistance is associated with NF-κB and STAT-1α activation and inhibition of PI3-K/Akt signaling pathway [26, 145, 146]. Thus, TNF-α represents the causal factor of insulin resistance [147]. It appears that the role of IL-6 in muscle decays as pro- or anti-inflammatory cytokine is less clear as indicated by conflicting data [141, 142, 149, 172, 177]. Observations indicate that IL-6-deficient animals (knockout mice) have higher levels of circulating TNF-α [119], while IL-6 inhibits TNF-α formation [55, 110]. Additionally, IL-6 stimulates formation of anti-inflammatory cytokines IL-1ra and IL-10 [177]. IL-1ra is of particular importance as it weakens inflammatory response being the efficient blocker of IL-1α and IL-1β binding to cognate receptors [45]. Once it is established that IL-6 has anti-inflammatory rather than pro-inflammatory characteristics, the rationale for protective role of physical activity against muscle wasting is to be revealed. Nonetheless, there is no direct relationship between exercise and reduced CSI although several studies have reported improved status of patients with CMS [67, 180]. To decipher the physiological role of IL-6, further studies are needed, as IL-6 levels are augmented both in patients with metabolic disease featured by insulin resistance and hyperlipidemia [37, 129] and after strenuous exercise which improves insulin-stimulated glucose uptake and plasma lipid profile [212]. This actually contradicting observations might be explained by simultaneous alterations in TNF-α concentration as increased TNF-α and decreased IL-6 transcription lead to higher incidence of diabetes [89]; however, the exact etiology of the CMS has at least few scenarios including elevated IL-6 as a systemic feedback mechanism to adipocyte-secreted TNF-α or alternative route to sensitize skeletal muscle to insulin or perhaps it results from "IL-6 resistance." None of these hypotheses have sufficient substantiation.

For decades TGF-β superfamily of cytokines (growth factors) has been acknowledged as inhibitors of both myoblasts proliferation and differentiation [3, 4, 23, 79]. Among several members myostatin (MSTN) also identified as growth and differentiation factor-8 (GDF-8) attracts special interest as *MSTN* knockout mice have unusually large shoulder and hip muscles [114, 184, 222]. "Loss of function" muta-

tions in *MSTN* gene were demonstrated to enlarge skeletal muscles by increased number and size of muscle fibers in other species including human beings [71, 81, 82, 113, 164]. When secreted, MSTN protein decreases myoblast proliferation by stimulating p21 protein, a well-known cyclin-dependent kinase Cdk2 inhibitor [80, 187, 188]. Cdk2 in turn inhibits G1 to S phase transition. Molecular mechanism of MSTN activity seems to hamper satellite cell proliferation through activin type II receptor (Act RIIB) as dominant negative form of Act RIIB in transgenic mouse lines exhibited dramatic increases in muscle mass comparable to those seen in *MSTN* knockout mice [97]. MRF genes are transcriptionally repressed by MSTN because signal transduction from Act RIIB directly activates Smad2/3 proteins with resultant fall in *MyoD* and *myogenin* gene expression [92, 105]. Moreover, MSTN is associated with increased Smad2/3 phosphorylation levels and decline in *MyoD* and *myogenin* gene transcription [206]. Furthermore, satellite cell proliferation is noticeably elevated in *MSTN*-null mice [111]. MSTN is produced by skeletal muscle and adipose tissue [6], even though its expression levels are subject to regulation following muscle damage [215]. Fasting and glucocorticoids were indicated as possible *MSTN* gene incentives [11, 159], and both C/EBP-δ and fewer miR-27a/b seem to mediate the respective muscle wasting effects [5, 8]. Additionally, the FoxO1 and TGF-β/Smad3, MyoD, and JNK/p38 kinase pathways are involved in MSTN secretion [9, 74, 169]. The former, FoxO1-dependent muscle atrophy results from insulin resistance, as insulin inhibits FoxO1 in skeletal muscle cells [104]. Additionally, constitutive FoxO1 activity causes *MSTN* gene disinhibition [9] in insulin-resistant states being followed by elevated MSTN expression [7]. In contrast, *MSTN* is downregulated during muscle regeneration and its levels are returned to normal in mature fibers [116, 193, 197, 222]. To some extent, there is also contribution of MSTN to aberrant regeneration in *mdx* mice (animal model of Duchenne muscle dystrophy) as improved muscle regeneration was observed in *mdx:MSTN*$^{-/-}$ mice [202].

5.6 Myogenic Myokines

Different cytokines are produced and released by skeletal muscle, while the exact role played by each is not fully elucidated. Despite IL-6 which is fairly well described in the context of skeletal muscle as endocrine organ, IL-8 is acknowledged as potent chemoattractant for neutrophils during acute phase of inflammatory reaction [17]. IL-8 belongs to distinct family of CXC chemokines where the acronym's C stands for cysteine while X is any amino acid that separates two cysteine residues at the NH_2 terminus. Interestingly, IL-8 plasma concentration is altered by physical activity but barely moderate-to-exhaustive eccentric exercise rouses *IL-8* gene expression in skeletal muscle and its blood plasma levels [125, 126]. A number of reports corroborated elevated IL-8 plasma levels following training during and after marathon run [127, 128, 133]. While accurate physiological

IL-8 function in skeletal muscle is not known, some authors ponder IL-8 as locally acting and associated with mild inflammatory response associated with single muscle fiber damage. This assumption is substantiated by the miniature IL-8 observed during and following concentric muscle contractions that in general are not injurious to skeletal muscle. Some studies indicate that IL-8 is angiogenic. Summing up, IL-8 by binding to its receptor CXCR1 induces chemotactic effect, whereas it stimulates endothelial epithelium for neovascularization through CXCR2 [20, 87, 130].

Besides *IL-6* and *IL-8*, also *IL-15* gene was shown to be induced in skeletal muscle during strength training [124]. The IL-15 signaling peptide acts on target cells by attaching to heterotrimeric membrane receptors (IL-15Rαβγ) with Janus kinases (JAK1 and 3) and STAT-3 and STAT-5 [65]. Since IL-15 cytokine is present as intracellular and secretory form, its detection is not easy carrying inconsistent reports [124, 125, 132, 135]. Anyway, several "in vitro" studies carried out on myogenic cells revealed IL-15 as anabolic factor capable to increase MyHC in differentiating muscle cells autonomously from IGFs [63, 151]. Similarly, this cytokine was demonstrated to have anabolic effects on skeletal muscle "in vivo" [150]. Furthermore, IL-15 participates in cross talk between muscle fibers and adipocytes [14]. It reduces adipose tissue mass with concurrent skeletal muscle overgrowth [30], and this is a unique muscle-to-fat endocrine axis with high therapeutic potential to combat muscle wasting in cachexia. The distinctive function of IL-15 is manifested in fully differentiated muscle cells where this cytokine represses proteolysis in muscle cachexia [150]. Accordingly, the IL-15 mRNA was markedly upregulated in differentiated skeletal myotubes but not undifferentiated myoblasts [152]. Summing up, IL-15 could be regarded as skeletal muscle growth-promoting myokine underestimated in prevention of muscle wasting.

5.7 Adipokines in Skeletal Muscle Growth

There is more than 30 different adipokines produced by adipocytes, making adipose tissue a significant source of regulatory factors with some being essential for fat-to-muscle communication. Leptin peptide, a "satiety hormone" is probably the most interesting amongst adipokines. It acts on target cells similarly to IL-6 as both leptin receptor (LRb) and IL-6 receptor (gp130Rβ) share a sequence homology liable for JAK recruitment and STAT phosphorylation needed for gene regulation although LRb and gp130Rβ are ligand specific without cross-reactivity [50]. Can a common signaling pathway for leptin and IL-6 evoke distinct cellular responses? The answer is yes, as IL-6 binds initially to IL-6Rα and as multifunctional cytokine exerts its biological activities through two molecules: IL-6Rα (IL-6 receptor) and gp130Rβ. When IL-6 binds to mIL-6Rα (membrane-bound form of IL-6R), homodimerization of gp130β is induced and a high-affinity functional receptor complex of IL-6, IL-6Rα, and gp130β is formed. Interestingly, sIL-6R (soluble form of IL-6R) also binds with IL-6, and the IL-6–sIL-6R complex can then form a complex with gp130β

[117]. The complex activates JAKs that then phosphorylate tyrosine residues in the cytoplasmic domain of gp130β. The gp130β-mediated JAK activation by IL-6 triggers two main signaling pathways: the gp130β Tyr759-derived SHP-2 (Src homology 2 domain-containing protein tyrosine phosphatase-2)/ERK (extracellular signal-regulated kinase) MAPK (mitogen-activated protein kinase) pathway and the gp130β YXXQ-mediated JAK/STAT pathway [171]. In contrast to LRb, gp130β has more tyrosines for phosphorylation ($Y^{767, 814, 905, 915}$) which is functionally important to negate feedback mechanism mediated by suppressor of cytokine signaling (SOCS) proteins [205]. SOCS3 binds to phosphorylated LRb through its SH2 domain to stop JAK-mediated phosphorylations [22] and additionally recruits ubiquitin-transferases for JAK/STAT degradation [2]. Consequently, leptin-dependent regulation of skeletal muscle is transient, whereas IL-6 seems to induce long-term effects. It was noticed that both cytokines possibly stimulate AMP-activated protein kinase (AMPK) by T^{172} phosphorylation mediated by AMPK kinase (LKB1) [213]. Alternatively, by increased cellular ATP turnover, leptin and IL-6 may activate AMPK with elevated levels of allosteric activator AMP. This system is fundamental for energy sensing and fatty acid oxidation in skeletal muscle, so leptin and IL-6 are mighty directors in delivery of substrates for energy production (acetyl-CoA). Recently, we observed other metabolic effects of leptin. In cultured C2C12 mouse myogenic cell line, this cytokine stimulated myoblast mitogenicity and inhibited muscle cell differentiation through JAK/STAT and MEK signaling pathway where MEK is an upstream ERK kinase (MAPKK) [145, 146]. Leptin through MEK-dependent manner caused GSK-3β phosphorylation (Y^{216}-GSK-3β) with resultant drop in myoblast viability and fusion. Overall, more research on adipokines is required in order to shed light on fat-to-muscle influences.

5.8 Conclusions and Perspectives

Skeletal muscle fibers as permanent cells do not divide, so upon injury this organ regeneration is entirely reliant on stem cell subpopulations including satellite cells which are activated by a myriad of signals where cytokines play the prime role. Nowadays, it is widely accepted that cytokines which were initially specific growth/differentiation factors of leukocyte origin are also synthesized and secreted by other tissues including skeletal muscle (myokines). It appears, that myokine profile is dependent on the physiologic (type and intensity of exercise, muscle fiber type) or pathologic (the magnitude of injury) conditions. Thus, it is obvious the pattern of myokines controls skeletal muscle growth and decline in health and disease. Skeletal muscle growth is regulated by mitogenic and survival signals (HGF/SF, FGF, IGFs, LIF), whereas damaged skeletal muscle is characterized by necrosis and activation of pro-inflammatory (TNF-α, IFN-γ, IL-1β, IL-6) followed by anti-inflammatory cytokines (IL-1ra, soluble TNF-α receptors (sTNF-R), and IL-10) targeting muscle satellite cells and controlling skeletal muscle degeneration

and regeneration phase, respectively. Finally, TGF-β superfamily of cytokines, greatly MSTN, stops further growth and muscle tissue regeneration. As skeletal muscle satellite cells are decisive for skeletal muscle regeneration, understanding the molecular mechanisms of their activation by myokines seems to be essential to combat aberrant muscle growth and repair.

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Chapter 6
The Role of Ribosome Biogenesis in Skeletal Muscle Hypertrophy

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Abstract The regulation of muscle size is primarily determined by protein metabolism. During periods of rapid muscle wasting, protein degradation has a major role, whereas during muscle hypertrophy the main force driving the increase in myofiber size is muscle protein synthesis. Although the majority of studies to date have focused on the short-lived responses in protein synthesis following an acute bout of resistance exercise, accumulating evidences in recent years have convincingly demonstrated that ribosome biogenesis as a main source of translational capacity plays an important role in muscle growth. This chapter will focus on muscle ribosome biogenesis in relation to the biology of muscle growth and its important implications for clinical studies.

Keywords Ribosomal RNA • Translational capacity • Muscle growth • mTOR

6.1 Background

Skeletal muscle is a highly plastic tissue, responding to the demand it is subjected to and adapting whenever conditions are permissive. The regulation of muscle size relies on protein metabolism, in which protein synthesis and protein degradation are the forces driving the resultant outcome, i.e., accumulation or loss of muscle proteins. Protein synthesis, or the mRNA translation into the corresponding polypeptide chain, is catalyzed by the ribosomes. The mature human ribosome (or 80S) is a

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complex formed from 4 different ribosomal RNAs (rRNA) and ~80 ribosomal proteins. The ribosome is composed of two subunits: the 40S or the small subunit, containing the 18S rRNA and 33 proteins, and the 60S or large subunit, consisting of 28S, 5.8S, and 5S rRNAs and 47 proteins [25].

The anabolic processes demand cellular resources and energy [61]. During skeletal muscle growth, muscle cells – as any other cell type – the synthesis of new proteins, and new ribosomes need to be tightly adjusted to the amount of substrate, such as nucleotides, amino acids, and nucleosides triphosphate. Thus, protein synthesis and ribosome biogenesis, as the major ATP-consuming processes, are constantly kept in check by many cellular cues and signaling pathways. The focus of this chapter is on the basic regulation of ribosome biogenesis, including the signaling modulating ribosomal DNA transcription, and the recent evidence on the role of ribosome biogenesis in muscle mass regulation.

6.2 Translation Capacity and Efficiency

Although the central dogma of molecular biology states that information flows from DNA to mRNA to protein, the relationship between each of these macromolecules in the cell is not linear or straightforward. This observation reflects the complex interplay between transcription, translation, mRNA, and protein stability (half-life) and the multiple points of regulation involved in gene expression [12, 14]. The transcription of DNA into mRNA and the translation of mRNA into protein (or protein synthesis) are key molecular nodes in the regulation of gene expression. While there does exist a relatively good correlation between mRNA and protein levels, the majority of gene expression appears to be regulated at the level of translational [49]. The rate of protein synthesis is primarily influenced by two different, but related, characteristics: translational efficiency and translational capacity [23, 37, 40]. Translational efficiency is described as the rate of translation per unit of cellular RNA or ribosome whereas translational capacity is defined as the total ribosome content of the cell. Given that the vast majority of cellular or tissue RNA (~80%) is comprised of ribosomal RNA [41, 61], total RNA has been used as a proxy measurement of ribosomal mass and, therefore, translational capacity.

The energetic and resource cost involved in translation – both the efficiency and the regulation of capacity, i.e., ribosome biogenesis – has been shown to require more energy and resources than any other cellular process [33, 34, 61]. Thus, the allocation of both cellular energy and resources to translation at rest and during growth is highly regulated in an effort to avoid the emergence of a futile cycle. Furthermore, the regulation of translation efficiency and capacity is strongly influenced by the energy status of the cell as well as nutritional, hormonal, and mechanical cues.

6.2.1 *Translation Capacity in Skeletal Muscle: There and Back Again*

Translation capacity and efficiency are not new concepts to the field of skeletal muscle physiology. For decades, researchers have been interested in better understanding the relationship between translational efficiency, capacity, and skeletal muscle growth. Some of the earliest studies reported that during skeletal muscle growth or atrophy, there were changes in both translational efficiency and capacity as reflected by changes in the ratio of protein to total RNA and the total RNA per unit of tissue, respectively [15–17, 24, 40].

Despite this early recognition of the importance of both translational efficiency and capacity in muscle growth and atrophy, most recent studies in the field of muscle physiology have focused on translational efficiency in the regulation of muscle growth based on the notion that acute changes in the rate of protein synthesis in response to nutrition and/or resistance exercise are the primary determinant of skeletal muscle hypertrophy. However, efforts to demonstrate that acute changes in the rate of protein synthesis are predictive of long-term skeletal muscle hypertrophy have yielded conflicting results [3, 36, 39]. The reason for this discrepancy remains to be resolved, but it has been suggested the acute change in the rate of protein synthesis is more an indicator of repair and remodeling processes following resistance exercise rather than an indicator of hypertrophic growth [2]. Conversely, human studies have reported an increase in the rate of protein synthesis at rest after resistance exercise training [4, 26, 64], and more recently, these changes have been attributed to a greater translational capacity as a consequence of ribosome biogenesis [9]. Therefore, it is likely that muscle growth is a combination of both changes in translation efficiency following each bout of resistance exercise training but also, and perhaps more important, changes in translation capacity observed with chronic training.

6.3 Overview of Ribosome Biogenesis

The 18S, 5.8S, 28S, and 5S rRNAs that form the mature ribosomes are produced by the transcription of two genes. The first three rRNAs – 18S, 5.8S, and 28S – arise from a single transcript, the 45S precursor rRNA (pre-rRNA). The 45S gene (the so-called ribosomal DNA) is transcribed by the dedicated RNA polymerase I (Pol I) [8]. The last rRNA, the product of the 5S gene, is transcribed by the RNA polymerase III (Pol III). The Pol III transcribes not only the 5S gene but also other small RNAs (<400 base pairs), such as the tRNA [48].

Ribosome biogenesis is the de novo synthesis of the two subunits of the mature ribosome. The biogenic pathway is a multi-step and extensive processing

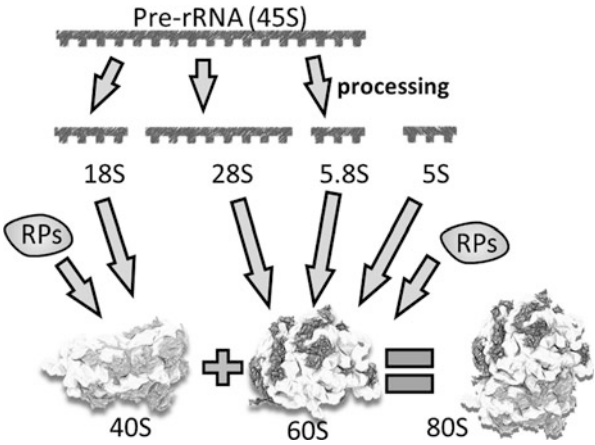

Fig. 6.1 Overview on the synthesis of the large (60S) and small (40S) subunits of the ribosome. *RPs* ribosomal proteins

of precursor rRNA, followed by assembly with ribosome proteins, leading to the formation of the macromolecular machine that will translate the genetic coding sequence of mRNA into the polypeptide chain of the protein, the mature ribosome (Fig. 6.1). In contrast to other cellular enzymes, which are proteins, the mature rRNAs are the enzymatic entities of the ribosome, also called ribozymes. Ribosome biogenesis involves the transcription of ribosomal DNA (rDNA), processing of the newly synthesized 45S pre-rRNA, and maturation of the pre-subunits with assembly of ribosomal proteins with their respective ribosomal subunit. Each of these steps are tightly regulated by the cell, thus due to the high energetic cost, many regulatory pathways converge on the first step of ribosome biogenesis, the rDNA transcription.

Ribosome biogenesis is intimately linked to cell growth and proliferation in organisms from yeast to higher eukaryotes. Accordingly, the rate of cell growth in both yeast and cancer cells is highly correlated to the level of ribosome biogenesis [8, 65]. Given that ribosome biogenesis is critical for the viability of the organism or cell, the production of ribosomes is highly conserved, thus allowing the process to be extensively studied in a more tractable model system such as yeast [65]. Although the relationship between cell growth and translational capacity is well established in yeast, much less is known regarding the role of ribosome biogenesis in multinucleated cells such as skeletal muscle.

6.3.1 rDNA Transcription

The rDNA gene is organized into tandem repeats that are spread throughout the mammalian genome with somatic cells containing approximately 200 copies of the rDNA gene (Fig. 6.1); however, not all rDNA copies are actively transcribed

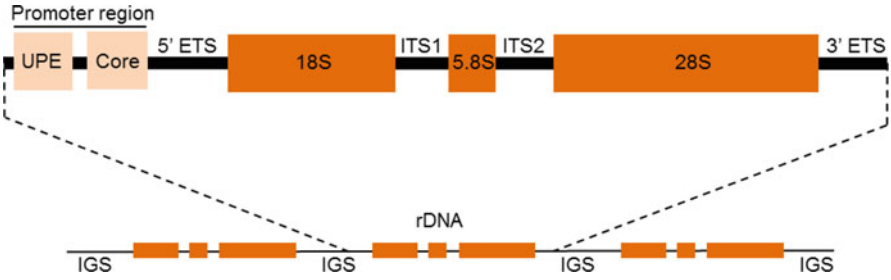

Fig. 6.2 Organization of the ribosomal DNA (rDNA) gene. Ribosomal DNA gene is arranged in approximately 200 tandem repeats. The intergenic spacer (IGS) regions are non-transcribed regions separating rDNA gene copies and where the promoter regions – core promoter region (Core) and the upstream promoter element (UPE) – are located. The pre-rRNA is transcribed as a single transcript containing the mature rRNAs in addition to both internal and external transcribed spacers (ITS, ETS)

[30]. The regulation of the rDNA repeats' conformation is important for ribosome biogenesis [19]. The nucleolus is an extremely prominent region within the nucleus where rDNA transcription occurs and is heavily packed with proteins responsible for synthesis of the 45S pre-rRNA and ribosome assembly (Fig. 6.2).

The Pol I enzyme requires the formation of a protein complex – the preinitiation complex (PIC) – at the rDNA promoter region. The upstream binding factor (UBF) is the first protein attaching to the rDNA. UBF binds physically to the promoter region (both UPE and the core promoters) via high mobility group (HMG) box domains, which confers DNA-binding activity of UBF. This association is the basic mechanism for the formation of the enhanceosome at the rRNA gene. The ribosomal enhanceosome is an almost 360° loop on the promoter elements allowing PIC formation for subsequent Pol I transcription [53]. Following enhanceosome formation, selectivity factor 1 (SL1) complex (formed by several TATA-binding proteins) associates with UBF and the rDNA [13]. The SL1 complex binds to the promoter region alongside UBF and recruits TIF-IA (also known as RRN3) and Pol I to initiate transcription [45]. TIF-IA, via protein-protein interactions, associates with SL1 and Pol I enzyme complex [5, 6], serving as a bridge between PIC and Pol I [8, 18, 38].

Following synthesis, the 45S pre-rRNA will be processed into the mature rRNAs for maturation with ribosomal protein into the 40S and 60S subunits. First, the ETS and ITS regions are removed by nucleases and then degraded [20]. The majority of the processing occurs while pre-rRNA is still in the nucleolus, although some processing also occurs in the nucleoplasm and cytoplasm [20]. In addition to the processing of the 45S, the 5S rRNA transcribed by the Pol III and the ribosomal proteins translated in the cytoplasm will also join the rRNA processing and maturation to form the ribosomal subunits [29].

6.3.2 *Upstream Pathways*

The majority of total cellular RNA is by far rRNA. Depending on the cell, as much as 80% of extracted RNA in tissue or cells can be rRNAs [61]. In proliferating cells, the majority of new transcripts are rRNA to make new ribosomes and support growth [61]. Due to the high cost of production, the process of making ribosomes is tightly regulated at many levels by nutritional, hormonal, and energy-sensing cues. It is widely known that many growth-related pathways, such as PI3K-Akt-mTOR axis and MAPK/ERK, trigger cell growth via increased rates of translation, especially at initiation and elongation steps. Those same pathways can also alter the rate of pre-rRNA synthesis. The transcription of rDNA is the central process governing ribosome biogenesis, in which the formation of the preinitiation complex (PIC) at the rDNA promoter is regulated by those pathways, such as mTOR and MAPK (Fig. 6.3).

The mammalian target of rapamycin (mTOR) is a master kinase controlling ribosome activity and its biogenesis. mTOR complex 1 (mTORC1) controls protein synthesis by phosphorylating many targets, especially the 4E-BP1 and p70S6K at the TOS motif sequence [47]. Hyperphosphorylation of 4E-BP1 releases the inhibitory effect of 4E-BP1 on translation initiation of 5'TOP mRNAs. Members of this selected group of mRNAs encode proteins involved in cellular growth. The majority of mRNA translation that 4E-BP protein inhibits encodes ribosomal proteins, mitochondrial ribosomal proteins, and proteins that associate with ribosomes to drive translation, the eukaryotic initiation, and elongation factors (eIFs and eEfs) [55]. mTORC1 also regulates the transcription of the RNA polymerases I, II, and

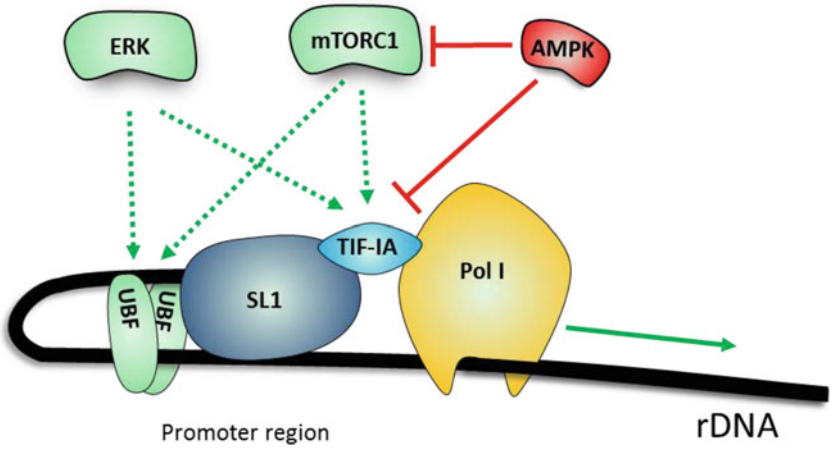

Fig. 6.3 Assembly of PIC at the rDNA promoter and pathways affecting components of PIC formation

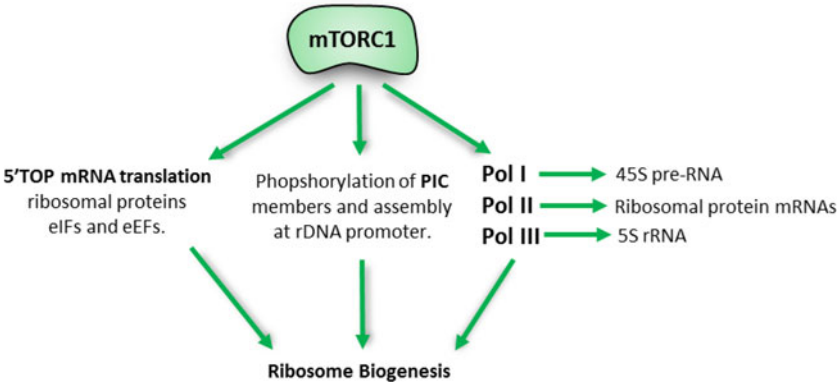

Fig. 6.4 mTORC1 promotes ribosome biogenesis and increase in translational capacity at multiple levels

III (Pol I, II, and III) [34, 56]. In addition to these effects, mTOR also promote the phosphorylation of TIF-IA [35]. Therefore, activation of mTORC1 increases translation efficiency and transcription of the RNA polymerases toward augmentation of translational capacity, supporting cell growth in addition to modulating PIC formation at the rDNA promoter (Fig. 6.4).

In addition to mTORC1, the MAPK pathway also promotes ribosome biogenesis. Different proteins of MAPK pathway also directly phosphorylate key proteins promoting assembly of the PIC components at the rDNA promoter. ERK and RSK proteins can phosphorylate TIF-IA and UBF proteins and promote rDNA transcription [52, 54, 66]. In addition, as ribosome biogenesis is also important in cell proliferation, cell cycle regulators, such as the cyclin-dependent kinases (CDK), have also been shown to modulate ribosome biogenesis. CDKs can phosphorylate UBF directly [57, 58] and TIF-IA [35]. In particular, CDK4, when complexed with cyclin D1, can directly phosphorylate UBF protein [58]. Moreover, the proto-oncogene protein c-Myc, known for its role in cell cycle and proliferation, also promotes ribosome biogenesis. The c-Myc protein has been shown to directly interact with PIC components at the rDNA promoter [1].

Ribosome biogenesis is a highly metabolic demanding cellular process, and as such, AMPK has been found to be an important factor linking energy balance with rDNA transcription. Not only can AMPK interfere with ribosome biogenesis via its regulation at mTORC1 and translation initiation level [31], but AMPK has also been found to interact with PIC components. AMPK can directly phosphorylate and destabilize TIF-IA association with SL1 [21] impacting negatively in the rate of pre-rRNA synthesis (Fig. 6.3).

6.4 Ribosome Biogenesis in Skeletal Muscle Hypertrophy and Atrophy

6.4.1 Hypertrophy

In animal models of muscle growth, ribosome biogenesis has been shown to precede muscle growth. Mechanical load of the plantaris muscle, via synergistic ablation of the plantar flexor muscles, markedly increases the expression of UBF, c-Myc, TIF-IA, and SL-1 components in addition to promoting the association of UBF, c-Myc, and Pol I with the rDNA promoter [59]. These events lead to a robust increase in rDNA transcription and accumulation of mature rRNAs [27, 28, 59]. Importantly, the degree of changes in mature rRNA has been shown to correlate with changes in muscle mass using this model of hypertrophy [43].

Following these key studies in muscle ribosome in animal models, a few studies have also investigated muscle ribosome biogenesis in humans. Specifically, the response to resistance exercise has been evaluated in recent years. Consistent with the animal studies, a single bout of resistance exercise is capable of increasing expression of 45S pre-rRNA [11, 42, 51]. Chronically, the repeated increases in ribosome biogenesis induced by resistance exercise lead to accretion in mature rRNA [9, 50]. Moreover, recent independent investigations using resistance exercise in rodents have also clearly demonstrated that a single bout and chronic training leads to pre-rRNA synthesis and accumulation of mature rRNAs [44, 63].

The mechanism leading to increased rDNA transcription following a bout of resistance exercise has been not fully understood; however, some recent studies have shed light on the pathways involved in promoting ribosome biogenesis in response to resistance exercise. Given the importance of both MAPK and mTORC1 signaling pathways in the regulation of ribosome biogenesis, it is not surprising these pathways are activated following resistance exercise. For instance, UBF, as a central regulator of rDNA transcription, is subjected to phosphorylation via both MAPK and mTORC1 pathways. Recent studies have shown that resistance exercise promotes a strong activation leading to UBF phosphorylation at multiple residues [11, 63]. In addition to this, another component of PIC formation, TIF-IA, has also been shown to rapidly increase in phosphorylation levels shortly after resistance exercise at an MAPK-pathway-dependent site [9, 11].

Overall, these studies support the notion that muscle hypertrophy in adults observed with mechanical stimulation is a result of greater muscle translational capacity, achieved via increased ribosome biogenesis.

6.4.2 Atrophy

Muscle atrophy is usually associated with an increase in muscle protein degradation rates [7, 46]. This is especially true for a highly atrophic condition in which

significant muscle loss is observed in a short period of time, such as in sepsis, cachexia, immobilization/bed rest, and denervation. However, the contribution of protein synthesis also has been brought to attention, as recent studies have shown that, for instance, disuse can also decrease protein synthesis rates at basal and postprandial state [60]. Moreover, smaller basal protein synthesis but potentially undetectable by current methods could contribute to the loss of muscle mass in cancer patient [22]. Therefore, impairment in muscle ribosome biogenesis could also contribute to both acute and chronic muscle wasting conditions. A few studies so far have tried to elucidate the roles of muscle ribosome biogenesis in muscle atrophy.

Machida et al. [32] studied the effects of muscle denervation on muscle atrophy. Both 45S pre-rRNA and mature rRNAs were markedly reduced following denervation. Similarly, a rodent model of chronic inflammation also caused an impairment in muscle ribosome biogenesis, as 45S, 28S, 18S, and 5.8S rRNAs were decreased following the onset of muscle inflammation, leading to decreased muscle mass and size [10].

Moreover the inability to mount a significant increase in pre-rRNA and mature rRNA expression in response to resistance exercise in humans [51] and mechanical loading in mice [27] has also been shown recently in aged in comparison with young cohorts. Thus, impaired ribosome biogenesis may also contribute to the blunted response to resistance exercise in older individuals.

Combined, these data suggest that muscle disuse and other muscle atrophic conditions and decreased translational capacity due to impaired ribosome biogenesis can be a major contributor to muscle wasting. Moreover, these data also highlight the potential links between ribosome biogenesis and sarcopenia.

6.5 Conclusion and Future Directions

Many recent studies have shown that changes in ribosome biogenesis and translational capacity correlate well with changes in muscle mass in both growth and wasting conditions [9, 10, 43, 50]. Thus, muscle ribosome biogenesis appears to be necessary for muscle hypertrophy [62]. However, more mechanistic and proof-of-principle evidences are necessary to stablish a causal relationship between ribosome biogenesis and muscle growth.

The regulation of ribosome biogenesis and translational capacity is an open venue for muscle biology researchers with interest in both the fundamental understanding of the molecular mechanisms of muscle growth and also of great applicability for clinical research. Novel aspects of regulation of muscle ribosome biogenesis may help elucidate the mechanism of muscle adaptation to different stimuli, providing tools to better design interventions of clinical interest. In addition to resistance exercise, endurance training studies will also further our understanding regarding the different exercise stimuli on ribosome biogenesis and the potential influence on skeletal muscle phenotypes. Moreover, of clinical relevance, novel

therapeutic approaches to ameliorate the loss of muscle mass during muscle disuse, chronic inflammatory conditions, cancer cachexia, and sarcopenia can be developed through the elucidation of the role of ribosome biogenesis in skeletal muscle size regulation.

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Chapter 7
Comprehensive Approach to Sarcopenia and Cachexia Treatment

Hidetaka Wakabayashi and Kunihiro Sakuma

Abstract Sarcopenia is characterized by a progressive and generalized loss of skeletal muscle mass and strength and is categorized into primary, or age-related, sarcopenia and secondary sarcopenia that can be activity, nutrition, or disease related. Resistance training is the best approach for the treatment of age-related sarcopenia. Increasing physical activity and avoiding prolonged and unnecessary bed rest or a sedentary lifestyle are good general strategies for mitigating activity-related sarcopenia. The treatment goals for nutrition-related sarcopenia involve the maintenance of a positive energy and protein balance.

Early recognition and treatment of cachexia is important and differs depending on the cachectic stage (precachexia, cachexia, or refractory cachexia). Physical exercise may counteract both precachectic and cachectic stages by virtue of its anti-inflammatory effects. Less well-established approaches include nutrition therapy for which the evidence is limited, pharmacologic treatment which largely is still in development, and psychosocial interventions which may have positive effects. A comprehensive approach, including rehabilitation nutrition, psychosocial interventions, and pharmacologic therapies, may be used for treating sarcopenia and cachexia. Rehabilitation nutrition can help to maximize functionality in people with sarcopenia and cachexia. Further research on the treatments for sarcopenia and cachexia, especially those that are multimodal, is necessary.

Keywords Sarcopenia • Cachexia • Disability • Rehabilitation nutrition • Multimodal treatments

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7.1 Introduction

Sarcopenia is characterized by a progressive and generalized loss of skeletal muscle mass and strength and is associated with an increased risk of adverse outcomes such as physical disability, poor quality of life (QoL), and death [1]. Cachexia is a complex metabolic syndrome associated with an underlying illness and is characterized by a loss of muscle with or without loss of body fat [2]. Although cachexia may be a component of sarcopenia and have a similar adverse event profile, the two conditions are not the same [3]. Sarcopenia and cachexia continue to be important public health issues, especially considering the increased prevalence of sarcopenia in populations with an increased older population. Research interest in sarcopenia and cachexia, evaluated by the number of times these words have been used as search terms in PubMed, has increased in recent years (Fig. 7.1).

To date, pharmacologic therapies for the treatment of sarcopenia and cachexia are largely still in development [4, 5]. When medications designed to treat sarcopenia are used, they can be enhanced by a regimen that also includes, when appropriate, resistance training, early ambulation, nutrition management, protein and amino acid supplementation, and smoking cessation [4]. For older people with sarcopenia and/or cachexia, the concept of rehabilitation and nutrition care management in combination is considered a useful approach to treatment [6]. In this review, we address the epidemiology, diagnostic criteria, causes, and therapies focusing on physical exercise/activity and nutrition as a comprehensive approach for the treatment of sarcopenia and/or cachexia.

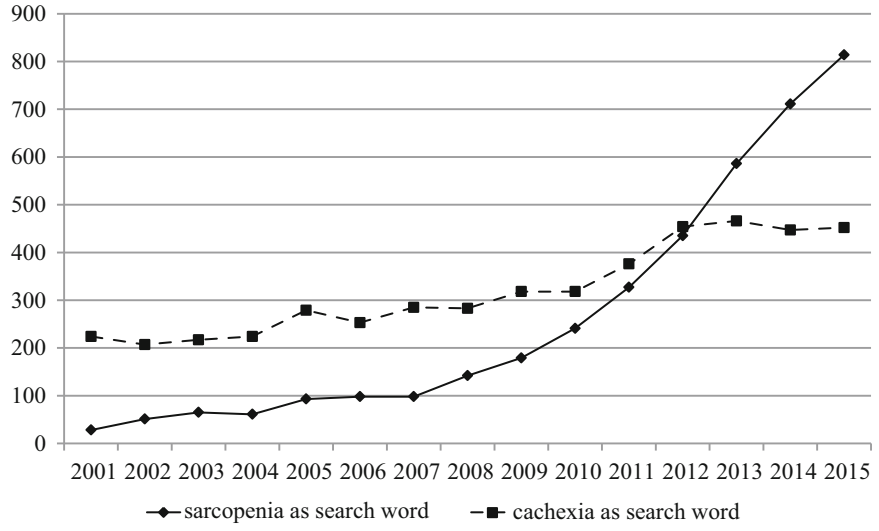

Fig. 7.1 Number of PubMed entries for sarcopenia and cachexia as respective search words (Assessed on 1 February 2016 from www.pubmed.gov)

7.2 Sarcopenia

7.2.1 Definition and Epidemiology

The term sarcopenia, first used by Rosenberg in 1989 to describe an age-related decrease in muscle mass, originated from the Greek words *sarx*, meaning flesh, and *penia*, meaning loss [7, 8]. Initially, this term denoted only the loss of muscle mass. In 2010, the European Working Group on Sarcopenia in Older People (EWGSOP) described sarcopenia as a syndrome characterized by the progressive and generalized loss of skeletal muscle mass and strength, associated with a risk of adverse outcomes such as physical disability, poor QoL, and death [1]. In 2011, the International Working Group on Sarcopenia defined sarcopenia as an age-associated loss of skeletal muscle mass and function [3]. Decreased muscle strength and physical function are also now included in the definition of sarcopenia.

According to a systemic review, the prevalence of sarcopenia in older adults, using the EWGSOP definition of sarcopenia, was 1–29% in community-dwelling populations, 14–33% in long-term care populations, and 10% in the acute care hospitalized population [9]. The prevalence of sarcopenia in patients who underwent surgery for gastrointestinal and hepatopancreatobiliary malignancies was 17–79% [10]. In another systematic review of patients with pancreatic ductal adenocarcinoma, the prevalence of sarcopenia among individuals of normal weight (body mass index [BMI] 18.5–24.9 kg/m^2) and those who were overweight or obese (BMI >25 kg/m^2) was 9.7–65% and 16.2–67%, respectively [11]. The prevalence of sarcopenia in older people in rehabilitation settings was 40–46.5% [12, 13].

7.2.2 Diagnostic Criteria

Sarcopenia is diagnosed by low muscle mass and either low muscle strength or low physical performance according to the EWGSOP definition [1]. This definition is similar to the criteria put forth by the Asian Working Group for Sarcopenia (AWGS) [14]. The International Working Group on Sarcopenia (IWGS) considers that sarcopenia is diagnosed by low muscle mass and low gait speed [3], whereas the Foundation for the National Institutes of Health (FNIH) sarcopenia project indicates that sarcopenia is diagnosed by low muscle mass and low muscle strength [15]. Sarcopenia is no longer diagnosed on the basis of low muscle mass alone, although the diagnostic criteria of sarcopenia are different between research groups. Presarcopenia is characterized by low muscle mass without considering the effects on muscle strength or physical performance, and severe sarcopenia is characterized by low muscle mass and low muscle strength, in addition to low physical performance as per the EWGSOP definition [1].

7.2.2.1 Muscle Mass

Appendicular muscle mass is most commonly assessed using dual X-ray absorptiometry (DXA) and bioimpedance analysis (BIA). However, the cutoff reference values for appendicular muscle mass are different between research groups. The AWGS divides appendicular muscle mass by height squared (DXA, 7.0 kg/m^2 for men and 5.4 kg/m^2 for women; BIA, 7.0 kg/m^2 for men and 5.7 kg/m^2 for women) [14], whereas the IWGS cutoff reference values are 7.23 kg/m^2 for men and 5.67 kg/m^2 for women [3]. In contrast, the FNIH sarcopenia project uses appendicular lean mass adjusted for BMI for cutoff values (<0.789 for men and <0.512 for women) [15], and the EWGSOP does not specify cutoff values.

Abdominal computed tomography (CT) is an accurate, practical approach to quantifying whole-body and regional skeletal muscle mass [16]. Cross-sectional imaging using CT is the preferred method for analyzing muscle mass in cancer patients, because CT is performed clinically for staging and diagnostic purposes [17, 18]. Core muscle size, measured as the total psoas cross-sectional area (TPA) at the third or fourth lumbar vertebra, is a precise indicator of total skeletal muscle mass [19]. The skeletal muscle index is calculated as the TPA divided by height squared and is associated with the severity of dysphagia in cancer patients [18]. Preoperative psoas muscle mass on the contralateral side is associated with postoperative gait speed following total hip arthroplasty for osteoarthritis [20]. The cutoff value when assessing the risk for the development of liver failure in Japanese cancer patients was defined as <5.67 cm^2/m^2 in males and <3.95 cm^2/m^2 in females [21].

7.2.2.2 Muscle Strength

Muscle strength is mainly assessed by grip strength; it is the default evaluation given the low cost, availability, and ease of use of this equipment during evaluations. The cutoff values for grip strength are as follows: EWGSOP, <30 kg for men and <20 kg for women [1]; AWGS, <26 kg for men and <18 kg for women [14]; and FNIH sarcopenia project, <26 kg for men and <16 kg for women [15]. The IWGS diagnostic criteria for sarcopenia do not include muscle strength assessment [3].

Knee extension is another method used to assess muscle strength; assessed in relation to body weight, it correlates with physical function [22]. A threshold ratio of knee extension strength/body weight between 2.78 kPa/kg and 2.86 kPa/kg is considered a potential cutoff value by which to identify women presenting with greater functional impairments [22]. Knee extension strength was determined to be a better predictor of functional performance than was handgrip strength, but only among residents in assisted living facilities [23].

7.2.2.3 Physical Performance

Physical performance is primarily assessed by usual gait speed. The EWGSOP and the AWGS cutoff values for usual gait speed are <0.8 m/s [1, 14], whereas the IWGS cutoff value is <1 m/s [3]. In the FNIH sarcopenia project, physical performance assessment is not included in the diagnostic criteria for sarcopenia [15]. The Short Physical Performance Battery that includes gait speed, ability and time to rise from a chair five times, static balance tests, the timed up and go test that measures the time to rise from a chair and walk a short distance, and the 6-min walk test is another method to assess physical performance [24].

7.2.3 Causes

The EWGSOP has categorized sarcopenia into primary or age-related sarcopenia and secondary sarcopenia that is activity, nutrition, or disease related [1].

Activity-related sarcopenia can result from prolonged bed rest, a sedentary lifestyle, and/or deconditioning, for example, in low- or zero-gravity conditions. Nutrition-related sarcopenia can result from the inadequate dietary intake of energy and/or protein, and it may also be associated with either malabsorption or gastrointestinal disorders. Disease-related sarcopenia can be associated with advanced organ failure (heart, lung, liver, kidney, and brain), inflammatory disease, malignancy, or endocrine disease. However, the etiology of sarcopenia in older people is often multifactorial so that it may not be possible to precisely characterize strictly primary or secondary sarcopenia in certain individuals [1]. For example, older patients with aspiration pneumonia tend to be prescribed nil by mouth and bed rest during treatment, and peripheral parenteral nutrition is usually administered during fasting periods of 1–2 weeks. Under such circumstances, older patients with aspiration pneumonia can simultaneously experience age-, activity-, disease-, and nutrition-related sarcopenia (Table 7.1).

Table 7.1 Causes of sarcopenia

Causes	Examples
Age-related sarcopenia	No other cause evident except aging
Activity-related sarcopenia	Bed rest, a sedentary lifestyle, and deconditioning or zero-gravity conditions
	Hospital-associated deconditioning
	Unnecessary non-eating and bed rest sarcopenia in hospitals (iatrogenic sarcopenia)
Nutrition-related sarcopenia	Inadequate dietary intake of energy and/or protein
	Inappropriate nutrition management in hospitals (iatrogenic sarcopenia)
Disease-related sarcopenia	Advanced organ failure, inflammatory disease, malignancy, or endocrine disease
	Invasion, cachexia, neuromuscular disorders

7.2.3.1 Age-Related Sarcopenia

The etiology of age-related sarcopenia can be multifactorial in that it can include decreased activity (bed rest), low protein intake, chronic inflammation, increased oxidative stress, lipotoxicity, reduced neuronal stimulation, hormonal changes, increased myostatin concentrations, and/or decreased blood capillary flow [25]. The ubiquitin-proteasome system (atrogin-1 and MuRF-1), which is important in protein degradation in acute muscle atrophy, seems not to be implicated in age-related sarcopenia [26]. In contrast, the dysfunction of autophagy-dependent signaling seems to be profoundly involved in age-related sarcopenia [27]. Sarcopenic conditions appear to be characterized by a marked defect of autophagy signaling, because p62/SQSTM1, but not LC3, accumulates in the sarcopenic muscle of mice [28]. It remains to be elucidated whether the myostatin-Smad pathway is fundamental to the development of sarcopenia [27].

7.2.3.2 Activity-Related Sarcopenia

Activity-related sarcopenia can result from prolonged bed rest, a sedentary lifestyle, and/or deconditioning or zero-gravity conditions [1]. For this type of sarcopenia, the loss of skeletal muscle mass and strength occurs at an approximate rate of 0.5% (range, 0.3–4.2%) total muscle mass per day [29]. Prolonged disuse (>10 days) can result in a decline in basal and postprandial rates of muscle protein synthesis, without an apparent change in muscle protein breakdown [29].

Hospital-associated deconditioning is characterized by a functional decline that occurs during acute hospitalization due to illness or injury and is unrelated to a specific neurological or orthopedic insult [6]. Hospital-associated deconditioning includes activity-related sarcopenia. In our previous cohort study [30, 31], 88–91% of patients with hospital-associated deconditioning were malnourished, and the decline in nutritional status resulted in a decline in the rehabilitation outcome.

7.2.3.3 Nutrition-Related Sarcopenia

Nutrition-related sarcopenia results from inadequate dietary intake of energy and/or protein and can be associated with either malabsorption or gastrointestinal disorders [1]. Adult malnutrition is classified as malnutrition in the context of acute illness or injury or invasion, chronic illness or cachexia, and/or social or environmental circumstances that can lead to starvation [32]. Nutrition-related sarcopenia includes starvation, and disease-related sarcopenia includes invasive injury and cachexia.

Older patients with hospital-associated deconditioning can be at risk for an inadequate energy intake that does not meet their basal energy expenditure. A total of 75 (44%) out of 169 patients fell into this category based on one report [31]. The energy intake of older patients with hospital-associated deconditioning seems to often be suboptimal, resulting in nutrition-related sarcopenia. Nutrition-related

sarcopenia causes hepatic glycogen depletion. Glycogen is stored only in the liver and muscles in humans. Muscle glycogen is stored as a readily available fuel source for muscle tissue; it is not released into the blood as a source of glucose following glycogenolysis. Therefore, glucose is synthesized from glycogenic amino acids derived via skeletal muscle catabolism in nutrition-related sarcopenia. A chronic negative energy and/or a protein imbalance result in nutrition-related sarcopenia.

7.2.3.4 Disease-Related Sarcopenia

Disease-related sarcopenia is usually associated with advanced organ failure (heart, lung, liver, kidney, and brain), inflammatory disease, malignancy, and/or endocrine disease [1]. It is characterized by invasion, cachexia, and neuromuscular disorders. An intensive care unit-acquired weakness [33] can be classified as disease-related sarcopenia. Acute inflammatory crises triggered by events such as sepsis, pulmonary decompensation, emergency surgery, and/or trauma can result in profound muscle wasting via mechanisms of action that may include tumor necrosis factor-α-mediated catabolism, mitochondrial dysfunction, and/or a reduction of muscle protein synthesis [34].

Phases of acute inflammation or invasion can be classified as catabolic and anabolic. In a catabolic phase, both muscle and adipose tissue are degraded by proinflammatory cytokines. In an anabolic phase, both muscle and adipose tissue can be synthesized and increased by an appropriate nutritional management and exercise. Clinically, a catabolic phase features a muscle that is more degraded than adipose tissue. In patients with a critical illness, the loss of muscle mass can reach 1 kg/day due to conditions causing severe inflammatory responses [35]. Disease-related sarcopenia can be further complicated by the other causes of secondary sarcopenia outlined previously that can compound the sarcopenic effect in the face of hospital-associated deconditioning.

7.2.3.5 Complications of All Causes of Sarcopenia

Older patients with a hip fracture and hospital-associated deconditioning can complicate all causes of sarcopenia [6, 36]. Activities of daily living are independent in frail older people with only age-related sarcopenia. However, the functional reserve in frail individuals is considered limited. For example, if they develop a hip fracture, their skeletal muscle mass can rapidly deteriorate as the other secondary causes of sarcopenia come into play.

Most patients with a hip fracture undergo surgery, and during the perioperative period, they tend to be prescribed bed rest and fasting. In acute hip fracture inpatients, the total 24-h energy and protein intakes before a multidisciplinary nutritional care intervention were 707 kcal and 33.8 g, respectively [37]. Collectively, extended

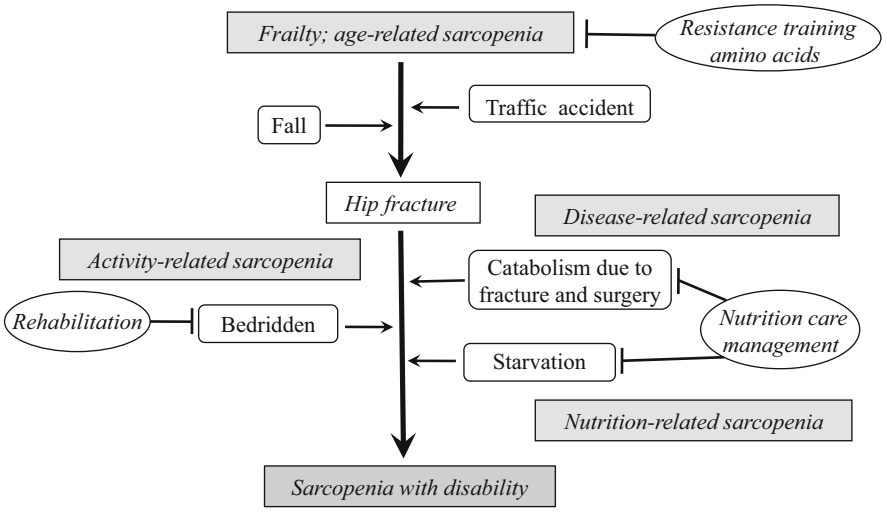

Fig. 7.2 Mechanism of sarcopenia with disability in frail older people with hip fracture. Frail older people with hip fracture become sarcopenia with disability because of extended bedridden, catabolism due to fracture and surgery, and poor nutritional support. They can complicate activity-, nutrition-, disease-related sarcopenia

bed rest, poor nutritional support, and the invasive nature of a surgery with its inflammatory sequelae can contribute to the type of skeletal muscle catabolism indicative of secondary sarcopenia (Fig. 7.2).

7.2.4 Treatment for Sarcopenia

The treatment for sarcopenia can differ depending on its primary and/or secondary classification and its etiology (Table 7.2).

7.2.4.1 Age-Related Sarcopenia

Resistance training has been shown to be the most effective intervention when striving to combat age-related sarcopenia, because it induces skeletal muscle hypertrophy which can enhance muscle strength [4], even in older people over 75 years [38]. In the Cochrane Database of Systematic Reviews, progressive resistance strength training was also reported as an effective intervention for improving physical functioning in older people by increasing muscle strength and gait speed [39].

Protein and amino acid supplementation can contribute to counteracting sarcopenia, because low protein intake is known to be associated with declining muscle

Table 7.2 Sarcopenia treatment

Causes	Treatment
Age-related sarcopenia	Resistance training
	Protein and amino acid supplementation?
	Pharmaceutical therapies?
Activity-related sarcopenia (iatrogenic sarcopenia due to unnecessary non-eating and bed rest)	Avoid bed rest, sedentary lifestyle
	Promote early activity, mobilization, exercise, rehabilitation
	Combination of early rehabilitation and nutritional intervention
Nutrition-related sarcopenia (iatrogenic sarcopenia due to inappropriate nutrition management)	Keep positive energy and protein balance
	Aggressive nutrition care management considering daily energy accumulation
	Light load activity and exercise
Disease-related sarcopenia	Treat and prevent diseases
	In catabolic phase, modest (15–30 kcal/kg/day) nutrition care management, light load activity, and exercise
	Nonsmoking

mass in older adults [40]. In a systematic review, nutritional supplementation was reported to improve both muscle mass and muscle strength in older people with sarcopenia [41]. A meta-analysis showed that protein supplementation can also increase muscle mass and strength gains during prolonged resistance-type exercise training in sarcopenia [42]. However, another meta-analysis reported that amino acid/protein supplements did not increase lean body mass and muscle strength significantly in older people [43]. Furthermore, in a recent meta-analysis, combining protein supplementation with resistance training did not seem to increase muscle mass or strength [44]. Considering these results, whole-protein supplementation failed to show a consistent effect on muscle mass, strength, or function [45].

7.2.4.2 Activity-Related Sarcopenia

Increasing physical activity and avoiding prolonged and unnecessary bed rest or a sedentary lifestyle are good general strategies for mitigating activity-related sarcopenia, and if needed, rehabilitation may be advised. Early rehabilitation may be safe and feasible for critically ill patients in the intensive care unit when medically prescribed [46, 47]. Therapies to combat the loss of muscle mass have focused largely on strategies using resistance exercises and/or nutrition, but with mixed success [48]. Iatrogenic sarcopenia due to unnecessary fasting and bed rest in hospitalized older people should be avoided.

In a recent randomized controlled trial, an early nutritional intervention using nutritional supplements (600 kcal, 20 g/d protein added to a standard diet) together with early rehabilitation preserved the muscle mass and independence of ill, older patients hospitalized for acute diseases [49]. A combination of early rehabilitation

and nutritional intervention seemed to be a highly effective method to treat and ameliorate activity-related sarcopenia, because many patients with hospital-associated deconditioning can be malnourished [30, 31].

7.2.4.3 Nutrition-Related Sarcopenia

Recommendations for the treatment of nutrition-related sarcopenia focus on the maintenance of a positive energy and protein balance. Daily energy requirements in cases of nutrition-related sarcopenia can be calculated as daily energy expenditure plus daily energy accumulation (200–750 kcal/day). The excess energy required to gain 1 kg of body weight was between 8856 and 22,620 kcal/kg in malnourished nursing home patients [50]. Gaining weight is more difficult in older compared with younger people. Therefore, aggressive nutrition care management is often necessary to improve nutritional status.

Prevention of nutrition-related sarcopenia is important, especially in some older inpatients with hospital-associated deconditioning who are prescribed peripheral parenteral nutrition that furnishes less than 300 kcal/day in the absence of an oral caloric intake and enteral nutrition [51]. Identification of iatrogenic sarcopenia in older hospitalized patients due to nutritional management issues should be addressed as soon as possible to determine if additional nutritional support can be provided.

Resistance training to improve muscle mass should be avoided if energy and protein balance is negative, because this type of exercise, as well as endurance training, exacerbates the negative energy balance and can worsen nutrition-related sarcopenia. Resistance training to improve muscle mass should be performed only if energy and protein are balanced or positive. However, prolonged bed rest should be avoided if energy and protein balance is negative, because inactivity enhances catabolic responses to calorie restriction [52]. Light activity and exercise should be performed to prevent activity-related sarcopenia.

7.2.4.4 Disease-Related Sarcopenia

The most important factor in disease-related sarcopenia is treatment of the disease. Appropriate treatment of disease-related sarcopenia is different in the catabolic phase compared to the anabolic phase. In a catabolic state, muscle degradation and endogenous energy production are usually increased, and disease-related sarcopenia can develop rapidly. Nutrition care management under these circumstances should be modest (15–30 kcal/kg/day), and daily energy expenditure beyond energy intake should be curtailed because of increasing endogenous energy production. Light load activity and exercise should be performed to prevent activity-related sarcopenia. Anabolic phase is the state during which muscle mass and adipose tissue can be gained as a result of physical exercise and activity and appropriate nutrition care management. Nutrition care management in the anabolic phase should be tailored

to the patient's daily energy expenditure plus daily energy accumulation (200–750 kcal/day) to improve muscle mass and strength. Resistance training to improve muscle mass is encouraged in anabolic phase.

In critically ill patients, neuromuscular electrical stimulation added to standard treatment has been proved to be more effective than the standard treatment alone in the prevention of skeletal muscle weakness [53]. In the Cochrane Database of Systematic Reviews, it has been shown that no harm has been associated with the following related to muscle disease: moderate-intensity strength training in myotonic dystrophy and facioscapulohumeral muscular dystrophy and aerobic exercise training in dermatomyositis, polymyositis, and myotonic dystrophy type I [54]. However, there is insufficient evidence to conclude that they offer any benefit. In mitochondrial myopathy, aerobic exercise combined with strength training appeared to be safe and may be effective at increasing submaximal endurance capacity [54].

Smoking cessation can be recommended for the prevention and treatment of sarcopenia. Cigarette smoking may contribute to the development of sarcopenia according to a meta-analysis [55].

7.3 Cachexia

7.3.1 Definition and Epidemiology

Cachexia, as defined at the Washington Cachexia Consensus Conference in 2006 [2], is a complex metabolic syndrome associated with an underlying illness that is characterized by a loss of muscle with or without a loss of fat mass. The prominent clinical feature of cachexia is weight loss (corrected for fluid retention) in adults or growth failure (excluding endocrine disorders) in children. Anorexia, inflammation, insulin resistance, and increased muscle protein breakdown are frequently associated with wasting disease. Wasting disease, which is distinct from starvation, age-related loss of muscle mass, primary depression, malabsorption, and hyperthyroidism, is associated with increased morbidity [2]. This definition can be applied to all causative diseases of cachexia.

The Special Interest Groups (SIGs), "cachexia-anorexia in chronic wasting diseases" and "nutrition in geriatrics" in the European Society for Clinical Nutrition and Metabolism (ESPEN), proposed that cachexia should be classified in accordance with its stage as either precachexia or cachexia in 2010 [56]. In 2011, cancer-induced cachexia was defined by international consensus as a multifactorial syndrome characterized by an ongoing loss of skeletal muscle mass (with or without loss of fat mass) that cannot be fully reversed by conventional nutritional support and leads to progressive functional impairment [17]. The pathophysiology of cancer cachexia is characterized by a negative protein and energy balance driven by a variable combination of reduced food intake and abnormal metabolism [17].

Cachexia is commonly referred to as protein-energy wasting in the context of renal diseases. The International Society of Renal Nutrition and Metabolism (ISRNM) defines protein-energy wasting as the state of decreased body stores of protein and energy fuels (i.e., body protein and fat masses) [57]. Cachexia is a severe form of protein-energy wasting that occurs relatively infrequently in kidney diseases [57].

The prevalence of cachexia ranges from 5 to 15% in end-stage chronic heart failure to 50–80% in advanced cancer [58]. The overall prevalence of cachexia is approximately 1% of the patient population; it affects an estimated nine million people and mortality rates of patients with cachexia range from 10 to 15% per year in chronic obstructive pulmonary disease to 20–30% per year in chronic heart failure and chronic renal disease and up to 80% in cancer patients [58].

In older inpatients with hospital-associated deconditioning, 88% were malnourished and 30% had precachexia [31]. Among them, nutritional status, low concentration of serum albumin, and precachexia were associated with poor rehabilitation outcomes as assessed using the Barthel index score at discharge [31]. Cachexia may be common in older patients who need rehabilitation, and it can adversely affect rehabilitation outcomes. Therefore, cachexia assessment is imperative in patients with wasting disorders.

7.3.2 Diagnostic Criteria

Several diagnostic criteria for cachexia have been proposed in consensus papers [2, 17, 56]. At the Washington Cachexia Consensus Conference, a weight loss of at least 5% of body weight within a period not exceeding 12 months, without the presence of edema or with a BMI <20 kg/m^2 in the presence of wasting diseases, was necessary for cachexia diagnosis. Furthermore, three of the following five criteria are necessary for a definitive diagnosis of cachexia: decreased muscle strength, fatigue, anorexia, low muscle (fat-free) mass, and biochemical abnormalities characteristic of inflammation, anemia, or hypoalbuminemia verified by a blood test [2]. This diagnosis can be applied to all causative diseases of cachexia.

Cachexia can be classified into three stages that include precachexia, cachexia, and refractory cachexia. The ESPEN SIGs indicate a diagnosis of precachexia has the following criteria: an underlying chronic disease, an unintentional weight loss $\leq$5% of usual body weight during the last 6 months, a chronic or recurrent systemic inflammatory response, and anorexia or anorexia-related symptoms [56]. This precachexia diagnosis is applicable to all wasting disorders.

There is international consensus that the diagnosis of cancer-induced cachexia is different depending on the stage. Precachexia is diagnosed by a weight loss <5% over the past 6 months [17]. Cancer-induced cachexia is diagnosed by weight loss >5% over the past 6 months in the absence of energy intake reduction, or a BMI <20 kg/m^2 and weight loss >2%, or an appendicular skeletal muscle index consistent with sarcopenia (i.e., males, <7.26 kg/m^2; females, <5.45 kg/m^2) and any

degree of weight loss >2% [17]. A diagnosis of refractory cancer cachexia includes the following criteria: fulfillment of the criteria by which cachexia is defined, a prognosis of <3 months, an Eastern Cooperative Oncology Group performance status of 3 or 4, being unresponsive to anticancer therapy, ongoing catabolism at an increasing rate, and a status of unsuitable for artificial nutritional support [17]. These diagnoses can only be applied to cancer patients.

Protein-energy wasting is diagnosed if three of the four following characteristics are present based on the ISRNM criteria: blood test data showing low concentrations of serum albumin, transthyretin, or cholesterol, low body mass (i.e., a low BMI, unintentional weight loss over time, low percentage of total body fat), low muscle mass (muscle wasting or sarcopenia, reduced mid-arm muscle circumference area, creatinine appearance), and low dietary intake (unintentional low protein or energy intakes) [57]. The ISRNM diagnosis is usually only applicable in renal disease cases.

7.3.3 Causative Diseases

Causative diseases of cachexia are cancer, chronic heart failure, chronic renal failure, chronic respiratory failure, chronic liver failure, connective tissue diseases and autoimmune disease including rheumatoid arthritis, chronic infectious diseases such as tuberculosis and acquired immune deficiency syndrome, and inflammatory bowel diseases.

Cancer is the most frequent disease cause of cachexia. Cancer-induced cachexia affects approximately 50–80% of cancer patients and may account for up to 20% of cancer deaths [59]. Cachexia was associated with an increased length of hospital stay, increased health cost, and more severe loss of function in patients compared with those unaffected by cachexia [60]. Cancer-induced cachexia impairs patients' QoL, as assessed by the Functional Assessment of Anorexia-Cachexia Therapy scale [61]. In a systematic review of qualitative research, the multidimensionality of weight loss and anorexia experience, patients' and caregivers' responses to coping with weight loss and anorexia, and clinical assessment and management of weight loss and anorexia were three major concerns [62]. Early detection of malnutrition and cachexia in cancer patients is important because malnutrition due to low energy intake may be able to be improved by nutritional support [63].

Chronic heart failure results in cardiac cachexia at the end stage [64]. Cardiac cachexia reduces the much needed delivery of macro- and micronutrients to fuel the heart and as a result leads to a decline in cardiac function [65]. Weight loss of more than 6% was previously used to define the presence of cachexia in patients with chronic heart failure [66]. Currently, consensus diagnostic criteria should be used. The prevalence of cardiac cachexia was 21.8% as determined at the Washington Cachexia Consensus Conference [67]. Survival analysis did not show reduced survival in patients with cardiac cachexia [67]. In contrast, body weight loss was an independent prognostic factor for mortality in chronic heart failure [68].

A multidisciplinary disease management program should be implemented in the early stages of heart failure and cardiac cachexia [69].

Protein-energy wasting is common in end-stage renal disease. Etiologies of protein-energy wasting are insufficient food intake, uremia-induced alterations such as increased energy expenditure, persistent inflammation, acidosis, multiple endocrine disorders, comorbidities, and the dialysis procedure [70]. The prevalence of protein-energy wasting as determined using the ISRNM criteria was 37% in hemodialysis patients, and the criterion of muscle mass loss was associated with increased mortality [72]. The ISRNM diagnostic criteria can be used to identify patients at higher risk of death in chronic hemodialysis patients [71]. A higher lean tissue index was associated with a better QoL in end-stage renal disease patients [73]. Prevention and treatment of protein-energy wasting should involve individualized and integrated approaches [74].

7.3.4 Treatment

Cachexia treatment differs depending on the stage of cachexia. In all patients with precachexia or cachexia, ensuring sufficient energy and protein intake, maintaining physical activity levels to retain a healthy muscle mass, and, if present, reducing systemic inflammation should be a high priority [63]. In contrast, palliative care is the top priority for treatment in cases of refractory cachexia. Therefore, early recognition and proactive treatment of precachexia and cachexia in wasting disorders is important. We address exercise, nutrition, pharmacologic therapies, and psychosocial interventions focusing on precachexia and cachexia.

7.3.4.1 Exercise

Exercise may be undertaken to treat precachexia and cachexia. Exercise can attenuate or even reverse the process of muscle wasting, by exerting anti-inflammatory and antioxidative effects that are able to affect an attenuation of signaling pathways associated with protein degradation and activate molecules associated with protein synthesis [75, 76]. Exercise represents a function-preserving, anti-inflammatory, and metabolism-modulating strategy in cachectic conditions that is associated with low medical costs [77]. Endurance exercise may have an anti-inflammatory effect, even when performed at a low intensity [78]. However, most studies investigating the exercise effect on cachexia have been limited to animal models [75].

Evidence of the effect of exercise on cachexia is insufficient. In the Cochrane Database of Systematic Reviews, no trials met the inclusion criteria of the systematic review, and there was insufficient evidence to determine the safety and effectiveness of exercise under the circumstances [79]. Another systematic review in cancer patients during their treatment revealed that aerobic and resistance exercise improved upper and lower body muscle strength more than usual care [80].

However, most studies were performed in patients with early-stage breast or prostate cancer, and evidence in cancer patients with advanced disease is lacking [80]. In a recent randomized clinical trial in heart failure patients, improvements in physical and functional capacities were attributed to their resistance exercise program, but not to the branched-chain amino acid supplementation regimen [81]. In adults with progressive diseases such as chronic obstructive pulmonary disease, chronic heart failure, and cancer, neuromuscular electrical stimulation appeared to be effective for improving muscle weakness according to the Cochrane Database of Systematic Reviews [82]. Exercise seems to be effective for the prevention of disuse muscle atrophy, when the patient is not too disabled or ill. However, further clinical research is necessary in order to verify the effects on cachexia.

7.3.4.2 Nutrition Therapy

Nutrition therapy for cachexia is very important; it has been shown that 46% of patients with incurable cancer report some amount of weight loss [83]. However, the current evidence in support of nutrition therapy for cachexia is limited. Nutritional interventions for cancer-induced cachexia improved weight and energy intake and some aspects of QoL (emotional functioning, dyspnea, loss of appetite, and global QoL) based on a meta-analysis [84, 85]. However, nutritional interventions did not improve mortality and/or cancer treatment [84, 85]. In another systematic review, dietary counseling for the management of weight loss and to improve energy intake did increase body weight and energy intake in patients with advanced cancer and cachexia [86]. Only in chronic obstructive pulmonary disease with precachexia and in early cachexia was there stronger evidence that nutritional support improved outcomes [87].

Using fish oils containing omega-3 polyunsaturated fatty acids for treating cachexia remains inconclusive. Omega-3 polyunsaturated fatty acids have been shown to inhibit nuclear factor kappa B, a proinflammatory transcription factor, and to reduce TNF-α production in vitro [4]. In a systematic review of the European Palliative Care Research Collaborative Cachexia Guidelines project, there was not enough evidence to support a net benefit of omega-3 polyunsaturated fatty acids in advanced cancer cachexia [88]. However, another preliminary study suggested that omega-3 polyunsaturated fatty acids are safe and have a positive effect on clinical outcomes and survival in pancreatic cancer patients [89]. The safety profile of omega-3 polyunsaturated fatty acids reveals infrequent adverse events with no severe toxicities [88]. Therefore, fish oil containing omega-3 polyunsaturated fatty acids might be used for cachexia, although their evidence remains inconclusive.

7.3.4.3 Pharmacologic Therapy

Pharmacologic therapy for cachexia is largely still in development. Megestrol acetate has been shown to improve appetite and was associated with a slight weight

gain in cancer, in AIDS, and in patients with other underlying pathologies in the Cochrane Database of Systematic Reviews [90]. However, edema, thromboembolic phenomena, and deaths were more frequent in patients treated with megestrol acetate [90]. Another review revealed that there was inadequate evidence to recommend thalidomide for cancer cachexia in clinical practice [91]. Nonsteroidal anti-inflammatory drugs for cancer-induced cachexia have not been recommended for use outside of clinical trials as highlighted in some systematic reviews [92, 93].

Recently, the selective androgen receptor modulator enobosarm was tested in patients with cancer who had muscle wasting in phase 3 trials. However, one trial demonstrated that there was no clinical improvement in patients performing the stair climb power test [94]. Another phase 3 trial of the ghrelin receptor agonist anamorelin has shown a significant benefit in terms of gains in lean and fat body mass, but not for handgrip strength [94]. These results indicate that pharmacologic therapy for cachexia can improve muscle mass, but cannot also effectively improve muscle strength and physical function. Therefore, adopting a multimodal approach that includes pharmacologic therapy as well as exercise and nutrition therapy may provide a better approach to treatment for cachexia than relying on a single approach.

7.3.4.4 Psychosocial Interventions

Psychosocial interventions may have a positive effect on cachexia. The concept of psychosocial effects in cancer cachexia can sensitize healthcare professionals to cachexia-related issues [95]. Psychosocial interventions may have small, short-term beneficial effects on the physical component of general health-related QoL and cancer-related QoL [96]. Other reports in the Cochrane Database of Systematic Reviews showed limited evidence of psychosocial interventions for patients with head and neck cancer [97], for recently diagnosed cancer [98], and for reducing fatigue during cancer treatment in adults [99]. However, several psychosocial interventions, particularly those based on cognitive behavioral therapy, demonstrated good economic value in cancer care in a systematic review [100]. Therefore, psychosocial interventions for cancer-induced cachexia should be included in the multimodal approach.

In patients with chronic heart failure, psychosocial interventions improved QoL in a meta-analysis [101]. Another meta-analysis showed that psychosocial interventions had a positive effect on psychological and physical health outcomes in chronic obstructive pulmonary disease [102]. In patients with rheumatoid arthritis, disclosure therapy and cognitive behavioral therapy with adjunct maintenance therapy were effective as reported in a systematic review [103]. Psychosocial interventions for cachexia seem to be promising, although firm evidence is limited and further studies are required.

7.4 Comprehensive Approach

A comprehensive approach to sarcopenia treatment should include, as is appropriate on a patient-by-patient basis, pharmacologic therapies for sarcopenia and diseases, resistance training, early ambulation, nutrition management including protein and amino acid supplementation, and smoking cessation [4]. In people with a disability complicating sarcopenia and/or cachexia, the concept of rehabilitation nutrition can be useful [6, 104, 105]. Rehabilitation nutrition can be implemented using the International Classification of Functioning, Disability and Health guidelines to evaluate nutrition status and to maximize functionality in people with a disability [6].

Several randomized controlled trials showed the effectiveness of rehabilitation nutrition as adjunct treatment for reducing the risk of and treating sarcopenia. A nutritional intervention in conjunction with resistance training during convalescent rehabilitation has shown improvement in skeletal muscle mass and activities of daily living in older inpatients with decreased skeletal muscle mass [106]. Exercise, together with amino acid supplementation, can increase muscle mass and walking speed in older women with sarcopenia [107]. A protein-rich diet, combined with progressive resistance training, has enhanced lean tissue mass and muscle strength in older women [108].

A comprehensive approach to cachexia treatment should include exercise, nutrition therapy, psychosocial interventions, and pharmacologic therapies for cachexia and any underlying diseases. The combination therapy for cancer cachexia should target secondary causes of anorexia, reduced food intake, inflammation-related metabolic changes, and reduced physical activity [109]. Sarcopenia and cachexia that are treated using a comprehensive approach in people with a disability should also include the concept of rehabilitation nutrition, psychosocial interventions, and pharmacologic therapies (Fig. 7.3). However, no randomized controlled trials incorporate a fully structured regimen that includes an exercise program, nutritional support, and pharmacologic treatment of altered metabolism [110].

7.5 Conclusions

The prevalence of sarcopenia and cachexia is likely to increase in a society in which the older people are making up a larger proportion of the population than has been the case previously. The major causes of disability, including stroke, hip fracture, and hospital-associated deconditioning, are often complicated by sarcopenia and cachexia [6]. Furthermore, sarcopenia and cachexia increase the risk of adverse outcomes, such as functional disability and death. However, current pharmacologic therapy for sarcopenia and cachexia has not shown a clear benefit. Therefore, a comprehensive approach, including rehabilitation nutrition, psychosocial interventions,

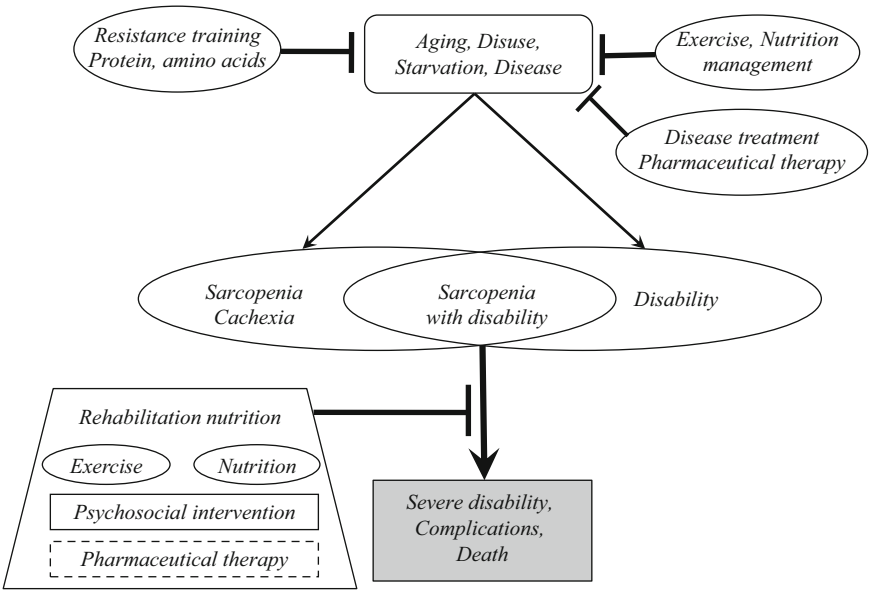

Fig. 7.3 Comprehensive approach to sarcopenia and cachexia treatment. Older people with age-, activity-, nutrition-, disease-related sarcopenia and cachexia can result in sarcopenia with disability and higher risk of adverse outcomes such as severe disability, complications, and death. In people with a disability complicating sarcopenia and cachexia, the concept of rehabilitation nutrition, which is implemented using the International Classification of Functioning, Disability and Health guidelines to evaluate nutrition status and to maximize functionality in people with a disability, can be useful. Comprehensive approach to sarcopenia and cachexia treatment in people with disability should include the concept of rehabilitation nutrition, psychosocial interventions, and pharmaceutical therapies

and pharmacologic therapies, is necessary for treating sarcopenia and cachexia. Further research is required on sarcopenia and cachexia treatments, especially for multimodal approaches in people with a disability.

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Chapter 8
The Role and Regulation of PGC-1α and PGC-1β in Skeletal Muscle Adaptation

Séverine Lamon and Aaron P. Russell

Abstract Members of the peroxisome proliferator-activated receptor gamma (PPARγ) coactivator-1 (PGC-1) family of proteins regulate mitochondrial biogenesis and function in the cell. PGC-1α and PGC-1β are highly expressed in skeletal muscle where they partner with numerous transcription factors to regulate energy metabolism. PGC-1α and PGC-1β not only control muscle oxidative capacity by influencing substrate metabolism and fibre type but also regulate essential cell processes in the muscle including angiogenesis, inflammation and neuromuscular junction formation. Positive adaption to exercise increases the expression of the PGC-1 family members in muscle, while conditions associated with muscle atrophy and perturbed metabolic function are often associated with a reduction in PGC-1 levels.

Keywords Peroxisome proliferator-activated receptor gamma (PPARγ) coactivator-1α (PGC-1α) • Peroxisome proliferator-activated receptor gamma (PPARγ) coactivator-1β (PGC-1β) • Skeletal muscle • Mitochondria • Energy metabolism • Exercise

8.1 Introduction

The first peroxisome proliferator-activated receptor gamma (PPARγ) coactivator-1 (PGC-1) family member, PGC-1α, was first discovered in 1998 [61]. Since then its family has been extended to consist of several other PGC-1α isoforms, including PGC-1α-a, PGC-1α-b and PGC-1α-c [51], and, more recently, PGC-1α4 [65]. Additional family members also include PGC-1β [43] and PGC-1-related coactivator (PRC) [1]. Both PGC-1α and PGC-1β are highly expressed in tissues requiring a large energy demand, such as brown adipose tissue, heart and skeletal muscle [1]. As such they were initially identified as potent regulators of tissue energetics and mitochondrial metabolism [1].

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The enriched expression of PGC-1α and PGC-1β in skeletal muscle has stimulated extensive research into their roles in regulating numerous processes important for healthy skeletal muscle metabolic and contractile function. Beyond the role of mitochondrial biogenesis and metabolic control, PGC-1α and PGC-1β have been implicated in numerous adaptive process including muscle fibre-type switching, angiogenesis, inflammation and neuromuscular junction formation. The use of transgenic gain and loss of function mouse models have played a key role in identifying the potential roles of PGC-1α and PGC-1β. However, the exogenous impact of PGC-1α and PGC-1β overexpression or ablation appears to be influenced by the promoters used, the tissues affected and the intensity of the exogenous regulation. Therefore, the translation of findings from transgenic PGC-1α and PGC-1β models, in relation to the potential role of endogenous PGC-1α and PGC-1β, needs to be considered carefully.

From a perspective of health and disease, PGC-1α is robustly upregulated following acute endurance or resistance exercise [76] or endurance training [66]. In human conditions and diseases associated with impaired skeletal muscle size and function, such as ageing [46], Huntington's disease [15], type II diabetes [54, 58] and amyotrophic lateral sclerosis (ALS) [68], PGC-1α and PGC-1β levels are reduced. However, a causal role for attenuated PGC-1 levels and disease remains equivocal. To advance the field, the development of pharmacological interventions to regulate PGC-1 family member, in a tissue-specific manner, is required.

8.2 The PGC-1 Family of Transcriptional Coactivators

The peroxisome proliferator-activated receptor gamma (PPARγ) coactivator-1 (PGC-1) family of proteins is a group of transcriptional coactivators central to the regulation of numerous cellular metabolic processes. PGC-1, now referred to as PGC-1α, was initially discovered in brown adipose tissue where it acts as a thermogenic regulator in response to cold exposure [61]. From the *Pgc-1α* gene, three tissue-specific transcripts are generated, referred to as PGC-1α-a, PGC-1α-b and PGC-1α-c [51]. Lately, a fourth truncated but functional isoform, PGC-1α4, has been identified in skeletal muscle where it may be involved in the regulation of rodent skeletal muscle mass [65]. Homologue members of the family include PGC-1β [43] and PGC-1-related coactivator (PRC) [1]. All family members share structural and functional similarities and possess a common N-terminal activation domain containing three LXXLL motifs that mediate interactions with other transcription factors [43]. Both PGC-1α and PGC-1β are enriched in tissues characterized by a high-energy demand, including brown adipose tissue, heart and skeletal muscle, whereas PRC expression is more stable across tissues [1]. All PGC-1 proteins are central regulators of tissue energetics and mitochondrial metabolism while also exerting individual tissue-specific effects. PGC-1 proteins partner with numerous transcription factors, including members of the peroxisome proliferator-activated receptors (PPAR), oestrogen-related receptor (ERR) and

nuclear respiratory factor (NRF) families, liver X receptor (LXR), retinoid X receptor (RXR), myocyte enhancer factor 2 (MEF2), forkhead box (FOXO) and hepatocyte nuclear factor 4 (HNF4), to coordinate the expression of genes involved in energy metabolism in the cell [22, 53, 72, 89, 94]. Beyond their role in the regulation of nuclear-encoded genes, PGC-1 proteins also directly mediate the expression of mitochondrial-encoded genes [26, 94] via their interaction with the transcription factor A mitochondrial (Tfam) and transcription factor B (TFB). PGC-1α and PGC-1β are the preferentially expressed isoforms in skeletal muscle, a tissue characterized by high-energy needs and remarkable structural and functional plasticity.

As a consequence of their multifactorial role in the regulation of energy metabolism in the cell, numerous signalling pathways modulate the expression and function of PGC-1 proteins in response to intra- and extracellular stress signals (Fig. 8.1). Signalling via the p-38 mitogen-activated protein kinase (MAPK) pathway activates PGC-1α [37]. Similarly, exercise induces PGC-1α in skeletal muscle. By increasing calcium concentration in the cell, exercise activates MAPK and indirectly enhances PGC-1α gene expression via the phosphorylation of the activating transcription factor-2 (ATF-2) that binds to PGC-1α promoter [91, 93]. PGC-1α activity is also regulated post-translationally. The 5′ AMP-activated protein kinase (AMPK) molecular pathway is triggered by exercise-induced ATP depletion. In contrast to p-38 MAPK, AMPK activation directly phosphorylates and activates PGC-1α [34]. PGC-1α is targeted by numerous other kinases, including protein kinase B (PKB, also known as Akt) [42], ribosomal protein S6 kinase beta-1 (S6K1, also known as p70-S6K) [48] and glycogen synthase kinase 3 beta (GSK3β) [57], which directly or indirectly regulate its biological activity. Further post-translational modifications include acetylation by acetyltransferases or deacetylases [12, 25] and ubiquitination by E3 ubiquitin ligases [57, 80]. Finally, PGC-1α expression is also regulated by DNA methylation of its promoter region. Situations activating PGC-1α such as exercise reduce promoter methylation [7], while situations decreasing PGC-1α such as diabetes enhance promoter methylation [45]. These enzymatic and epigenetic modifications are essential to fine-tune PGC-1α activity by enhancing or repressing its binding affinity for a specific co-transcription factor. PGC-1β is also post-translationally modified via lysine acetylation [36].

8.3 microRNA Regulation of PGC-1α

microRNAs (miRNAs) are small non-coding RNAs that play a major role in skeletal development and adaptation to various physiological and pathophysiological situations [67, 96]. miRNAs inhibit protein expression [38, 56] either by degrading specific mRNA species or by repressing their protein translation. The miRNA inhibitory activity most commonly occurs through the binding of the 5′ end of the miRNA with the 3′ UTR of the target mRNA, although miRNA targeting the 5′ UTR may also occur. Overexpression of miR-696 in C2C12 myotubes negatively

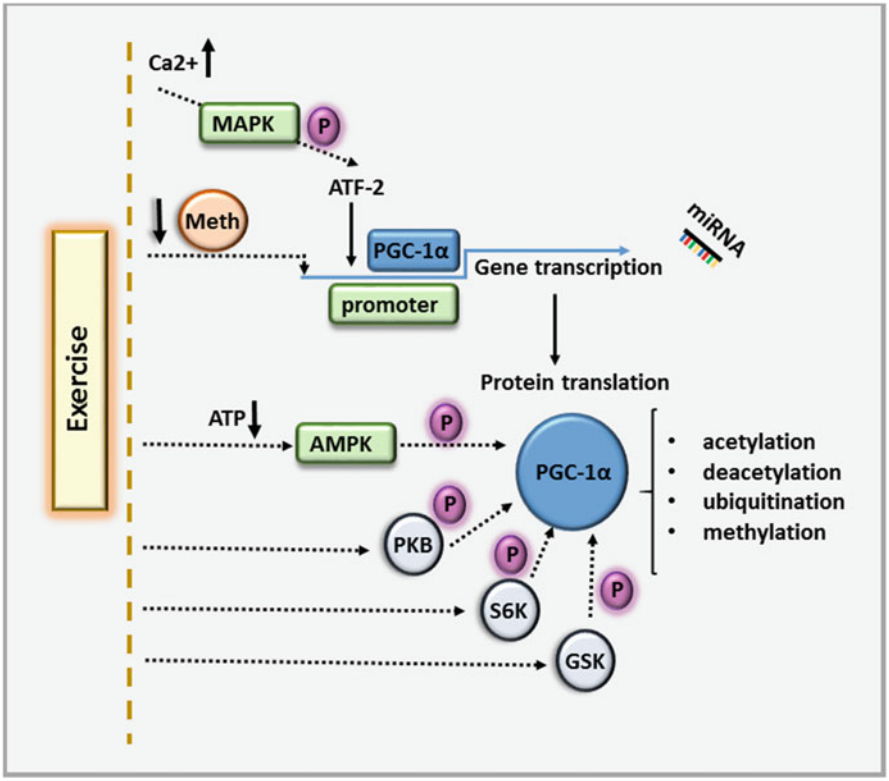

Fig. 8.1 Transcriptional and translational control of skeletal muscle PGC-1α. Exercise is a potent regulator of PGC-1α in muscle. Increases in calcium concentration (Ca2+) activate the p-38 mitogen-activated protein kinase (MAPK) pathway. This indirectly enhances PGC-1α gene expression via the phosphorylation (P) of the activating transcription factor-2 (ATF-2) that binds to PGC-1α promoter. Additionally, PGC-1α gene expression is increased by the exercise-induced attenuation of promoter methylation (Meth). miRNA binding to the 3′ untranslated region of the PGC-1α gene can result in mRNA degradation or inhibit protein translation. Post-translational regulation of PGC-1α activity can occur via the exercise-induced depletion of ATP and consequent activation of 5′ AMP-activated protein kinase (AMPK), which can directly phosphorylate and activate PGC-1α. PGC-1α is also targeted by other exercise-activated kinases, including protein kinase B (PKB, also known as Akt), ribosomal protein S6 kinase beta-1 (S6K1, also known as p70-S6K) and glycogen synthase kinase 3 beta (GSK3β). Further post-translational modifications include acetylation, deacetylation, ubiquitination and methylation

regulates PGC-1α protein with a corresponding decrease in two PGC-1α-regulated genes, pyruvate dehydrogenase kinase 4 (PDK4) and cytochrome c oxidase subunit 2 (COXII) as well as fatty oxidation [2]. However, it was not established if miR-696 directly regulates PGC-1α via 3′ UTR binding.

miR-23a binds directly to the PGC-1α 3′ UTR in C2C12 myotubes, repressing its mRNA levels, while transgenic mice overexpressing miR-23a have a reduction in PGC-1α, cytochrome b and cytochrome c oxidase complex IV (COX IV) protein

levels [68]. In miR-23a transgenic mice, when compared with wild-type mice, protein markers of mitochondrial content, including PGC-1α and COX IV, are significantly decreased in the slow soleus muscle, but not the fast plantaris muscle [85]. However, the miR-23a reduction in PGC-1α did not affect the adaptation to 4 weeks of voluntary wheel running, with increases in endurance exercise capacity, muscle mass, capillary density and the protein content of myosin heavy chain IIa, PGC-1α, COX IV and cytochrome c observed. It appears that miR-23a targets PGC-1α and regulates basal metabolic properties of slow but not fast-twitch muscles, but does not influence whole-body endurance capacity or exercise-induced muscle adaptations in the fast plantaris muscle [85].

8.4 Roles of the PGC-1 Family Members in Skeletal Muscle

Skeletal muscle has a rare capacity to continually adapt to stress exerted by its external environment. With such a high level of expression and rapid regulation in skeletal muscle, PGC-1α and PGC-1β are important players in numerous adaptive processes. These include mitochondrial biogenesis, muscle fibre type, substrate metabolism, angiogenesis, inflammation and neuromuscular junction formation (Fig. 8.2). The majority of our understanding of the potential role of PGC-1α and PGC-1β has come from transgenic gain and loss-of-function mouse models. However, the physiological impact of exogenous PGC-1α and PGC-1β overexpression appears to be influenced by the promoters used, the tissues affected and the intensity of the transgenic regulation. Therefore, a considered approach should be taken when interpreting certain findings based on transgenic PGC-1α and PGC-1β models, in relation to the potential role of endogenous PGC-1α and PGC-1β.

8.5 Mitochondrial Biogenesis

In vitro, the overexpression of PGC-1α [94] or PGC-1β [77] in myotubes increases mitochondrial mass. In mice with a muscle-specific transgenic overexpression of PGC-1α [44, 90] or PGC-1β [5], there is an increase in genes involved in mitochondrial biogenesis, function and oxidative metabolism. In skeletal muscle cells and tissue, PGC-1α and PGC-1β exert their positive effect on mitochondrial biogenesis through its direct interaction and coactivation of ERR and NRF family members [22, 53, 73, 75]. It appears that PGC-1α is not required for basal mitochondrial biogenesis as PGC-1α knockout mice have a very mild attenuation in mitochondrial function [27, 41]. In contrast to PGC-1α knockout mice, the ablation of PGC-1β, specifically in mouse skeletal muscle, decreases in expression of genes controlling mitochondrial protein import, translational machinery and energy metabolism, leading to impaired mitochondrial structure and function [24].

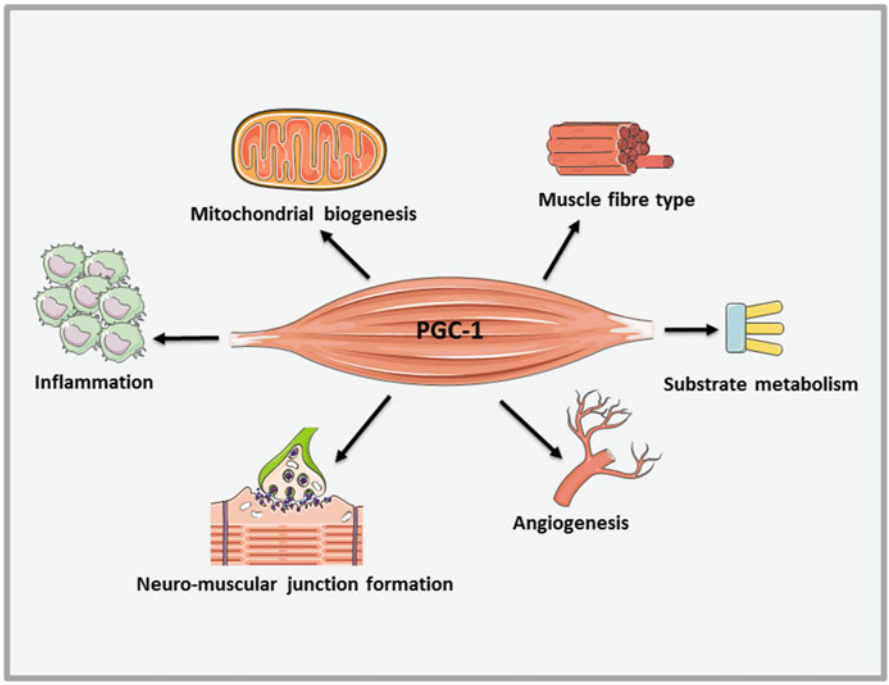

Fig. 8.2 Various processes regulated by PGC-1α and/or PGC-1β in skeletal muscle

8.6 Muscle Fibre Type

Transgenic overexpression of PGC-1α in fast type IIb glycolytic muscles results in a conversion to the more oxidative slow-twitch type I and IIa fibres [44]. PGC-1β increases the fast-oxidative type IIX muscle fibres via its coactivation of the MEF2 transcription factor family [5]. The high oxidative capacity of these muscle fibres is due to their enrichment in the mitochondria that is influenced by the PGC-1α and PGC-1β coactivation of ERRα and NRF1. However, as PGC-1α and PGC-1β knockout mice appear to have a normal muscle fibre composition, other factors also play a major role in fibre-type determination [4, 24].

8.7 Substrate Metabolism

Both PGC-1α and PGC-1β regulate the expression of many genes involved in energy metabolism and substrate oxidation [30], and many of these effects are dependent on ERRα [10, 73, 89]. PGC-1α or PGC-1β overexpression in rodent skeletal muscle increases glucose transporter type 4 (GLUT4) levels as well as insulin-stimulated

glucose transport [8, 36]. Their overexpression also increases the expression of numerous genes involved in fat oxidation, such as the fatty acid transporter, CD36 [8, 33, 81]. PGC-1α or PGC-1β overexpression in L6 myotubes also increases creatine transporter mRNA levels and creatine uptake via ERRα [10].

8.8 Angiogenesis

Increase in skeletal muscle capillary density, a process referred to angiogenesis, is an important adaptation to exercise and plays a role in improved endurance performance. This allows for increased blood flow and nutrients to enter the muscle. PGC-1α and PGC-1β, through hypoxia-inducible factor (HIF)-independent but oestrogen-related receptor-α (ERRα)-dependent activation, increase the gene expression of vascular endothelial growth factor (VEGF), a key player in angiogenesis [3, 63]. PGC-1α also positively influences angiogenesis via its upregulation of secreted phosphoprotein 1 (SSP1) [64]. This PGC-1α/SSP1 regulatory axis stimulates macrophage activation and recruitment. As observed in many other situations, it appears that PGC-1α [16] is not required for basal angiogenesis. However, it is required for exercise-induced angiogenesis [40].

8.9 Neuromuscular Junction Formation

The neuromuscular junction (NMJ) is a synapse that forms a connection between the motor neuron and the muscle fibres. In doing so the NMJ mediates signals that are important for muscle activity. PGC-1α overexpression positively regulates NMJ formation and stability [6, 29]. Ablation of PGC-1α reduces gene expression of a number of NMJ-related genes such as acetylcholine receptor (AChR) and utrophin [29]. As such the manipulation of PGC-1α levels has been considered as a potential therapeutic approach for diseases with impaired NMJ function such as Duchenne muscular dystrophy (DMD) and amyotrophic lateral sclerosis (ALS). In the *mdx* mouse model of human DMD, transgenic overexpression of skeletal muscle PGC-1α improves muscle histology, running performance and plasma creatine kinase levels [29]. Similarly, viral-induced overexpression of PGC-1α in neonatal *mdx* mice increased utrophin and type I myosin heavy chain expression. There was also an increase in mitochondrial protein content as well as resistance to contraction-induced damage and fatigue [74]. Viral-induced overexpression of PGC-1α in 1-year-old *mdx* mice increases muscle tension and force production, when measured 3 months postinjection. However, histopathology was not improved [31]. Overexpressing PGC-1β in skeletal muscle of *mdx* mice also improves muscle function, without significantly impacting the NMJ [14]. Skeletal muscle overexpression of PGC-1α in fast-twitch muscles of $SOD1^{G37R}$ ALS mouse model

improves skeletal muscle function and mitochondrial biogenesis, but does not preserve neurodegeneration or extend survival [18].

8.10 Inflammation

In multiple conditions of disuse and inactivity, as well as in disease conditions, systemic inflammation is often observed [23, 49], a response commonly associated with reduced levels of skeletal muscle PGC-1α and PGC-1β [50, 58, 68]. Often the inflammatory response is stimulated by a discordant metabolic and immune response resulting in the accumulation of pro-inflammatory macrophages that release pro-inflammatory cytokines such as tumour necrosis factor alpha (TNF-a), interlukin-6 (IL-6) and monocyte chemotactic protein 1 (MCP-1) [55]. Skeletal muscle ablation of PGC-1α results in an increase in both local skeletal muscle and systemic inflammation [28]. In C2C12 muscle cells, the overexpression of PGC-1α and PGC-1β attenuates the upregulation of pro-inflammatory cytokine expression following treatment with TNFα, a response mediated via the PGC-1 reduction in p65 phosphorylation (NF-κB family member) [21]. In vivo, mice with a skeletal muscle transgenic overexpression of either PGC-1α or PGC-1β have an altered anti-inflammatory profile characterized by increases in C-C motif chemokine ligand 1 (CCL1), CCL22, interleukin 1 receptor antagonist (IL-1Ra), transforming growth factor β (TGFβ) or interlukin-10 (IL-10). This was also associated with a decrease in interlukin-12 (IL-12) [20].

8.11 Expression of the PGC-1 Family Members in Models of Skeletal Muscle Use, Disuse and Disease

The multifactorial role played by PGC-1α and PGC-1β in skeletal muscle makes them susceptible to high levels of regulation as the metabolic environment of the muscle changes. One important aspect of the plasticity of skeletal muscle is its ability to rapidly increase or decrease its mass in response to anabolic and catabolic stimuli. Expression of the PGC-1 proteins is reduced in conditions associated with skeletal muscle atrophy and perturbed metabolic function. Normal ageing is typically associated with a decrease in muscle mass and function that is exacerbated by physical inactivity [84]. PGC-1α and PGC-1β mRNA levels were higher in young (28 ± 0.2 years) than in elderly healthy adults (62.4 ± 0.2 years) [46], and muscle PGC-1α protein content was decreased in sedentary old adults (63 ± 3.2 years) when compared to active old adults (70 ± 1.6 years) and to young adults (22 ± 0.6 years) [70]. Immobilization, a model of muscle disuse, partially mimics the effects of age-related muscle wasting and induces accelerated muscle atrophy. In both young (21–30 years) and older (60–72 years) subjects,

immobilization decreased PGC-1α and PGC-1β gene expression after 2 days and 4 days, while a decrease in PGC-1α mRNA was still visible after 14 days of muscle disuse [78]. Similarly, 5 days of one-legged knee immobilization decreased PGC-1α mRNA expression in healthy young (22 ± 1 years) subjects [86]. Muscle atrophy is also a secondary outcome of numerous metabolic or genetic disorders, which are frequently associated with attenuation in the activity and expression levels of the PGC-1 family members. When compared to healthy controls, PGC-1α and PGC-1β gene levels were reduced in muscle from patients with Huntington's disease [15], a disease characterized by impaired oxidative phosphorylation [17], and from patients with type II diabetes [54, 58]. A collective attenuation of PGC-1 signalling genes is observed in the muscle of patients with amyotrophic lateral sclerosis (ALS). In addition to a decrease in PGC-1α and PGC-1β protein levels, their downstream targets ERRα, NRF1, MFN1, MFN2 and COX IV were downregulated, reflecting a profound disruption of the whole mitochondrial network [68]. Finally, comparable findings have been reported in animal models. PGC-1α protein levels were notably reduced in a rat model of congestive heart failure [82], while PGC-1α gene levels were attenuated in rats displaying cancer cachexia, chronic renal failure or submitted to denervation [69, 72]. Overexpressing PGC-1α in cultured muscle fibres provided an insight into the molecular mechanisms underlying its role in muscle atrophy. It was proposed that PGC-1α protects muscle mass by attenuating the transcriptional activity of FOXO3a, a central activator of muscle protein degradation [9, 72]. In line with this hypothesis, overexpressing PGC-1α in rodent muscle limited muscle atrophy induced by age [90] and immobilization [35].

Endurance exercise is a potent activator of mitochondrial biogenesis and oxidative metabolism in muscle [32]. As such, a single bout of exercise induces the PGC-1 family members in skeletal muscle, with little distinction between exercise types and regimes. An early study showed that PGC-1α mRNA increased at 2 and 6 h following 3 h of two-legged knee extensor exercise [60]. Ten kilometre cycling time trial induced mRNA expression of PGC-1α and its coactivator ERRα 2 hours postexercise while activating the mitochondrial regulators Mfn1, Mfn2, NRF-2 and COX IV 24 hours postexercise [13]. Ninety minutes interval and continuous cycling exercise at a similar intensity equally increased PGC-1α mRNA [88]. Interestingly, resistance exercise also has the ability to induce the PGC-1 family members. Following a single session of endurance or resistance exercise, there was an increase in PGC-1α mRNA 30 min and 180 min after exercise, respectively, with no change in PGC-1β [76]. Finally, resistance exercise potentiated the endurance-induced activation of PGC-1α by increasing its mRNA levels by twofold more than following endurance exercise alone [87].

Exercise training stimulates the expression of the PGC-1 family, suggesting a sustained role of the PGC-1 proteins in muscle adaptation to exercise and remodelling. PGC-1α mRNA expression increased in healthy men undergoing 6 weeks of endurance training, with a greater relative increase observed in type IIa fibres than in type I and IIx fibres [66]. Eight weeks of either endurance or resistance training independently enhanced PGC-1α mRNA expression, but as it is the case for a single bout of exercise, combined training regimes potentiated this activation [65].

While PGC-1α mRNA expression consistently increases with endurance training, one study reported an increase in mitochondrial function that was not associated to an increase in PGC-1α protein expression in response to 2 weeks of high-intensity interval training (HIT) [83], while 6 weeks of HIT increased PGC-1α protein content by 100 % [11]. Such discrepancies may result from differences in PGC-1α protein subcellular localization. At rest, PGC-1α is localized in the cytosol, but its presence in the nucleus increases following endurance exercise [71, 92]. There is also evidence suggesting that PGC-1α can shuttle to the mitochondria to allow Tfam coactivation [71]. As a result, total PGC-1α protein content may not always be reflective of PGC-1α activation [92].

While the roles of the PGC-1 family members in suppressing protein degradation and enhancing mitochondrial metabolism have been independently established, it is less clear whether the PGC-1 proteins have the capacity to trigger muscle hypertrophy by enhancing muscle protein synthesis. PGC-1α did not transiently increase protein synthesis in C2C12 myoblasts [9], and one study showed that the hypertrophy response induced by chronic overload of skeletal muscle was independent of PGC-1α [59]. A recently described truncated spliced variant of PGC-1α, PGC-1α4, was expressed in resistance-trained mice and humans [65]. In humans, 8 weeks of resistance training increased PGC-1α4 expression, and its expression levels were correlated with leg press exercise performance. Rather than regulating genes involved in cell bioenergetics, PGC-1α4 stimulated muscle hypertrophy in vitro and in mice by inducing insulin growth factor-1 (IGF-1), the primary stimulator of the PI3/AKT pathway, one of the major protein synthesis signalling routes in muscle [62]. It also repressed the myostatin pathway, an inhibitor of AKT and negative regulator of muscle mass [79]. Induction of PGC-1α4 was however not resistance exercise specific [47, 95], and exercise-induced PGC-1α4 expression did not correlate with changes in muscle size or muscle strength, questioning the role for PGC-1α4 in human muscle hypertrophy.

8.12 Conclusion

Since the discovery of the PGC-1 family, a considerable amount of work has been performed to identify molecules that activate and inhibit its function, as well as the downstream transcription factors that they coactivate. The development of several gain- and loss-of-function mouse models has identified the pleiotropic roles that the PGC-1 family plays, especially in skeletal muscle. This has established the PGC-1 proteins as potential targets to treat diseases characterized by metabolic and neuromuscular impairments, as well as conditions such as ageing and immobilization. As the PGC-1 family are expressed in numerous tissues and are involved in the physiological adaptation to multiple stress situations, their endogenous levels are often downregulated in many pathological conditions. However, a causal role for attenuated PGC-1 activity and disease remains equivocal. Presently the development of pharmacological interventions to regulate PGC-1 family members has eluded

the field and remains a research priority into the future. With this in mind, caution is required as excessive upregulation of PGC-1α can cause cardiomyopathy [39], skeletal muscle atrophy [52] and neurodegeneration [19]. Therefore, the ability to modulate the amount of PGC-1 activity in specific tissues will also present a challenge to the field.

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Chapter 9
Characteristics of Skeletal Muscle as a Secretory Organ

Wataru Aoi

Abstract Growing evidence has shown that skeletal muscle cells can secrete bioactive proteins into the extracellular milieu. Secretion of many of those proteins is accelerated in response to exercise and muscle contraction and can regulate functions of several organs via autocrine, paracrine, and endocrine routes; this is referred to as the myokine theory. Habitual exercise leads to various health benefits such as metabolic improvement, anti-inflammation, and muscle building, which are at least partly caused by myokines including specific interleukins. Comprehensive analysis suggests that skeletal muscle cells can secrete over 100 proteins, many of which remain unknown. However, recent studies have identified additional novel myokines. Some secreted proteins can improve nutrient metabolism and muscle/bone mass; others exert anti-inflammatory and anti-tumorigenesis effects. The concept of myokines could be expanded to not only include high molecule weight proteins but also small peptides and noncoding RNA and applied to various fields including nutrition and medicine.

Keywords Myokine • Skeletal muscle • Energy metabolism • Inflammation • Cancer

9.1 Introduction

Skeletal muscle supports the body, mediates physical activity with muscle contraction, and functions as a major nutrient consumer, consuming over 70 % of blood glucose [1]. In addition, growing evidence indicates that several bioactive proteins and low molecular weight peptides are secreted by muscle cells and elevated in response to exercise. These secreted factors can regulate muscle itself in addition to other organs via an endocrine, autocrine, or paracrine routes. This is referred to as the myokine theory [2]. Such secreted proteins regulate metabolic capacity, muscle mass, bone density, hormone secretion, cognitive function, and tumorigenesis (Fig. 9.1).

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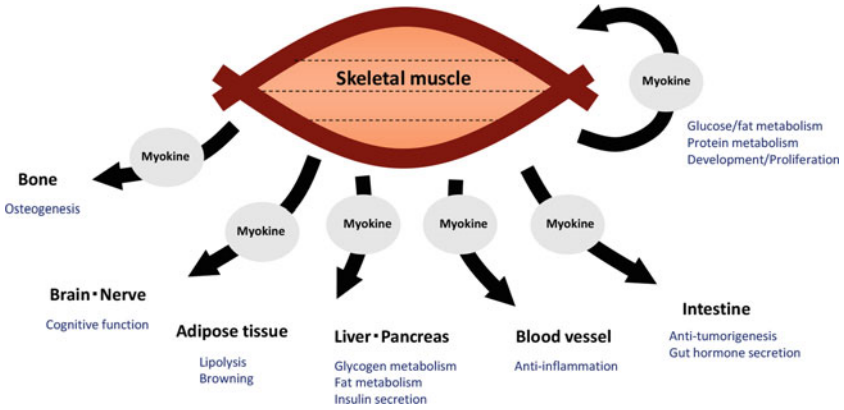

Fig. 9.1 Skeletal muscle is a secreted organ. Several proteins and low molecular weight peptides are secreted by muscle cells. These secreted factors can regulate muscle itself in addition to other organs via an endocrine, autocrine, or paracrine routes. Such secreted proteins can regulate metabolic capacity, muscle mass, bone density, hormone secretion, cognitive function, and tumorigenesis

Accumulating evidence has demonstrated the mechanisms underlying the benefits of acute and regular exercise. One bout of exercise drastically changes various physiological parameters such as hormone production, blood flow, and the activity of the nervous and immune system, in addition to altering the expression or activity of certain genes and proteins in skeletal muscle. For example, improvement of glucose metabolism is observed not only during exercise but also several hours after, often persisting until the next day. Furthermore, habitual exercise adaptively improves various physiological functions including energy metabolism, nervous system, endocrine system, and immune function, even in the resting state. The expression or activity of several key proteins in skeletal muscle is involved in the development of this adaptation. Adequate regular exercise has numerous health benefits. Epidemiological studies have shown that habitual exercise reduces the risk of various common diseases such as type 2 diabetes, cardiovascular disease, and carcinogenesis [3–7]. In addition, regular exercise improves the prognosis of existing diseases, including diabetes, ischemic heart disease, heart failure, and chronic obstructive pulmonary disease. Secreted proteins are suggested to mediate the acute and chronic effects obtained by exercise and could promote health benefits.

9.2 Muscle-Secreted Proteins as Metabolic Regulators

Many secreted proteins have been suggested to be involved in the regulation of metabolic function of skeletal muscle itself and other metabolic organs. Interleukin (IL)-6 is a representative myokine that is transiently elevated in muscle

following one bout of exercise [8]. IL-6 may act locally within the contracting skeletal muscle in a paracrine manner or be released into the circulation and may increase over tenfold, thus inducing systemic effects [9, 10]. IL-6 directly increases glucose metabolism of skeletal muscle in resting state [11] and can lead to further improvement of insulin sensitivity in response to exercise [12]. In addition, previous studies also showed that recombinant IL-6 infusion at normal physiological levels selectively stimulates lipid metabolism in skeletal muscle in healthy subjects [13] and in subjects with type 2 diabetes [14]. In addition, muscle-derived IL-6 has been suggested to play a role in increased lipolysis in adipose tissue through an endocrine mechanism [15]. In fact, recombinant IL-6 intra-lipid infusion elevates plasma fatty acid levels, which probably caused by adipose tissue lipolysis [12]. Furthermore, injection of IL-6 results in hepatic glycogen catabolism and accelerated circulatory glucose output in rats [16]. This may contribute to the maintenance of blood glucose and supply the required energy substrate during exercise. In addition, physiological elevation of IL-6 levels stimulates production of an insulin secretagogue, glucagon-like peptide-1, from intestinal L cells and pancreatic alpha cells, which ultimately promotes insulin secretion from pancreatic β cells [17].

In addition to IL-6, other muscle-secreted proteins including brain-derived neurotrophic factor, fibroblast growth factor (FGF) 21, and IL-15 have been shown to be produced in skeletal muscle in response to acute or chronic exercise and have been suggested to increase nutrient metabolism [18–21]. FGF21 is secreted from muscle cells with activation of Akt signaling and can stimulate lipolysis, glucose transporter (GLUT) 1-mediated glucose uptake in adipocytes [22], fatty acid utilization for energy, and ketone body production in the liver [23]. In muscle cells, FGF21 exposure increases glucose uptake in both absence and presence of insulin via GLUT-1 and GLUT-4 [19]. Additionally, FGF21 prevents palmitate-induced insulin resistance in skeletal muscle [24]. These observations suggest that FGF21 plays a role in lipolysis and glucose uptake in the liver, adipose tissue, and skeletal muscle. IL-15 has been shown to be increased in skeletal muscle and plasma by acute exercise [20, 25], although this remains controversial. IL-15 overexpression in muscle resulted in increased expression of oxidative metabolic factors including sirtuin 1, peroxisome proliferator-activated receptor (PPAR)-δ, PPAR-γ coactivator (PGC)-1α, and PGC-1β in skeletal muscle and adipose tissue [26, 27], which would contribute exercise-induced activation of glucose and lipid metabolism. The chemokine CXC motif ligand-1 (CXCL-1), referred to as keratinocyte-derived chemokine (KC) in mice and IL-8 in humans, is also elevated by one bout of exercise in skeletal muscle locally [28, 29]. CXCL-1 overexpression induces aerobic metabolism in skeletal muscle and suppresses diet-induced fat accumulation in adipose tissue. Myonectin has been also identified as a regulatory factor of lipid metabolism in the liver and adipose tissue [30].

A recent study showed that PGC-1α1 expression in muscle stimulates expression of FNDC5, a membrane protein that is cleaved and secreted as irisin [31]. PGC-1α1 has been shown to play a central role as part of transcriptional coactivators involved in aerobic metabolism; thus, a considerable amount of attention has been focused on it as a target for the prevention or treatment of metabolic syndrome

through activation of lipid metabolism. Acute and habitual exercise elevates PGC-1α1 expression in skeletal muscle [32, 33] and, consequently, the secretion of irisin from muscle into the circulation. Secreted irisin acts on white adipose cells and facilitates brown fatlike development, which may account for metabolic elevation and body fat reduction induced by exercise although it remains controversial how this factor actually contributes. More recently, the same group reported that another PGC-1α1-dependent small molecule myokine, ß-aminoisobutyric acid (BAIBA), is also increased by habitual exercise and stimulates browning of white adipose tissue along with hepatic fat oxidation via PPARα-mediated signaling [34]. Habitual exercise has been shown to increase circulating BAIBA along with a reduction of metabolic risk factors in mice. Additionally, meteorin-like (Metrnl) is also secreted from skeletal muscle with overexpression PGC-1α4 [35] which is known as a regulator of muscle hypertrophy but not aerobic metabolic capacity [36]. The elevation of circulating Metrnl induces oxygen uptake along with thermogenesis, which is caused by increasing IL-4 expression via eosinophils and then induces the browning of white adipose tissue indirectly via modulation of macrophage activity [35]. In contrast to BAIBA, the level of circulating Metrnl is increased in response to a single bout of exercise.

9.3 Muscle-Secreted Proteins and Myogenesis/Osteogenesis

It has been suggested that muscle-secreted proteins have additional functions, and some proteins contribute to muscle hypertrophy via autocrine or paracrine effects. Insulin-like growth factor-1 (IGF-1) is a well-known secreted protein contributing to muscle mass [37, 38]. It has been shown that overexpression of IGF-1 caused by transgenic and adeno-associated virus injection results in hypertrophy and higher strength and accelerates muscle regeneration in normal and dystrophic mice [39–41]. The hypertrophic effect is mainly mediated by protein synthesis through increased Akt-mTOR-p70S6 K signaling. The insulin signaling pathways, PI3K/Akt and mTOR/p70S6 K, play important roles in glucose uptake as well as protein synthesis in muscle cells. In the synthesis cascade, Akt signals to p70S6 K via mTOR activation, resulting in phosphorylation of S6-rebosomal protein and increased translation of various proteins related to hypertrophy [42]. IGF-I overexpression can also prevent age-induced muscle atrophy in mice [43]; thus, upregulation of IGF-I would be an appropriate method for inhibiting sarcopenia. However, there are controversial findings regarding the effect of IGF-I administration to the elderly on muscle mass and function because decrease in sensitivity to IGF-I occurs in aged muscle [44]. Leukemia inhibitory factor (LIF) is another secreted protein that accelerates muscle mass. The expression level is regulated by intracellular Ca^{2+} induced by exercise in skeletal muscle, and LIF is released into extracellular fluid but not seem to reach the circulation [45]; thus, it acts locally in muscle tissues. LIF stimulates satellite cell proliferation via mainly activation of the JAK2-STAT3 signaling pathway and promotes muscle hypertrophy and regeneration [46].

In contrast, growth and differentiation factor 8, known as myostatin, which belongs to the transforming growth factor-b (TGF-b) superfamily, is an established inhibitor of muscle hypertrophy. Myostatin is secreted and circulates in blood in an inactive complex with several proteins and peptides [47]. After binding activin receptor IIB on the cell membrane, myostatin stimulates Smad signals and decreases the level of myogenic regulatory factors including MyD and Pax3, leading to inhibition of myoblastic proliferation and differentiation during developmental myogenesis [48, 49]. In addition, it suppresses protein synthesis via Akt-mTOR signaling. As a result, myostatin leads to inhibition of cell cycle progression and reduction of both myogenesis and protein synthesis.

Follistatin-related factors directly bind to myostatin and inhibit its anti-hypertrophic effect [50]. Follistatin-like protein 1 (Fstl1), a muscle-secreted protein, is known as a representative inhibitor of myostatin. It has been shown that Fstl1 is secreted from muscle cells into circulation through activation of Akt-mTOR signal in response to IGF-I [51]. In addition, inflammatory cytokines such as interferon gamma and IL-1β stimulate secretion of Fstl1. Because the level of Fstl1 in circulation is increased by a single bout of exercise in humans [51], the effect of this protein would spread to the entire body in an endocrine manner. Decorin, a leucine-rich proteoglycan, is secreted from contracting muscle into the circulation in response to a bout of exercise and can inhibit function of myostatin via not only direct binding but also stimulating expression of Mighty, a downstream factor of the myostatin cascade [52]. In decorin overexpression muscles, Myod1 and follistatin were increased, whereas ubiquitin ligases, atrogin1 and MuRF1, were decreased [52]. Thus, decorin secreted from muscle cells is involved in hypertrophy and atrophy of skeletal muscle. The expression of decorin has been shown to be increased by strength training in humans and mice, which could contribute adaptation to muscle mass.

More myokines have been reported to induce muscle proliferation and differentiation of satellite cells. Chitinase-3-like protein 1 is elevated in the circulation and muscle tissues by acute exercise and activates myoblast proliferation via protease-activated receptor 2 [53], which is involved in restructuring of skeletal muscle in response to exercise training. IL-7, identified in media from primary cultures of human myotubes differentiated from satellite cells, stimulates satellite cell migration [54]. Interestingly, strength training markedly increases IL-7 expression in skeletal muscle, which suggests that IL-7 is associated with muscle hypertrophic adaptation induced by training. IL-6 also has an aspect that contributes exercise-induced proliferation of satellite cells after muscle damaging exercise [55]. STAT3 signaling induced by IL-6 has been suggested to act as a regulator of the proliferation.

Several proteins secreted by skeletal muscle can regulate bone metabolism; namely, there is a cross talk system between muscle and bone. Conditioned medium from cultured muscle cells stimulates differentiation of bone marrow stromal cells, promotes bone healing, and results in osteoblast formation [56, 57]. It has been suggested that candidate effectors among secreted proteins include the growth factors IGF-I and TGF-β and cytokines such as IL-6 and IL-15 [58–60]. In addition, irisin

also serves to increase formation of osteoblasts [61]. This effect is mainly exerted through Wnt/β-catenin signaling that can protect osteocytes from glucocorticoid-induced apoptosis [62] and through MAP kinase signaling that promotes osteoblast proliferation and differentiation [63]. Recently, connective tissue growth factor was found as a novel osteogenic factor which secreted from skeletal muscle in bioinformatics analysis [64], and its expression has been shown to be increased in response to exercise [65].

9.4 Muscle-Secreted Proteins and Anti-inflammation/Anti-tumorigenesis

Muscle-secreted proteins may also have anti-inflammatory properties, and muscle-derived IL-6 likely contributes to reduced inflammation when in circulation [66]. IL-6 can increase the levels if anti-inflammatory factors including IL-10, IL-1 receptor agonist, and C-reactive protein in neutrophiles and the liver [67]. Indeed, recombinant IL-6 infusion inhibits the endotoxin-induced increase in circulating levels of tumor necrosis factor (TNF-α), a representative pro-inflammatory cytokine [68]. IL-6 is also recognized as a pro-inflammatory cytokine. In severe systemic infection, circulating IL-6 is drastically elevated and may reach over 10,000-fold the level in resting healthy state. In contrast, chronic low-grade elevation of IL-6 (below tenfold of that in resting healthy state) is induced by sedentary life, obesity, and dietary habits, which are associated with the development of metabolic diseases, although regular physical activity reduces the elevation of circulating IL-6 in the resting state along with metabolic improvement [69, 70]. Therefore, it is necessary to consider separately the exercise-induced secretion of IL-6, which is a transient/moderate elevation, and the pathological states, which are transient/high or chronic/low elevations. Other myokines such as Fstl1 also exert anti-inflammatory activity as a systemic effect [71].

A number of epidemiological studies have showed the average individual's level of physical activity and its relationship to the incidence of cancer in Europe, the United States, and Japan. The general consensus among the authors of these studies is that physical activity can prevent cancer in the colon, breast, uterus, pancreas, and lungs [72–75]. In particular, almost all studies clearly demonstrate that physical activity significantly reduces the incidence of colon cancer. A review of these epidemiological studies by the World Cancer Research Fund/United States Cancer Research (WCRF/AICR) showed that physical activity was the only lifestyle change that would convincingly reduce an individual's risk of colon cancer [76]. Although the exact mechanism underlying the beneficial results obtained in epidemiological studies remains unclear, various potential mechanisms such as activation of the immune system and antioxidant status, anti-inflammatory signals, improved insulin sensitivity, proportion of different bile acids, and exercise-induced increases in gastrointestinal transit have been suggested [77–81]. Previously, we reported that

regular exercise prevents the formation of aberrant crypt foci (ACF), which are the precursor lesions of colon adenocarcinoma, associated with anti-inflammatory activity on the mucosal surface of the mouse colon [82]. However, the endogenous defense system, such as antioxidants and chaperone proteins remained unchanged, suggesting that the anti-tumorigenesis effect of habitual exercise is affected by the levels of circulating factors such as myokine, rather than endogenous proteins in the colon.

Secreted protein acidic and rich in cysteine (SPARC) was identified as a muscle-derived protein that suppresses tumorigenesis, using comprehensive transcriptome approach of muscle tissue in sedentary and exercised young and old mice [83]. SPARC in the muscle tissue was elevated in response to one bout of exercise. However, circulating SPARC does not adaptively elevate by habitual exercise in the resting state, suggesting that increased SPARC levels in muscle tissues due to regular exercise do not contribute substantially to the circulating concentration while at rest. However, habitual exercise significantly promoted the transient exercise-induced increase in the circulating SPARC levels.

SPARC belongs to the matricellular protein family, which is primarily involved in development, remodeling, and tissue repair through modulation of cell-cell and cell-matrix interactions [84–86]. In addition, SPARC has been reported to have more unique functions such as angiogenesis regulation, collagen production/fibrillogenesis, chaperoning, inhibiting adipogenesis, and further exerting anti-tumorigenic effects [87–90]. Previous studies have revealed that a lack of SPARC promotes pancreatic and ovarian tumorigenesis in vivo [91, 92]. In addition, the presence of exogenous SPARC in cancer cell lines reduces cell proliferation in vitro [93]. Furthermore, epigenetic silencing of the *SPARC* gene via promoter hypermethylation is frequently identified in colon cancers and is linked to rapid progression of the tumor [94, 95]. Moreover, modulation of SPARC expression affects the sensitivity of colorectal tumors to radiation and chemotherapy [96, 97]. Intriguingly, a clinical study showed that the 5-year survival of patients with tumors that expressed high levels of SPARC was significantly better than that of those with tumors that did not express SPARC [95]. Based on these studies, we examined the effect of the myokine SPARC on the onset of colon tumors by using SPARC-null mice. In azoxymethane (AOM)-induced colon cancer model in mice, habitual low-intensity exercise for 6 weeks reduced the formation of ACF in the colons of wild-type mice [83]. In contrast, more ACF were found in AOM-treated SPARC-null mice than in wild-type mice, and exercise did not have an inhibitory effect. We also examined the effect of exogenous SPARC on ACF formation in the colon by injecting AOM-treated mice with recombinant SPARC. Furthermore, in a cell culture experiment, addition of recombinant SPARC to colon carcinoma cells inhibited cell proliferation in a dose-dependent manner. However, addition of conditioned medium from short interfering RNA (siRNA)-treated muscle cells to the colon carcinoma cells accelerated their proliferation. These results indicated that secreted SPARC suppresses colon tumorigenesis.

A cause of the precursor lesions of tumor formation is dysregulation of apoptosis [98]. The terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL)

assay showed that regular exercise increased the number of apoptotic colon cells in wild-type mice; however, the number did not differ between sedentary and exercised SPARC-null mice [83]. Furthermore, the levels of cleaved caspase-3 and caspase-8 were higher in wild-type mice than in SPARC-null mice, and regular exercise further increased the levels of these apoptosis markers in wild-type mice but not in SPARC-null mice. These findings indicated that SPARC mediates exercise-induced colon reduction via caspase-3- and caspase-8-dependent apoptosis. In addition, we identified an effect of exogenous SPARC on colon tumor by using colon carcinoma cells and found that apoptosis of these cells was elevated by addition of recombinant SPARC in a dose-dependent manner. This in vitro result supports the hypothesis that SPARC prevents proliferation of colon tumor cells via increased apoptosis.

Myokines might also mediate suppression of mammary cancer. Accumulated evidence in a review from WCRF/AICR has shown that a lifestyle of habitual physical activity probably reduces the risk of developing mammary cancer in both premenopausal and menopausal stages [76]. In addition, conditioned medium with serum obtained from mice immediately after exercise suppressed cell growth and enhanced caspase activity in the mammary cancer cell line MCF-7, which was reduced in the serum obtained at 2 h after exercise [99]. Thus, Hojman et al. tried to identify tumor suppressive myokines from candidates in contracting muscle from exercised mice, and identified the secreted protein oncostatin M, which is increased in both muscle tissues and serum in response to one bout of exercise. This myokine inhibits proliferation of MCF-7 cells by activating caspase, suggesting it may inhibit tumor [99].

9.5 Perspective

As discussed above, the novel concept that skeletal muscle is a secretory organ has recently been developed. Currently, over 20 proteins and peptides have been identified and one secreted protein has multiple functions (Table 9.1), for example, IL-6 induces nutrient metabolism in metabolic tissues, insulin secretion from pancreas, and anti-inflammatory activities in blood vessels. This shows that exercise exerts physiological changes and adaptations through complex mechanism. In addition, multiple studies have suggested that several other proteins secreted from muscle have not been identified. For example, a bioinformatics study showed that the secretome of human muscle cells includes over 300 proteins [100]. In addition, an in vitro study demonstrated that myocytes secrete many proteins into the medium during differentiation [101, 102]. Furthermore, transcriptome and proteome studies of human and rodent muscle tissue have demonstrated that the expression of many genes and proteins increases in response to exercise [103–106]. Except proteins and peptides, exercise also releases various metabolic factors from skeletal muscle into circulation. For example, lactate is generated from carbohydrates via glycolytic metabolism and the amount is linked to the intensity of exercise. After its release into blood, lactate is carried to other tissues and is utilized as a substrate for aerobic

Table 9.1 Major proteins and peptides secreted from skeletal muscle

Protein/peptide	Function	Target organs	References
IL-6	Glucose metabolism, lipid metabolism, insulin secretion, anti-inflammation muscle proliferation	Skeletal muscle, adipose tissue, liver, intestine, bone	[9, 11, 12, 13, 15, 16, 17, 55, 60, 67, 68]
IL-7	Muscle hypertrophy	Skeletal muscle	[54]
IL-15	Glucose metabolism, lipid metabolism, muscle hypertrophy	Skeletal muscle, adipose tissue	[20, 21, 25, 26, 27]
CXCL1	Glucose metabolism, lipid metabolism	Skeletal muscle	[28, 29]
BDNF	Glucose metabolism	Skeletal muscle	[18]
FGF-21	Glucose metabolism	Skeletal muscle, liver, adipose tissue	[19, 22, 23, 25]
Myonectin	Lipid metabolism	Adipose tissue, liver	[30]
Irisin	Lipid metabolism, osteogenesis	Adipose tissue, bone	[31, 61, 62, 63]
BAIBA	Lipid metabolism	Adipose tissue	[34]
Metrnl	Lipid metabolism	Adipose tissue	[35]
LIF	Muscle hypertrophy	Skeletal muscle	[45, 46]
IGF-1	Muscle hypertrophy, osteogenesis	Skeletal muscle, bone	[39, 40, 41, 58]
Fst/Fstl-1	Muscle hypertrophy, endothelial function	Skeletal muscle, endothelium	[50, 51, 71]
Myostatin	Muscle anti-hypertrophy	Skeletal muscle	[47, 48, 49]
Decorin	Muscle hypertrophy	Skeletal muscle	[52]
CHI3L1	Muscle proliferation	Skeletal muscle	[53]
CTGF	Osteogenesis	Bone	[64]
Oncostatin M	Anti-tumorigenesis	Breast	[99]
SPARC	Anti-tumorigenesis	Colon	[83]

IL-6 interleukin 6, *IL-7* interleukin 7, *IL-15* interleukin 15, *CXCL1* CXC motif ligand 1, *BDNF* brain-derived neurotrophic factor, *FGF-21* fibroblast growth factor 21, *BAIBA* β-aminobutyric acid, *Matrnl* meteorin-like, *LIF* leukemia inhibitor factor, *IGF-1* insulin like growth factor 1, *Fst* follistatin, *Fstl-1* follisation-like 1, *CHI3L1* Chintase-3-like protein 1, *CTGF* connective tissue growth factor, *SPARC* secreted protein acidic and rich in cysteine

metabolism or gluconeogenesis. Recently, studies into further roles of such muscle-mediated metabolites, including mitochondria biogenesis and as energy substrates in the brain [107, 108], have suggested that lactate and other substances such as amino acids and ions should be reconsidered as endocrine bioactive factors. In addition, microRNAs (miRNAs) may be secreted from muscle into the circulation and function in an endocrine manner. Some miRNAs are taken into intracellular vesicles (e.g., exosomes) and released into circulation without being degraded by RNase [109, 110]. In addition, the circulating miRNAs (c-miRNAs) can move from the circulation into other cells and regulate their function. This occurs via regulation

of gene expression at the posttranscriptional level through translational inhibition or mRNA degradation. Several miRNAs are highly enriched in skeletal muscle [111–113] and may be secreted from muscle into circulation [114]. In the future, many other muscle-secreted bioactive factors including metabolites and microRNA could be identified, which may accelerate the understanding of the effect of exercise on improvement of physical performance and prevention of diseases.

In addition, we may begin to better understand the mechanisms by which myokines are secreted from muscles into extracellular space or circulation. Some myokines are secreted with the increase of transcription. In contrast, the secretion of others is activated through post-transcription mechanisms such as activation of translation and exocytosis. However, the detailed mechanism is still unclear, despite recent functional findings. Furthermore, it is uncertain whether myokines travel in blood as free molecules or bound to certain blood proteins and the mechanism by which they affect and recognize their target cells through target receptors and are taken up into recipient cells. In the future, the dynamism of myokines between skeletal muscle, extracellular fluid, and recipient cells must be clarified.

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Chapter 10
Biological Role of TRPC1 in Myogenesis, Regeneration, and Disease

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Abstract The transient receptor potential canonical (TRPC) family consists of seven isoforms that have been proposed as molecular components essential for nonselective calcium (Ca^{2+}) entry. TRPC1 proteins are expressed in the sarcolemma of skeletal muscles, and TRPC1 is important and necessary for stretch-activated and store-operated channels for Ca^{2+} entry. Studies have established the essential role of TRPC1 in maintaining Ca^{2+} homeostasis, regulating myoblast migration and differentiation, regenerating muscle, and contributing to the pathogenesis of muscular dystrophy. This chapter summarizes the evidence for the regulation of TRPC1 to fulfill specific physiological functions in skeletal muscles.

Keywords Transient receptor potential canonical type 1 • Calcium • Mechanosensation • Myogenesis • Muscle regeneration • Duchenne muscular dystrophy

10.1 Introduction

Skeletal muscles exhibit high plasticity in response to mechanical stimuli. Calcium (Ca^{2+}) signaling plays a crucial role in translating mechanical signals into intracellular signaling cascades that control transcription and translation of phenotype-specific genes that affect muscle growth and development [1]. Of particular interest, the transient receptor potential canonical (TRPC) proteins are a family of Ca^{2+}-permeable nonselective cation channels that participate in mechanotransduction in different cell types [2]. TRPC1 is widely expressed in skeletal muscle [3], myoblasts, and myotubes [4, 5]. Even though the physiological function of TRPC1 channels is not yet fully understood, they may contribute to or form stretch-activated (mechanosensitive) channels (SACs) as well as store-operated channels (SOCs) for Ca^{2+} entry [3, 6, 7]. In addition to interacting with other members of the TRPC family to form heteromeric channels [8], TRPC1 also associates with a

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range of cytoskeletal and scaffolding proteins to form a macromolecular complex based at the costamere [4, 9]. These associations are critical for the localization, activation, and function of the channel and, subsequently, TRPC1-mediated Ca^{2+} influx. Moreover, TRPC1 regulates muscle development and regeneration [10]. In this chapter, we will review the biological functions of TRPC1 and its regulatory roles in skeletal muscle physiopathological processes.

10.2 Involvement of TRPC1 in Skeletal Myogenesis

Skeletal muscle stem cells, or satellite cells, were first identified in 1961 and are located underneath the basal lamina between the myofiber sarcolemmas [11]. Satellite cells are the predominant source of myoblasts for replenishing muscle fibers upon muscle injury and regrowth during postnatal life [12]. Satellite cells exist in heterogeneous populations that are able to differentiate and give rise to new myonuclei, as well as to undergo self-renewal to repopulate themselves. In normal, uninjured muscle, satellite cells remain in a quiescent state and are mitotically inactive, expressing the transcription factor Pax7. Upon mechanical or biochemical stimulation, a majority of the satellite cells become activated, express MyoD, and then undergo active proliferation to increase their population. After several rounds of cell division, the satellite cell progeny, which are now called myoblasts, exit from the cell cycle and undergo myogenic differentiation to become post-mitotic myonuclei. The newly differentiated myocytes then fuse with the nearby existing myofibers to form new muscle fibers [13, 14]. Unlike the majority of satellite cells (80%), which commit to myogenic differentiation, a subpopulation of satellite cells (20%) undergo asymmetric cell division, downregulate MyoD expression to replenish the satellite cell niche, and eventually become quiescent again [15, 16]. Adult postnatal myogenesis, sequentially comprising satellite cell activation, migration, and differentiation, as well as the self-renewal processes, is a tightly coordinated biological event that ensures successful skeletal muscle regeneration in response to injury. Extensive research has focused on the signaling cascades that regulate different stages of myogenesis, among which Ca^{2+} signaling has been a hotspot. This section presents an overview of the involvement of TRPC1-mediated Ca^{2+} entry in the myogenic program.

10.2.1 Activation of Satellite Cells

In intact muscle, satellite cells are mitotically inactive, expressing Pax7 but not MyoD or myogenin [17]. In the absence of muscle injury, satellite cells are tightly associated with the juxtaposed muscle fibers. In response to muscle injury,

biochemical factors released from the damaged tissue trigger the activation of satellite cells, causing the satellite cells to exit their quiescent state [18]. The satellite cells then begin to express the transcription factor MyoD, which is essential for progression from quiescence into S phase of the cell cycle, and thereby become proliferating myoblasts [19]. One soluble factor, fibroblast growth factor 2 (FGF2), is a strong activator that binds to FGF receptors on the satellite cells and triggers a downstream signaling pathway that ultimately leads to active proliferation [20]. Although the FGF-associated downstream signaling events are not fully understood, Ca^{2+} signaling mediated by TRPC channels has a role in regulating satellite cell activation. FGF2 induces an increase in $[Ca^{2+}]_i$ in satellite cells, and the influx of Ca^{2+} activates nuclear translocation of the members of the nuclear factor of activated T cell (NFAT) family, namely, NFATc2 and NFATc3. NFATc2-null mice display impaired muscle regeneration, while *NFATc3*$^{-/-}$ mice exhibit altered primary myogenesis [21, 22] resulting in a more than twofold increase in MyoD immunopositive cells and a concomitant increase in satellite cell proliferation [23]. FGF2-induced intracellular Ca^{2+} influx in satellite cells was suppressed by SKF 96365, a TRPC channel blocker. Furthermore, SKF 96365 attenuates the nuclear translocation of NFATc3 in satellite cells and the number of MyoD immunoreactive cells [23]. These data suggest that the TRPC channel plays a central role in FGF-2-mediated satellite cell activation. SKF 96365 is, however, a nonselective TRPC channel blocker; it is possible that other TRPC members are involved. We previously reported physical interaction of TRPC1-TRPC3 in C2C12 myotubes, but the absence of TRPC3 in myoblasts and in ex vivo satellite cells makes it unlikely to be directly related in satellite cell activation [23, 24]. Expression of TRPC4 or TRPC6 channel has not been demonstrated in satellite cells. TRPC1 channel is strongly expressed in satellite cells on freshly isolated adult FDB myofibers [23] raising the possibility that TRPC1 channel for responding to the effects of FGF during muscle regeneration.

In embryonic rat neural stem cells, the contribution of TRPC1 in stem cell activation has clearly been determined [25]. TRPC1 and FGF receptor 1 (FGFR-1) are co-expressed in a subpopulation of actively proliferating neuroepithelial cells in the lateral ventricle of the developing rat telencephalon, one of the known domains of neural stem cells. Co-immunoprecipitation experiments show that TRPC1 and FGFR-1 interact either directly or indirectly in a complex present in the telencephalic membrane fraction, where the active functional forms of FGF receptors are located. Basic FGF induces Ca^{2+} entry into embryonic neural stem cells and promotes stem cell proliferation. The influx of Ca^{2+} and the associated cell proliferation are antagonized by SKF 96365 and, more importantly, by antisense oligos specifically targeting TRPC1, suggesting that FGF-triggered stem cell proliferation occurs via TRPC1-mediated Ca^{2+} entry. Thus it is possible, and perhaps likely, that the activation of muscle satellite cells in response to FGF2-induced Ca^{2+} entry also occurs through TRPC1 channel.

10.2.2 Migration of Satellite Cells

During early stage of myogenesis, satellite cells activate from the quiescent state and commit to active proliferation to generate myoblasts that express MyoD. Although skeletal muscle damage usually occurs in a localized region, satellite cell activation is not restricted to only the site of muscle injury. In fact, satellite cells are capable of migrating not only from adjacent non-injured regions to damage sites on the same muscle fiber but also across the basal lamina between myofibers [26, 27]. Therefore, activated satellite cells, also termed MyoD-positive myoblasts, possess migratory features. In order to migrate, myoblasts must reorganize their contractile and cytoskeletal components to attain structural asymmetry or, in other words, polarity. Moreover, directional cell movement depends on tight spatiotemporal coordination of protrusion and retraction in different parts of the cell as well as the presence of mechanosensitive machinery, which is required to sense mechanical stimuli in the nearby environment. Cells experiencing different substrate rigidities exhibit fluctuations in $[Ca^{2+}]_i$, which is regulated by mechanosensitive channels, and Ca^{2+} gradients confer the cellular polarity necessary for the directional migration process [28, 29]. Repetitive Ca^{2+} transients, particularly mediated via mechanosensitive TRPC1 channels, are involved in myoblast migration.

TRPC1 gene silencing by gene-specific siRNA in the transformed renal epithelial cell line MDCK alters the morphology and the polarity of MDCK cells in a dose-dependent manner [30]. While mock-transfected or TRPC1-overexpressing MDCK cells exhibit clear polarity in the form of persistent directional protrusion, cells transfected with siRNAs targeting TRPC1 show a random orientation and a significant reduction in their distance displaced from original position. TRPC1-knockdown endothelial progenitor cells [31] and synovial fibroblasts from *TRPC1*$^{-/-}$ mice [32] give similar results, suggesting that TRPC1-associated Ca^{2+} transients have a regulatory role in cell migration across cell types.

TRPC1 complexed with STIM1 and Orai1 constitutes the core component of store-operated Ca^{2+} entry (SOCE), the Ca^{2+} entry mechanism that is activated by depletion of Ca^{2+} stores [33]. It has been demonstrated that SOCE-mediated Ca^{2+} signaling regulates cell polarity [28]. A study using multiple fluorescence labeling and siRNA strategy have demonstrated that TRPC1, but not Orai1, displays a high degree of co-localization with STIM1 and is responsible for regulating cell polarity. The crucial role of TRPC1 in regulating myoblast migration was revealed in a study showing that stimulation of C2C12 myoblasts with IGF-1 triggers Ca^{2+} influx, activates calpain, and accelerates migration [34]. The presence of a calpain inhibitor significantly impairs the effect of IGF-1 on myoblast migration. GsMTx-4, a specific inhibitor of SACs, completely abolishes IGF-1-induced Ca^{2+} entry. Similarly, when myoblasts are treated with TRPC1-specific siRNA and shRNA, the effect of IGF-1 on calpain activity is completely lost and the percentage of migrating cells diminishes significantly, confirming that Ca^{2+} influx through TRPC1 is responsible for calpain activity and myoblast migration [34].

In addition, calpain-dependent myristoylated alanine-rich C kinase substrate (MARCKS), an actin-binding protein expressed at the focal adhesion sites of myoblasts, is important in regulating cell adhesion and migration processes [35]. Treatment with calpain blocks MARCKS accumulation resulting in enhanced myoblast migration. Conversely, inhibition of calpain activity with siRNA leads to MARCKS accumulation, accompanied with retardation of myoblast migration [36]. TRPC1-knockdown myoblasts exhibit accumulated MARCKS expression in the cytosol, indicating that IGF-1 promotes myoblast migration by regulating calpain activity via TRPC1-mediated Ca^{2+} influx and possibly through inhibition of MARCKS accumulation [34].

10.2.3 Differentiation of Satellite Cells

TRPC1 has been implicated in satellite cell differentiation because of its upregulation during C2C12 myogenesis [24, 37]. During C2C12 differentiation, proliferative MyoD-expressing myoblasts switch on myogenin, a basic helix-loop-helix transcription factor that regulates myogenic differentiation through transactivation of muscle-specific genes such as myosin heavy chains (MHCs). Soon after expressing myogenin, the myoblasts exit from the cell cycle and commit to differentiating into myocytes. The myocytes become elongated, align with adjacent myocytes, and fuse to form multinucleated myotubes. We previously reported the dynamic expression of TRPC1 in C2C12 cells in which TRPC1 is initially expressed in all proliferating myoblasts but is soon restricted to multinucleated myotubes upon terminal differentiation and is totally absent from the undifferentiated mononucleated myocytes, suggesting its differential roles in myogenesis [24]. Indeed, a significant increase in Ca^{2+} influx is observed 24 h post-differentiation in both C2C12 and primary myoblasts [38]; however, this Ca^{2+} influx is abolished in TRPC1-knockdown C2C12 and in primary myoblasts derived from *TRPC1*$^{-/-}$ mice. Unlike *TRPC1*$^{+/+}$ myoblasts, *TRPC1*$^{-/-}$ myoblasts do not line up with each other [38]. In addition, other studies show that myocyte fusion in TRPC1-knockdown cells occurs much more slowly than in control cells [34, 37]. A possible explanation for these findings is that loss of TRPC1-mediated Ca^{2+} influx impairs the fusion process. In vitro myogenic cell fusion can be characterized as undergoing two phases: the first phase is the fusion between individual myoblasts to generate nascent myotubes, followed by the second phase, which is the incorporation of the newly differentiated myocytes into nascent myotubes [39]. In TRPC1-knockdown myoblasts, siTRPC1 treatment does not alter the fusion index but significantly reduces the number of nuclei per myotube, implying that the second fusion phase requires the regulation of TRPC1 [40]. Another possibility is a defect in cell migration or downregulation of MyoD and myogenin. The transcriptional activity of MyoD is known to be inhibited by a myogenic repressor prohibitin 2 [41]. It has been

demonstrated that phosphorylated Akt interacts specifically with prohibitin 2 and partially decreases the binding of prohibitin 2 to MyoD, thereby increasing MyoD transcriptional activity and subsequently increasing expression of myogenin and the MHC [38, 41]. In addition the phosphoinositide 3-kinase (PI3K)/Akt pathway plays a major role in the transcriptional activity of MyoD in muscle hypertrophy and regeneration. Inhibition of TRPC1 expression attenuates subsequent activation of Akt and the p85 unit of PI3K during early myogenic differentiation in myoblasts [38]. *TRPC1*$^{-/-}$ mice present delays in skeletal muscle regeneration in response to cardiotoxin-induced injury, with smaller fiber size and reduced myofibrillar protein content. Therefore, impairment of the Akt/PI3K pathway may account for the poor regenerative capacity in *TRPC1*$^{-/-}$ muscles.

TRPC1- and TRPC4-mediated SOCE are necessary for the expression of a crucial player in myogenesis, myocyte enhancer factor 2 (MEF2), in human postnatal myoblasts [40]. Overexpression of STIM1 with TRPC1 or TRPC4 increases SOCE and enhances myoblast fusion, leading to hypertrophic myotubes. Nevertheless, in cells that are deficient in TRPC1 or TRPC4 expression, overexpression of STIM1 or Orai1 to normalize Ca^{2+} concentration for SOCE is insufficient to restore the normal size of myotubes unless TRPC channels are reexpressed. This evidence further confirms the importance of TRPC1-mediated SOCE in myocyte fusion.

In yeast two-hybrid screening, TRPC1 is an interacting partner of the a-isoform of the inhibitor of the MyoD family (I-mfa) [42]. I-mfa directly represses myogenic regulator factors such as MyoD and myogenin. I-mfa binds MyoD and myogenin, allowing MyoD and myogenin to retain their subcellular localization in the cytosol, and inhibits the transactivation activity of the MyoD family, thus repressing myogenesis [43]. TRPC1 interacts with I-mfa both in vitro and in vivo [42]. Ectopic expression of I-mfa in CHO-K1 cells suppresses endogenous TRPC1-mediated SOCE. Conversely, inactivating endogenous I-mfa expression through RNAi enhances SOCE in A431 human epidermoid carcinoma cells. Although TRPC1 is present in A431 cells and the protein level appears unaltered after I-mfa knockdown, thapsigargin (TG)-induced SOCE is dramatically enhanced in I-mfa-knockdown cells, and such enhancement can be reduced substantially in the presence of TRPC1-neutralizing antibody. This suggests that I-mfa inhibits TRPC1-mediated SOCE not by modulating the transcription or translation of TRPC1 but instead most likely through physical interaction. Importantly, myogenin transfection reduces the association between TRPC1 and I-mfa in a concentration-dependent manner, suggesting that I-mfa may not bind to myogenin and TRPC1 simultaneously. Instead, myogenin appears to compete with TRPC1 for I-mfa. As such, we believe that during early differentiation, TRPC1 may physically interact with I-mfa, compete with myogenin for binding to I-mfa, and thus release myogenin for the subsequent myogenic differentiation program. We share this view with other investigators [38].

10.2.4 Regulation of Quiescent Satellite Cells

In an in vitro mass myoblast culture, whether it is a primary myoblast culture or a well-established myogenic cell line (e.g., C2C12), myoblasts are highly proliferative upon serum stimulation and express MyoD just like activated satellite cells in vivo [44, 45]. Nevertheless, the generation of differentiation-resistant, mononucleated cells that may be analogous to quiescent satellite cells in vivo always accompanies the formation of multinucleated myotubes [44]. This undifferentiated cell population, the reserve cells, is characterized by the absence of MyoD expression. When isolated, reserve cells retain the capacity to reexpress MyoD and reinitiate the myogenic program [44]. Therefore, generation of reserve cells is considered essential to maintaining a consistent, self-sustained population in the face of repeated injury [46].

We have previously revealed that, although TRPC1 expression in C2C12 increases upon differentiation, the channel is present only in multinucleated myotubes. The mononucleated cells that represent the majority (if not all) of the reserve cells in a differentiated C2C12 culture do not express the TRPC1 channel [24], raising the possibility that downregulation of TRPC1 may play a role in maintaining the reserve-cell population. In fact, as discussed previously, persistent expression of MyoD requires TRPC1 expression. Downregulation of TRPC1 may be required to suppress expression of MyoD in the reserve-cell population and prevent the cells from progressing into S phase, instead arresting in G0 phase. A cyclin-dependent kinase inhibitor, p27, is expressed at a high level in the quiescent reserve cells, whereas its expression decreases following mitogen stimulation [47, 48]. In a thyroid cancer cell model, p27 expression is significantly elevated in TRPC1-knockdown cells [49], raising the question of whether TRPC1 downregulation in reserve cells would also enhance p27 and downregulate MyoD. The underlying mechanism that governs the selection of either maintenance or downregulation of TRPC1 expression is entirely unknown, but asymmetric cell division that regulates differential cell fates in satellite cells may be involved [50]. Since TRPC1 channel activity and expression is responsive to growth factors such as IGF-1 and FGF2, the presence or absence of receptors for growth factors or even of the TRPC1 channel itself, as a result of asymmetric cell division, may alter the ultimate daughter cell fates, determining whether a cell fuses to myotubes or remains an undifferentiated reserve cell. Another possibility is that the absence of TRPC1 channels releases l-mfa that may then bind to MyoD and prevent transcriptional activity. The role of TRPC1-mediated Ca^{2+} signaling in potentially regulating cell fate, and its possible involvement in asymmetric cell division, remained to be explored.

10.3 Involvement of TRPC1 in Muscle Regeneration

Skeletal muscle demonstrates high adaptability to altered mechanical loading and also possesses a remarkable capacity for regeneration [51, 52]. Muscle disuse (or unloading) leads to muscle atrophy, fiber-type transition, and compromised contractile function, as seen in astronauts and patients on bedrest. Subjecting disused muscles to mechanical reloading can restore muscle mass, cross-sectional area, fiber-type transition, and contractile function. However, disused muscles are also prone to reloading-induced muscle injury, and improper rehabilitation may lead to further functional deterioration and impaired muscle regrowth [53].

Skeletal muscle regeneration is a coordinated process that involves an early phase of tissue inflammation to scavenge necrotic cell debris and establish a favorable environment for myogenic cell activities and a late phase of myofiber regeneration. Infiltrating inflammatory cells triggers the recruitment and activation of myogenic cells [54]. Activated myogenic cells proliferate and migrate to the injured site in order to repair injured myofibers. During the late stage, anti-inflammatory responses resolve inflammation and confer a favorable microenvironment for myoblasts to commit to myogenic differentiation and subsequent fusion [54, 55]. Regeneration is considered completed upon resolution of inflammation, absence of central nucleated myofibers at the injured area, and recovery of contractile functions. This section focuses on the role of TRPC1 in muscle regeneration following disuse and injury.

10.3.1 Muscle Regeneration Following Reduced Mechanical Load

Skeletal muscle is well known for its mechanosensitive responsiveness; sensors recognize mechanical stimuli and translate the inputs into biochemical signals that undergo complicated molecular cascades and ultimately elicit an appropriate cellular response. The mechanosensitive nature of skeletal muscle is best demonstrated by the response during gravitational unloading and reloading. In response to gravitational unloading, postural muscles, such as the soleus that is composed predominantly of slow-twitch fibers, display the most prominent phenotypic changes, including an increase in fast MHC fibers due to slow-to-fast fiber-type shifting, accompanied by a reduction in muscle mass and fiber cross-sectional area due to atrophy [56]. Such remodeling occurs only very mildly in fast-twitch muscles [57].

TRPC1 channel-mediated Ca^{2+} entry is mechanosensitive [28, 58–60]. Involvement of TRPC1 signaling in unloading-induced atrophy is evident in a C2C12 model exposed to simulated microgravity [59]. This condition retards myoblast proliferation and inhibits terminal differentiation concomitant with repressed TRPC1 expression. The TRPC blocker SKF 96365 retards proliferation and retains myoblast at the G2/M phase of the cell cycle in a dose-dependent manner, similar to microgravity conditions. Yet the role of TRPC1 in mechanical unloading was

unclear until our group reported dynamic TRPC1 expression in the soleus muscle when mice were subjected to hind limb unloading [61]. Despite a rather stable expression of TRPC1 mRNA transcript, the TRPC1 protein level significantly decreases after 14 days of hind limb unloading; we confirmed the reduction by immunohistochemistry, in which TRPC1 immunoreactivity was significantly diminished in the sarcolemma of the soleus muscle. These in vivo data echo the in vitro observation [59] that C2C12 cells under microgravity downregulate TRPC1, indicating that TRPC1 expression in skeletal muscle is mechanosensitive. This notion is further supported by the findings that TRPC1 transcripts in the soleus muscle were seven times higher than those in fast-twitch EDL muscle (unpublished data), corroborated by immunohistochemical staining. In contrast, the TRPC3 channel, which is the predominant TRPC isoform in EDL muscle [62], displays rather stable expression during hind limb unloading [61]. The preferential expression of TRPC1 in slow muscles rather than in fast muscles fits well with the response of the channel expression upon mechanical unloading.

Given the evidence that TRPC1 is mechanosensitive, we initially expected that when the unloaded mouse was allowed to resume normal weight bearing, TRPC1 expression would immediately revert. Surprisingly, TRPC1 expression decreases further in the first few days of reloading [61]. Restoration of TRPC1 expression commences at around 7 days of reloading; however, we are unsure by which day the expression of TRPC1 reaches its lowest point, because we have not examined the time points between day 3 and day 7 of reloading. The expression does not fully return to control level until day 28 of reloading. The time window of TRPC1 protein restoration correlates strongly with the recovery of muscle mass and fiber cross-sectional area and the fiber-type shifting that unloading alters [63]. This correlation prompted us to hypothesize that TRPC1 expression is related to reloading-induced muscle regrowth. The hypothesis led us to further experiments on in vivo TRPC1 silencing in soleus muscles immediately following reloading [63]. By employing focal electroporation of siRNA, we achieved specific downregulation of TRPC1 only within the target muscle, while the TRPC1 channel expression in other regions and even in adjacent muscles remained unaltered. Inhibition of TRPC1 expression lasted for at least 7 days post-electroporation, which allowed sufficient time to observe the effects of TRPC1 suppression in reloaded muscle regrowth. Our hypothesis was confirmed by the observation that silencing of TRPC1 in soleus muscle impairs the overall muscle regrowth process. We demonstrate significant reductions in muscle size, mass, and fiber cross-sectional area in TRPC1-knockdown soleus muscles, compared to control oligo-electroporated reloaded soleus muscles [63]. Moreover, TRPC1 silencing blocks the typical fast-to-slow fiber remodeling in the reloaded soleus, thus establishing the physiological relevance of TRPC1 in reloading-induced muscle regrowth. We subsequently correlated the expression of TRPC1 during the unloading-reloading process with expression of calcineurin (CaN) and NFATc1, which are well known for their roles in fiber-type conversion.

A temporal increase in CaN expression and NFAT nuclear translocation precedes the onset of TRPC1 upregulation during the reloading process [63]. CaN inhibitors (FK506 and cyclosporine A) suppress CaN activity and block the recovery of

the percentage of MHC I fiber and fiber cross-sectional area in reloaded mice, which is consistent with results from previous reports [64–66]. We considered the possibility that CaN/NFAT activity modulates TRPC1 expression in reloading muscle. Inhibition of CaN activity results in repression of TRPC1 compared with the vehicle control. This novel finding helps establish the possible role of CaN/NFAT in regulating TRPC1 expression in reloaded muscle, suggesting that the TRPC1 channel participates in CaN/NFAT-mediated muscle regrowth. SOCE regulates gene expression via TRPC1 when Ca^{2+} entry triggers the activation of CaN followed by dephosphorylation of NFAT, NFAT nuclear translocation, and transactivation of gene expression [67–70]. The possibility that TRPC1 expression being regulated by CaN/NFAT may represent a novel pathway deserves further investigation. The detailed pathway of CaN/NFAT leading to TRPC1-mediated SOCE and the involvement of SOCE in fiber-type conversion have yet to be explored, though it is possible that the PI3K/Akt pathway could be involved in the muscle mass and fiber cross-sectional area recovery mediated by TRPC1. Nevertheless, the research thus far strongly indicates that TRPC1 is a mechanosensitive channel and that Ca^{2+} entry via TRPC1 plays an important role in muscle remodeling during mechanical reloading [38].

10.3.2 Muscle Regeneration Following Injury

Under normal circumstances, muscle repair after injury follows a finely coordinated process that involves inflammation, removal of necrotic cellular debris, and regeneration. Intracellular signaling cascades that control transcription and protein translation of phenotype-specific genes regulate this process. Reloading from disuse induces muscle damage similar to that as in stretch-induced or eccentric muscle contractions [71]. Eccentric contraction-induced muscle damage is characterized by immediate muscle weakness with prolonged stiffness. Eccentric contractions cause leakage of soluble cytosolic proteins from the muscle into the plasma due to increased membrane permeability [72]. They also trigger a rise in resting $[Ca^{2+}]_i$, possibly caused by SACs [73, 74]. We previously demonstrated that the eccentric contraction-induced proteolysis of cytoskeletal proteins coincides with a reduction in muscle force, while the absence of extracellular Ca^{2+} or treatment with a calpain inhibitor attenuates the damage of cytoskeletal proteins and improves muscle tetanic force [75]. These data indicate that muscle cytoskeletal damage following eccentric contraction is due to an increase in Ca^{2+} influx that activates calpain and its associated proteolytic activity. In addition, the loss of immunogenicity of cytoskeletal proteins was reduced in muscles treated with SAC blocker streptomycin as well as in TRPC1 knockout muscles [7], suggesting that the channel responsible for Ca^{2+} entry after eccentric contraction is possibly the SAC-TRPC1 channel. All these evidence support the hypothesis that TRPC1 functions as SAC to increase Ca^{2+} influx and activate calpain-mediated proteolysis of cytoskeletal proteins.

Apart from the loss of cytoskeletal proteins by eccentric contractions, inflammation, such as infiltration of neutrophils into injured fibers, contributes a significant proportion of muscle damage during eccentric contractions or reloading following disuse atrophy [76]. Inflammation is a critical biological event and a prerequisite to muscle regeneration; blocking the inflammatory response results in poor or suboptimal muscle regeneration [77, 78]. Proinflammatory cytokines provide an important stimulus in the early phase of reloading that modulates satellite cell activation and the eventual recovery of muscle mass. There are two classes of macrophages that are implicated in muscle regeneration, M1 and M2 macrophages. The invasion of phagocytic M1 macrophages (in addition to neutrophils) regulates satellite cell activation, proliferation, and migration, while non-phagocytic M2 macrophages in the second phase of infiltration may modulate muscle regrowth during regeneration [79]. Tumor necrosis factor-α (TNF-α), which is primarily synthesized by macrophages, has been described as a "master regulator" of inflammatory cytokine production. *TNF-α receptor*$^{-/-}$ mice exhibit a reduction in MyoD expression and a slower recovery of muscle force [80], as well as suppression of transactivation of early myogenic markers and a consequent decrease in expression of myosin heavy chain [81]. TNF-α is important in promoting satellite cell proliferation and avoiding premature myogenic differentiation following injury [82]. In spite of the critical role of inflammation in muscle regeneration, prolonged inflammatory responses (e.g., responses to chronic injury) impair muscle regeneration by delaying myogenesis [54], suggesting that the cross talk between inflammatory cells and skeletal muscle helps tightly regulate the timing of muscle regeneration.

Despite the established involvement of TRPC1 in muscle regeneration upon injury, there is a lack of data supporting the participation of TRPC1-induced Ca^{2+} entry during the initial inflammatory response following skeletal muscle injury. Indirect evidence, however, can shed light on the potential interactions between TRPC1 and inflammatory responses during the regenerative process. For example, treatment of cultured rat hippocampal astrocytes with proinflammatory cytokines such as TNF-α, IL-1β, and LPS significantly suppresses endogenous TRPC1 expression [83]. Similarly, in gene manipulation studies, there is a negative association between TRPC1 and TNF-α, where inhibition of TRPC1 expression using siRNA impairs TRPC1-associated Ca^{2+} influx. This results in the enhancement of TNF-α-induced COX2 and prostaglandin E2 production, whereas overexpression of TRPC1 suppressed PGE2 release and inhibits COX-2 expression [84]. Blocking TRPC1-associated SOCE produces identical results [84]. Interestingly, TNF-α treatment drives TRPC1-mediated SOCE, suggesting that either the two processes are not entirely mutually exclusive or there might be a negative feedback mechanism in the immune response. In an induced inflammatory setting, antigen-induced elevation of systemic TNF-α levels is more profound in *TRPC1*$^{-/-}$-knockdown mice than in wild-type mice [85]. During the initial stage of muscle regeneration, the inflammatory response is mediated by proinflammatory cytokines such as TNF-α and IL-1β. Considering that TNF-α and IL-1β promote satellite cell activation but inhibit myogenic differentiation [55, 86, 87] and are dominant during the initial phase of muscle injury, TNF-α may possibly act on TRPC1-associated satellite cell activation

and proliferation. Following inflammation, TNF-α may trigger the activation of TRPC1 expression and its associated SOCE, and TRPC1-mediated SOCE, in turn, may suppress the activity of TNF-α and take charge of the subsequent regenerative muscle regrowth program. Another possibility is that activation of TRPC1 leads to inhibition of the TNF-α-mediated inflammatory response and thus initiates the onset of the regenerative process.

Though cardiotoxin-injured *TRPC1*$^{-/-}$ mice show deficient muscle regeneration, it is also essential to investigate whether they exhibit prolonged inflammatory responses that delay muscle regeneration. We believe that investigating the interaction between proinflammatory cytokines and TRPC1 during skeletal muscle injury will enrich our understanding of muscle plasticity and the role of TRPC1 in muscle regeneration. Nevertheless, this proposed hypothesis may be an oversimplification of the interaction between TRPC1-mediated SOCE and inflammatory responses, considering that the inflammation-muscle regeneration process is a very complicated biological concert involving multiple components (e.g., TRPC1 for migration and chemotaxis of neutrophils [88], macrophage phagocytosis [89], and caspase-dependent cytokine secretion [90]), all of which are important in the regulation of the muscle injury and regeneration process. Furthermore, the unidentified relationships between TRPC1 and other important inflammatory signals such as interferon-gamma (IFNγ), IL-6, and other immune chemokines limit our understanding and await further investigation.

10.4 Involvement of TRPC1 in Muscular Dystrophy

Duchenne muscular dystrophy (DMD), a devastating X-linked recessive disease, is a rapidly progressing form of muscular dystrophy. The pathogenic mechanisms are complex and there is currently no cure. This disease is caused by the lack of the cytoskeletal protein dystrophin [91] and is characterized by unremitting degeneration-regeneration cycles with chronic inflammation, progressive degeneration, and fibrosis. It has long been suggested that excessive intracellular Ca^{2+} directly initiates a cascade of pathological events causing activation of proteases and muscle degeneration in DMD [92].

The disruption of dystrophin alters the protein and activity levels of TRPC1, causing excessive Ca^{2+} influx into the dystrophic muscles. Dystrophin and its associated glycoprotein complex (DAGC) link the actin cytoskeleton to laminin in the extracellular matrix [93]. Apart from providing structural stability during muscle contraction, there is clear evidence that dystrophin and the DAGC anchor signaling molecules for various signaling pathways [94]. TRPC1 channels are likely part of the costameric macromolecular complex linked to cytoskeletal dystrophin-DAGC, and they interact with many scaffolding proteins such as Homer 1 and caveolin-3, among others [4]. The channels are regulated by the presence of this complex, and therefore the absence of dystrophin leads to channel dysregulation and increased Ca^{2+} influx, as observed in *mdx* (a mouse model of DMD) muscles.

TRPC1 is localized on the plasma membrane. TRPC1/TRPC4 channels interact with the α1-syntrophin-dystrophin complex and regulate the activity of SACs and SOCs [95]. α1-Syntrophin-deficient myotubes display abnormal Ca^{2+} influx dependent on TRPC1 [4]. The scaffolding protein Homer 1 is linked to and stabilizes TRPC1, and *Homer 1*$^{-/-}$ mice exhibit myopathy associated with aberrant Ca^{2+} entry due to TRPC1 overactivity [96]. The scaffolding protein caveolin-3 also regulates TRPC1 expression. Caveolin-3 expression assists in localizing TRPC1 to the plasma membrane and mediates SOCE [97]. Together, these results provide evidence that intact dystrophin-macromolecular complex is required for the expression, localization, and activation of TRPC1 channels to regulate Ca^{2+} influx.

Increased expression and function of TRPC1 in *mdx* muscles [97, 98] may contribute to disease onset and progression. TRPC1-mediated Ca^{2+} entry induces muscle damage in *mdx* mice. It appears that the channel can be directly or indirectly activated from membrane stretch or Ca^{2+} store depletion. Even though there is no clear conclusion about whether TRPC1 contributes to SACs in skeletal muscle [99, 100], the activation of SACs following stretch-induced muscle contractions provides evidence that the influx of Ca^{2+} is likely to be caused by SACs. A substantial increase in intracellular Ca^{2+} concentration follows eccentric contractions in *mdx* muscle fibers; more importantly, SAC blockers (Gd^{3+}, streptomycin, and GsMTx-4) prevent muscle damage and the early rise in Ca^{2+} concentration [6]. In vivo, streptomycin-treated *mdx* mice show less muscle damage and membrane permeability when subjected to downhill treadmill running [101]. Higher levels of TRPC1 protein in the *mdx* diaphragm muscles correlate with a more severe dystrophic phenotype, while daily injections of streptomycin significantly reduce Evans Blue uptake and blood CK levels [98]. These studies all support the view that TRPC1 contributes to SAC-Ca^{2+} entry and is part of the mechanism underlying the muscle damage process in dystrophic muscles.

The association of Orai1 and TRPC1 to form distinct STIM1-gated channels that are activated following store depletion may be a source of Ca^{2+} overload in *mdx* muscle. The contribution of TRPC1 channels to SOCE in DMD is supported by studies showing that SOCE is more active in *mdx* muscles and contributes to elevated intracellular Ca^{2+} [102, 103]. STIM1 and Orai1 are also upregulated in *mdx* muscles [103]. Furthermore, RNA silencing of either TRPC1 [3] or Orai1 [104] restores aberrant SOCE in *mdx* muscles. Other families of TRP channels, such as TRPC3, TRPV2, and TRPV4, are also sources of Ca^{2+} entry and stretch-induced muscle damage in *mdx* muscles [62, 105, 106]. An increase in reactive oxygen species (ROS) production could provide another pathway for SOCE activation, and details of the regulatory signaling pathways involved have been reviewed recently [107].

The inflammatory response to muscle injury is of considerable importance in physiological and pathological contexts. Inflammation is a major pathogenic feature that significantly contributes to disease progression in DMD [108–110]. Emerging evidence indicates that the complex roles myeloid cells play can influence DMD pathology. The temporal and spatial patterns of macrophage distribution are disturbed in dystrophic muscle; for instance, M1 macrophages persist in *mdx*

muscles and cause further muscle damage due to the continuous inflammatory response [111]. This disrupts the timing and balance of the M1-M2 transition and leads to aberrant regeneration [112]. Given the possibility that TRPC1 has roles in inflammation and the muscle regenerative process (as described earlier), further studies are needed to elucidate its regulation in DMD in order to attenuate the pathogenic process without affecting muscle regeneration.

10.5 Concluding Remarks

TRPC1 channel is reported to contribute to both store-operated and stretch-activated Ca^{2+} entry pathways. The channel functions on its own or may interact with other TRP channels and scaffolding proteins for numerous physiological functions in skeletal muscles. In vivo and in vitro studies demonstrate that TRPC1 contributes to muscle cell proliferation, differentiation, and regeneration. TRPC1 has also been implicated in muscle regeneration following disuse atrophy. Moreover, dysregulation of TRPC1 channel causes disturbances in Ca^{2+} homeostasis and is involved in the pathogenesis and progression of Duchenne muscular dystrophy. There are numerous studies that highlight the interactions between TRPC1 and inflammatory responses in other cell types during the regenerative process. It remains to be elucidated to explore the roles of inflammatory cells in modulating TRPC1 in muscle diseases. Further understanding of the mechanism in regulating the channel may provide therapeutic implications targeting TRPC1 or its upstream or downstream pathways to promote muscle regeneration.

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Chapter 11
ROS and nNOS in the Regulation of Disuse-Induced Skeletal Muscle Atrophy

Jeffrey M. Hord and John M. Lawler

Abstract Skeletal muscles are our biomechanical engine, generating force and power for movement. Therefore, skeletal muscles are also a primary center of metabolic activity, serving as a sink for glucose and substrate storage of amino acids. In order to respond to changing mechanical demands, skeletal muscles adapt their mass to overloading and unloading by altering the balance between protein synthesis and protein degradation. Mechanical unloading, or disuse, elicits rapid skeletal muscle fiber atrophy, where the underlying mechanisms regulation of protein synthesis and degradation appear to center around an Akt-FoxO3a axis, NF-kappaB, and proteolytic pathways including calpains and the ubiquitin- proteasome system. Recent research has focused on the process of mechanotransduction, the ability to sense and as contributory to unloading-induced muscle atrophy, as a trigger of muscle remodeling. A recently discovered mechanotransductive phenomenon is the translocation of the mu-splice variant of neuronal nitric oxide synthase (nNOSμ) from the cell membrane to the cytosol. Recently, our laboratory causally linked translocation of nNOSμ in unloaded skeletal muscle with elevated oxidative stress. Sources of reactive oxygen species (ROS) during unloading may include mitochondria, xanthine oxidase, and NADPH oxidase-2 (Nox2). The combination of increase oxidative stress and reduced stress response proteins (e.g., heat shock proteins) permits the rapid degradation of contractile proteins and removal of partially oxidized proteins. Detailed discussion of the pathways involved are discussed within our review.

Keywords Oxidative stress • Nitric oxide • Mechanotransducrion • Atrophy • Skeletal muscle

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11.1 Introduction

Skeletal muscle plays a significant role in our overall health, survival, and our quality of life. It comprises about 40% of the total body mass and is a vital component to basic biological functions such as metabolism, respiration, and locomotion. Characterized by its highly dynamic and adaptable nature, skeletal muscle contains the ability to respond to a variety of stressors to alter its size and function. For example, increasing the workload and mechanical loading causes skeletal muscle hypertrophy, whereas lessening or removing the load will result in muscle atrophy [21]. Skeletal muscle atrophy is also a biological response to chronic disease (i.e., cachexia, congestive heart failure, aging). The physiological impact of muscle atrophy is characterized by muscle weakness, fatigability, and increased mortality. Therefore, uncovering the critical molecules, proteins, and signaling cascades that regulate skeletal muscle atrophy is an important area of biological and physiological research. While the cell signaling underlying skeletal muscle atrophy across wasting phenomena remained mysterious, there has been encouraging progress in recent years.

The reduction or elimination of mechanical loading skeletal muscle is often synonymous with "disuse." Disuse-induced skeletal muscle atrophy results in physiological changes such as a reduction in muscle mass and strength. The extent of disuse-induced skeletal muscle atrophy depends on several factors such as age, degree of mechanical unloading, and the function and fiber type composition of the affected muscle. As previously mentioned, skeletal muscle wasting also occurs as a comorbidity to chronic disease and has been observed in pathologies such as cancer, congestive heart failure, diabetes, chronic obstructive pulmonary disease (COPD), severe burn injury, inflammatory bowel disease, etc. Muscle wasting in these pathologies is partly due to muscle disuse, but overriding effectors include oxidative stress, inflammatory signaling, and cytokines. Whether atrophy is primarily due to disuse or as a comorbidity with disease, there are common atrophic signaling events that stimulate reduction in muscle fiber cross-sectional area (CSA), decreased muscle protein content, and decreased mass. In addition, atrophy promotes a reduction in capillary density, architectural disruptions of the myofiber, phenotypic alterations in fiber type, reduction in force production, and eventual weakness that corresponds with easily fatigable muscles. Indeed, skeletal muscle weakness and fatigability are major factors leading to impairment in the quality of life of affected individuals.

As mentioned above, prolonged periods of disuse due to bed rest, limb immobilization, denervation, or hindlimb unloading result in skeletal muscle atrophy. Concomitant with reduction in muscle mass, disuse-induced atrophy also results in a shift in fiber type from the slower oxidative phenotype to the faster glycolytic phenotype. Skeletal muscle atrophy is due to an imbalance between synthesis and breakdown of muscle protein. While the majority of the published studies have focused on protein degradation, the impact of decreased protein synthesis is critical to the wasting process. Recent papers have emphasized cross talk between protein degradation and protein synthesis, centered around an Akt–FoxO axis.

The impact of disuse-induced protein degradation is a complex process, with new players and pathways having been identified in recent years. Major roles in the degradation process are taken on by the ubiquitin proteasome pathway (UPP), caspase-3/calpains, and the autophagic–lysosomal pathway.

There is direct evidence that elevation of oxidative stress occurs during disuse and may serve as a trigger to atrophic signaling by promoting muscle protein degradation and reducing muscle protein synthesis. However, the exact sequence of events from the onset of disuse to the increased production of reactive oxygen species (ROS) and subsequent atrophic signaling has not yet been established. In this review, we will provide (1) an overview of the various experimental models of disuse-induced atrophy, followed by (2) an overview of synthesis and degradation mechanisms and pathways involved in disuse-induced atrophy. Then, we will introduce the (3) evidence that ROS production plays a critical role during disuse-induced atrophy, followed by (4) the regulation of neuronal nitric oxide synthase (nNOS) localization and activity in response to disuse.

11.2 Models of Disuse

Disuse-induced atrophy has been investigated in several different models in both humans and rodents. Due to the invasiveness of procuring muscle samples from subjects, rodent models of disuse atrophy are often used to study the tissue adaptations, cellular mechanisms, and signaling cascades involved in disuse atrophy. A variety of models of disuse atrophy have been developed to mimic the various clinical atrophy-inducing events. For instance, the rodent model of limb immobilization is limb casting. Another common model of disuse is hindlimb unloading by way of rodent tail suspension, a common experimental model used to mimic the physiological response to prolonged periods of bed rest or spaceflight. In addition, animal models have been used to investigate the impact on skeletal muscle due to spinal cord injuries or denervation. A common model used to examine disuse of the respiratory muscles (e.g., diaphragm) is the mechanical ventilation rodent model.

While there are a number of animal models of disuse-induced skeletal muscle atrophy, human paradigms of disuse are often used for research purposes as well, despite the invasiveness. For example, human models of limb immobilization (i.e., casting or leg brace), prolonged bed rest, and unilateral lower limb suspension (ULLS; e.g., immobilizing one leg and walking with crutches or knee walker) are used to examine inactivity-induced atrophy in patients.

11.3 Mechanisms of Disuse-Induced Skeletal Muscle Atrophy

11.3.1 Decreased Protein Synthesis and Elevated Protein Degradation

To maintain a basal level of skeletal muscle mass, a balance between protein synthesis and protein degradation is necessary. In recent years, there has been a

significant ongoing debate regarding the roles of protein synthesis and degradation during disuse atrophy. This is partly due to the different models (humans vs. rodents) that have been used. In general, the major difference between human and rodent disuse models concerns the relative rate of atrophy, with atrophy occurring at a much faster rate in rats than in humans [57], although muscle fiber type plays a significant role. While most of the available data indicates that there is an initial drop in protein synthesis rates along with an increase in rates of degradation, there is a contrasting view that in humans, disuse atrophy occurs primarily in response to reduced synthesis rates [57]. This is derived from human studies that have found a reduction in synthesis, while data regarding breakdown is inconsistent [11, 13, 17, 20, 91] and, in some instances, the methodology has been deemed questionable or unreliable [88]. Data inconsistencies are due in part to the difficulties associated with measuring rates of protein degradation in humans, with most of the data collected relying on the expression of genes associated with specific protein degradation pathways. Obviously, additional studies are warranted to determine if reductions in protein synthesis are the driving force of atrophy or if there is simply a lack of data from human disuse studies. For a glimpse into this ongoing debate of decreased synthesis vs. increased degradation, refer to a recent *Journal of Physiology Crosstalk* series [58, 62].

Accumulating evidence indicates that disuse-induced skeletal muscle wasting is due to a combination of the reduction in the rate of protein synthesis and an elevation in protein degradation. For instance, in the hindlimb unloading model skeletal muscle, protein synthesis rates were reduced within the first 5 h of disuse [89], and within 48 h the decreased synthesis rate levels off at a steady state [89]. Prolonged periods of disuse are known to lead to an increase in protein degradation [9]. However, rapid increases in proteolysis have been reported during mechanical unloading as well [4, 69]. Together, these data indicate that the loss of skeletal muscle protein during disuse is most likely the outcome of declining synthesis rates and elevated degradation rates, with the latter playing the dominant role [16, 69].

The key degradation components that promote skeletal muscle wasting include ubiquitin proteasome pathway (UPP), caspase-3/calpains, serine protease, and autophagic–lysosomal pathways [60, 69]. Primary targets of degradation during disuse atrophy include myofibrillar proteins, although sarcoplasmic proteins are also degraded [51].

11.3.2 Disuse Atrophy Signaling Cascades

A variety of physiological stimuli trigger skeletal muscle atrophy. For example, atrophy that occurs as a comorbidity with chronic disease is considered to be caused by increased levels of pro-inflammatory cytokines, endotoxins, glucocorticosteroids, and tumor-derived factors [16, 69]. On the other hand, disuse-induced skeletal muscle atrophy occurs through a reduction in mechanical tension [29] and elevated levels of oxidative stress [60].

In part, skeletal muscle atrophy can occur as the result of blunting the anabolic insulin-like growth factor 1 (IGF-1) signaling pathway. Activation of IGF-1 requires signaling through the IGF receptor (IGFR) and the insulin receptor substrate-1 (IRS-1), which leads to phosphorylation of phosphatidylinositol-3 kinase (PI3K). Active PI3K then phosphorylates phosphatidylinositol-4,5-bisphosphate to phosphatidylinositol-3,4,5-trisphosphate in the sarcolemma, creating a binding site for Akt. Activation of Akt leads to active phosphorylation of mammalian target of rapamycin complex 1 (mTORC1). mTORC1 regulates synthesis through phosphorylation and activation of p70S6K and downstream inhibition of eukaryotic translation initiation factor 4E-binding protein 1 (4E-BP1). Data from numerous studies support the role of the IGF-1/PI3K/Akt/mTORC1 signaling cascade in the regulation of skeletal muscle hypertrophy and atrophy [16, 69]. For example, IGF-1 resistance has been reported after 14 days of hindlimb unloading in skeletal muscle of rats [3]. However, injections of growth hormone and IGF-1 during unloading have limited effect, possibly due to upregulation of IGF-1 binding proteins [5, 79]. In addition, activation of Akt/mTORC1 and the downstream effectors is repressed during hindlimb immobilization and hindlimb unloading [22, 30, 98]. A critical event in this signaling cascade is the activation of Akt, which is suggested to be a transition point between hypertrophy and atrophy [16, 69].

Degradation pathways in skeletal muscle include the ubiquitin proteasome pathway (UPP), calcium-dependent caspase-3/calpains, serine protease, and the autophagic–lysosomal pathway [60, 69]. Calpains are thought to initiate proteolysis via unzippering of the Z-disc [18, 65, 85] during periods of disuse, while the primary degradation pathway is believed to be the UPP [77, 84]. Recently, autophagic–lysosomal proteins [84, 86] have been cited as contributory to unloading-induced proteolysis.

As the major proteolytic pathway during disuse atrophy, the UPP serves to breakdown myofibrillar and sarcoplasmic proteins in muscle fibers. Distinct components of the UPP include the E1, E2, and E3 ubiquitin ligases. E1 ubiquitin ligases activate ubiquitin, the activated ubiquitin is transferred to the E2 conjugating ligase, and the E3 ubiquitin ligases regulate the transfer of the ubiquitin to the targeted protein. Two critical tissue-specific E3 ubiquitin ligases, MuRF1 and MAFbx/Atrogin 1, are upregulated in response to a variety of atrophy-inducing events. Bodine et al. [9] found that in response to various models of disuse (i.e., hindlimb unloading, limb immobilization, and denervation), transcription of MuRF1 and MAFbx/Atrogin 1 was increased [9]. Under resting conditions in skeletal muscle, these genes are expressed at relatively low levels; however, following reductions in mechanical load, expression of these genes is quickly induced. Expression of both MuRF1 and MAFbx/Atrogin 1 is rapidly elevated in numerous models of atrophy and suggested to significantly contribute to the initiation of atrophy [8].

FoxO and NFκB Signaling: Expression of both MuRF1 and MAFbx/Atrogin 1 is regulated by a variety of transcription factors, including members of the forkhead box O transcription factor family (FoxO1 and FoxO3a), nuclear factor kappa B (NFκB) transcription factors (p50 and Bcl3), and the glucocorticoid receptor (GR)

[64, 67, 94, 97]. During prolonged periods of disuse, alterations in the activity of glucocorticoids have not been shown; thus, elevated expression of MuRF1 and MAFbx/Atrogin 1 is not likely due to GR signaling [95].

However, during various atrophy-inducing states, including disuse, elevated expression of FoxO1/3a has been observed [39, 64, 72] along with FoxO1/3a transcriptional regulation of MuRF1 and MAFbx/Atrogin 1 [67, 80, 94]. Cytoplasmic dephosphorylation of FoxO proteins leads to nuclear translocation where they function as transcription factors acting to suppress growth and promote apoptosis [61]. FoxO activation can be regulated upstream by Akt, which can phosphorylate multiple sites on FoxO to prevent its nuclear translocation [10, 35, 67]. However, findings from human studies indicate there may be a dissociation between Akt signaling and synthesis/degradation, leaving the exact mechanism of FoxO1/3a regulation unclear [44].

As previously mentioned, NFκB-associated modulators (e.g., Bcl-3) and transcription factor subunit (e.g., p50) have been shown to upregulate MuRF1 and MAFbx/Atrogin 1 in response to mechanical unloading [28]. In the unloaded rat soleus, a significant increase in the nuclear concentration of NFκB/inhibitor of κB (IκB) proteins p50, c-Rel, and Bcl3 was observed [24]. However, activation of the canonical NFκB dimers p50–p65 was not found to be significantly activated [25]. Kandarian's group found that p50 and Bcl3 serve as transcription factors for MuRF1 and MAFbx/Atrogin 1 during rodent hindlimb unloading, and knockout of each transcription factor led to sparing of the muscle [24].

Recent data from the Kumar and Kandarian research groups indicates that NFκB signaling during disuse may be regulated in part by activation of the fibroblast growth factor-inducible 14 (Fn14) receptor. Fn14 transcription and protein levels are induced in skeletal muscle during immobilization, hindlimb unloading, and following denervation [50, 97]. In contrast, remobilization and resistance exercise both lead to a reduction in Fn14 transcription [50]. These data suggest a mechanosensitive role for Fn14 and its downstream NFκB signaling.

While FoxO1/3a and NFκB transcription factors have received much attention related to disuse-induced atrophy signaling, they are not the only mediators involved. Recent work by the Adams research group has unveiled two novel transcription factors upregulated with disuse: activating transcription factor 4 (ATF4) [15, 19] and the tumor suppressor p53 [19]. Ebert et al. [15] knocked out the ATF4 gene in a mouse model and observed a muscle sparing effect following 3 days of limb immobilization but not 7 days of immobilization [15]. In a follow-up study, their group reported increased expression of p53 with immobilization [19]. Muscle fiber atrophy even occurred in the absence of ATF4, while simultaneous forced expression of both factors exacerbated disuse atrophy [19]. Their data suggest that ATF4 and p53 signal through independent, yet additive, routes during skeletal muscle atrophy by stimulating p21 [15, 19]. Interestingly, neither ATF4 nor p53 act as transcription factors for MuRF1 or MAFbx/Atrogin 1 [15, 19], indicating that their transcriptional regulation of atrophy is through alternative ubiquitin ligases or other mechanisms. These novel data support the notion that attenuation of muscle atrophy is likely accomplished in several phases and that multiple transcription factors participate in the regulation of the atrophic process.

11.3.3 Novel Disuse-Induced E3 Ubiquitin Ligases

As indicated in the above paragraph, regulation of atrophy is not due to upregulation of a single transcription factor nor upregulation of a single E3 ubiquitin ligase. In recent years, independent investigators have identified novel E3 ligases that are linked regulation of skeletal muscle during catabolic states. For instance, expression of a HECT domain ubiquitin ligase referred to as Nedd4-1 was increased in skeletal muscle in response to denervation [6] and hindlimb unloading [31]. In addition, the muscle ubiquitin ligase of SCF complex in atrophy-1 (MUSA1) is another recently discovered F-box E3 ubiquitin ligase. Overexpression of MUSA1 promoted muscle loss, whereas RNA interference led to sparing of muscle mass [48, 68].

Moreover, atrophic signaling that leads to FoxO1/3a activation has been linked to increased expression of the mitochondrial E3 ligase 1 (Mul1) [41]. Mul1 participates in the promotion of mitochondrial fragmentation, depolarization, and mitophagy, all of which are associated with mitochondrial dysfunction [41].

Examination of the negative regulation of IGF-1 signaling has unveiled new E3 ubiquitin ligases: Cbl-b and mitsugumin 53 (MG53 or TRIM72). For instance, Cbl-b has been observed to negatively regulate IGF-1 signaling during prolonged periods of unloading [53]. MG53 targets the insulin receptor for degradation and subsequently ubiquitinates IRS-1 in skeletal muscle from mice with metabolic syndrome [78]. These are just a few of the E3 ligases that have been connected or possibly have connections to disuse atrophy. Advances in techniques, antibodies, genetic models, epigenetics, etc., will further our understanding of the players and cascades involved in atrophy signaling.

11.4 Reactive Oxygen Species (ROS) Production in Skeletal Muscle During Disuse-Induced Atrophy

11.4.1 Oxidative Stress and Disuse-Induced Skeletal Muscle Atrophy

It has been 25 years since the first study connecting elevated levels of oxidative stress to disuse-induced skeletal muscle atrophy [32]. Kondo et al. [32] found that immobilization-induced atrophy in rats was associated with increased oxidative stress. Furthermore, the antioxidant properties of vitamin E attenuated the degree of atrophy [32]. Since their novel findings were published, supporting data has accumulated suggesting the role/s of specific oxidant sources and antioxidants during the atrophic process. Furthermore, the manipulation of which can lead to muscle sparing effects during disuse [38, 49, 36]. Altogether, the growth and progress of this field of interest has driven the investigative efforts examining the roles of ROS, redox modifications, and associated signaling events that lead to skeletal muscle atrophy during prolonged periods of disuse.

11.4.2 ROS Production in Skeletal Muscles During Prolonged Periods of Disuse

Oxidative stress occurs when there is an imbalance between prooxidants and antioxidants, with the imbalance favoring the buildup of oxidized molecules within the cell or tissue [37]. An ever-growing body of evidence indicates that disuse leads to elevated levels of oxidized proteins and lipids within skeletal muscle. Increased cellular concentrations of oxidized proteins and lipids, which are essentially damaged, are the result of elevated activities of prooxidant sources and ROS, as well as a reduction in the antioxidant capacity of the myofiber [37]. To investigate this phenomenon, researchers have focused on the prooxidant sources within skeletal muscle fibers. Accumulating evidence suggests that skeletal muscle disuse leads to ROS production from various sources, including the mitochondria, xanthine oxidase (XO), and NADPH oxidase (Nox).

Strong evidence from mechanical ventilation and hindlimb immobilization studies indicates that a primary source of ROS during skeletal muscle disuse is the mitochondria [49, 86, 23]. For instance, mitochondrial release of hydrogen peroxide is increased more than twofold in the soleus and plantaris from rodents that endured 2 weeks of hindlimb immobilization [49]. However, it is currently unknown if both populations of skeletal muscle mitochondria, subsarcolemmal and intermyofibrillar, contribute to this elevation in ROS production.

In the series of pioneering work from Kondo et al. in the early 1990s, Kondo and colleagues suggested that XO may be the primary source of ROS in myofibers during disuse [33]. Since then, however, data inconsistencies have been reported concerning XO and disuse. Evidence from mechanical ventilation experiments indicates that XO plays a significant role in oxidative injury and contractile dysfunction during periods of disuse [96]. In agreement, XO inhibition blunted the severity of disuse-induced contractile dysfunction caused by hindlimb unloading, even though XO inhibition did not spare the soleus muscle from atrophy [46]. In contrast, XO inhibition during hindlimb unloading has been found to offer significant muscle sparing effects [14]. Altogether, the evidence implicates XO as having a regulatory role in the decline of contractile function associated with disuse, but it is still unclear whether or not XO promotes disuse atrophy.

Two Nox isoforms are expressed in skeletal muscle fibers, Nox2 [36, 71] and Nox4 [81]. The Nox2 isoform is a plasma membrane-associated, multi-subunit complex that is capable of actively producing superoxide when cytosolic subunits bind to the membrane-bound subunits. Sustained activation of Nox, in particular Nox2, has been associated with skeletal muscle atrophy in vitro and in vivo [1, 71]. Our group recently reported that 54 h of hindlimb unloading was associated with increased sarcolemmal localization of two Nox2 subunits, gp91phox and p47hox [36]. Moreover, oxidative stress in response to mechanical ventilation has been linked to contractile dysfunction and fiber atrophy, both of which were attenuated by the administration of a Nox inhibitor [47]. The limited evidence indicates that disuse-induced oxidative stress is partially the result of Nox activation. Links between Nox2 and mitochondrial ROS are currently unknown in disuse atrophy.

11.4.3 Mechanistic Links Between Oxidative Stress and Disuse-Induced Atrophy

Oxidative stress has been linked to the manipulation and slowing of the rate of muscle protein synthesis. Accumulating evidence from in vitro experiments suggests that elevated ROS concentrations interfere with the phosphorylation of Akt/mTORC1 signaling and, in effect, limit translational efficiency [56, 73, 87, 99]. As previously mentioned, activation of Akt is suggested to be a transition point between hypertrophy and atrophy. Decreased Akt phosphorylation often results in lower rates of protein synthesis and increased degradation, which lead to atrophy. Recent in vivo investigations have found that the disuse-associated increase in mitochondrial-derived oxidants led to reductions in Akt activity [86, 23]. For instance, elevated ROS in the immobilized soleus led to significant reductions in phosphorylation of Akt and mTORC1 [86]. Moreover, oxidative stress-associated inhibition of Akt, mTORC1, PRAS40, and 4E-BP1 was observed recently following mechanical ventilation [23]. These findings indicate that disuse-induced oxidative stress reduces protein synthesis by inhibiting Akt/mTORC1 signaling and limiting translation.

A growing body of evidence suggests that oxidative stress contributes to elevated rates of skeletal muscle proteolysis and atrophy through various routes. For instance, alterations in ROS production and signaling can regulate transcription factors and eventual gene expression of critical components of the UPP [86, 36]. For example, disuse-associated oxidative stress has been shown to lead to increased dephosphorylation of cytoplasmic FoxO3a, which is suggestive of elevated nuclear FoxO3a content and activity [36]. Moreover, increased ROS due to hindlimb immobilization has been shown to significantly increase expression of FoxO3a target genes: MuRF1 and MAFbx/Atrogin 1 [86]. Furthermore, disuse-induced oxidative stress promotes increased activity of the 20S proteasome [7].

Elevations in ROS production during disuse have also been implicated in regulating the expression and activity of components of the autophagic–lysosomal degradation pathway. For instance, inhibition of mitochondrial-derived ROS during disuse reportedly prevents the rise in autophagic vesicle formation and cathepsin expression [86]. Disuse-induced elevations in ROS production have also been linked to the activation of both calcium-mediated calpains and caspase-3 [49, 86]. Additionally, elevated levels of disuse-induced ROS can promote proteolysis in muscle fibers by oxidizing proteins thereby enhancing their susceptibility to proteolytic degradation [26, 76]. For example, antioxidant administration during hindlimb unloading effectively attenuates protein ubiquitination and fragmentation of myosin heavy chain proteins [26]. Altogether, these data strongly suggest a significant role for oxidative stress in the regulation of disuse-induced proteolysis.

Possible links between disuse-associated ROS and NFκB, ATF4, p53, and several of the recently discovered atrogenes leave many unanswered questions in atrophy signaling. The limited data that is available points toward the likelihood that many of

these transcription factors and atrogenes are altered in response to disuse-associated ROS. For example, antioxidant treatment during mechanical ventilation has verified oxidative stress as an upstream activator of NFκB and linked to diaphragm muscle weakness [75]. Furthermore, ATF4 expression was highly responsive to hydrogen peroxide stimulation in C2C12 myotubes [59]. In mechanically unloaded skeletal muscles, increased content of p53 has been suggested to lead to disturbances in the oxidative balance of the myofiber [74]. Elevated levels of ROS have also been shown to induce expression of NEDD4-1 in brains from patients with Alzheimer's, Parkinson's, and Huntington's diseases and spinal cords from amyotrophic lateral sclerosis (ALS) patients [34]. Additionally, recent evidence indicates that anthocyanin (delphinidin) treatment during hindlimb unloading suppressed the expression of genes associated with oxidative stress while concomitantly preventing the rise in Cbl-b expression [52]. Accumulating evidence suggests that ROS exerts a strong influence on transcriptional regulation during disuse atrophy, possibly beyond what is currently known. Refer to Fig. 11.1 for our proposed model of ROS regulation of atrophy gene transcription.

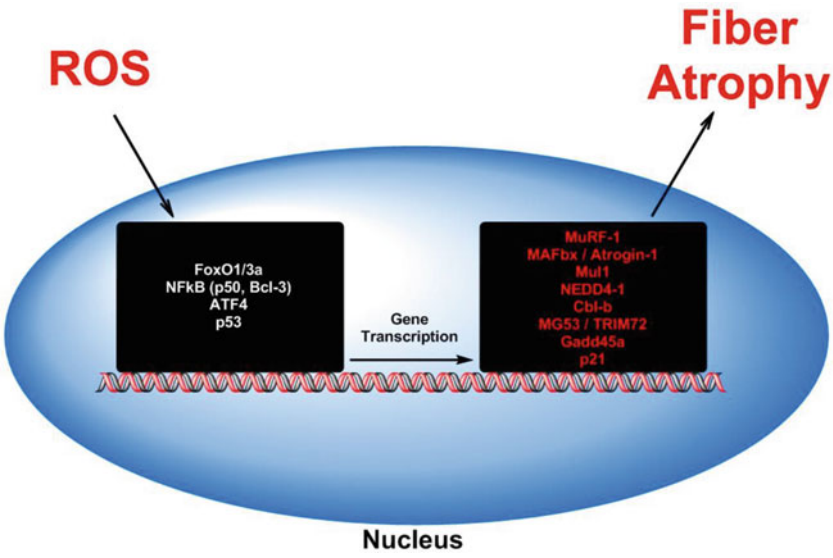

Fig. 11.1 ROS regulation of transcription factors and expression of proteins involved in promoting the reduction of fiber size due to disuse. Based on published findings, we propose a list of transcription factors along with E3 ligases and growth-arresting proteins known to directly promote atrophy or exert blunting effects on synthesis pathways, each of which is likely influenced by elevated myofiber ROS. This is a simplified figure illustrating ROS regulation of disuse fiber atrophy (refer to text for further discussion). Transcription factors listed do not necessarily correspond to the list of E3 ligases and growth-arresting factors. *Gadd45a and p21 are reportedly transcribed under the direction of ATF4 (Gadd45a & p21) and p53 (p21) [15, 19]

Altogether, a growing body of evidence suggests that oxidative stress can impede the rate of protein synthesis and accelerate protein degradation in a variety of ways, resulting in skeletal muscle atrophy. However, several gaps in knowledge are evident concerning ROS and disuse. Future studies should examine the specific mechanisms responsible for the rise in ROS production during disuse-induced skeletal muscle atrophy, as well as the notion of cross talk among oxidant sources. In the ROS-induced ROS-release model described by Andreas Daiber [12], activation of Nox and subsequent superoxide production could lead to depolarization of mitochondrial membrane ATP potassium channels. The reduction in mitochondrial membrane potential would then lead to increased mitochondrial-derived ROS, which could activate PKC. Active PKC has been shown to be capable of triggering Nox2 complex formation and activation. In this model, the initial ROS production from Nox2 signals for the augmentation and intensification of oxidative stress from the mitochondria [12]. In addition, activation of Fn14 has been shown to promote ROS production by stimulating Nox2 activity in macrophages [43]. Thus it is possible that Nox2 serves as an intermediate in an Fn14–Nox2–NFκB pathway. Clearly, there are many unanswered questions, and continuing research efforts will be required to elucidate our understanding of both the locations and regulation of ROS production during disuse-induced skeletal muscle atrophy.

11.5 Potential Role of Neuronal Nitric Oxide Synthase-mu During Disuse Atrophy

Currently, we do not have a full understanding of the complexities involved in oxidant production during disuse-induced skeletal muscle atrophy. Skeletal muscle wasting that occurs during pathological conditions, such as sepsis and chronic heart failure, has been associated with an overproduction of nitric oxide (NO) [2, 55]. However, upregulation of iNOS, rather than nNOSμ, was cited as causal in cachectic muscle atrophy.

However, during disuse-induced atrophy, it is unclear whether excess NO production, source, or localization accelerates the atrophic process. In a study by Suzuki et al. [83], 14 days of hindlimb unloading was associated with a sarcoplasmic translocation of the mu (μ) – isoform of neuronal nitric oxide synthase (nNOSμ) from the sarcolemma-localized dystrophin glycoprotein complex (DGC) – and this translocation correlated with elevated levels of sarcoplasmic NO [83]. Their group followed this experiment up with genetic and pharmacological nNOS inhibition studies that indicated translocation of nNOSμ activity during hindlimb unloading as a critical event promoting atrophy [83]. Dystrophin remained attached to the DGC, while dysferlin, a multifunctional protein involved in membrane repair, remained bound to nNOSμ. Inhibition of nNOS prevented activation of FoxO3a and ubiquitin ligases [83]. Cytosolic levels of •NO were elevated as determined via EPR (electron paramagnetic resonance). We found that nNOSμ translocation is an early event

in the mechanical unloading process, occurring within 3 days with the rat model [36]. In addition, Vitadello et al. [92] also found untethering of nNOSμ from the sarcolemma and translocation of nNOSμ with hindlimb unloading, linked to downregulation of grp94 [93]. Grp94 is an HSP90-like stress protein that chaperones protein folding [45]. Curcumin, the primary compound in the spice tumeric, with an antioxidant and anti-inflammatory properties mitigated nNOSμ translocation and atrophy [92].

In contrast, Lomonosova et al. [42] reported that L-arginine mitigated hindlimb unloading-induced reduction of nNOS, •NO, dystrophin, and HSP90 protein levels and mRNA transcripts gene expression. Heterogeneity in dystrophin sarcolemmal localization was found, suggesting disruption of the DGC. Furthermore, hindlimb unloading reduced levels of desmin [42], a Z-disc fiber protein that transmits loading to the DGC and surrounding extracellular matrix protein (e.g., laminin, collagen). Protein abundance was determined from serial frozen cross sections and thus assessed global levels, rather than site-specific changes. Efficacy of L-arginine may suggest a substrate limitation or decoupling of nNOSμ. In addition, a different spin trap was used for EPR in the Lomonosova study compared with Suzuki et al.: diethyl-dithiocarbamate (DETC) vs. *N*-methyl-D-glucamine-dithiocarbamate (MGD). Thus the global role of •NO and downstream nitrosative species remain uncertain in unloading-induced atrophy. However, we propose that (1) disruption of the DGC and membrane environment and untethering of nNOSμ from the sarcolemma, (2) subcellular location of nNOS and downstream effects on FoxO3a-ubiquitin ligase signaling, (3) substrate availability, (4) the role of other NOS isoforms, and (5) local •NO bioavailability may be more important than global •NO levels in regulating muscle fiber atrophy with mechanical unloading. However, additional research is crucial to unraveling these mysteries.

Interestingly, evidence indicates NOS and •NO are involved in the regulation of the type I MHC isoform [70, 82]. For instance, a recent report found that with 8 weeks of NOS inhibition, there was a significant decrease in the percentage of type I fibers and concomitant increase in the percentage of type IIa fibers in the soleus muscle [82]. Since disuse leads to a shift in fiber type from type I to type IIa, these data point toward the notion that a reduction in myofiber NO may be a critical event promoting the shift.

It is proposed that in healthy muscle, sarcolemma-localized nNOSμ is required for myofiber mechanotransduction. For example, muscle fiber hypertrophy with mechanical overloading was dependent upon intact sarcolemmal nNOSμ and NADPH oxidase 4 (Nox4) [27]. Genetic ablation or inhibition of nNOSμ early in the overloading process attenuated muscle hypertrophy by over 50% [27]. In support of these findings, the reduction in sarcolemma nNOSμ that occurs with unloading leaves the muscle fiber susceptible to sarcolemmal lysis by neutrophils upon reloading [54], thus slowing recovery, whereas mice expressing a muscle-specific nNOS transgene were significantly protected from sarcolemma damage and injury in response to reloading [54]. Clearly, nNOSμ plays a vital

role as a load-sensitive molecule in skeletal muscle. Translocation of nNOSμ has been reported to occur in response to various forms of disuse: hindlimb unloading [36, 83, 90], spaceflight [66], denervation [83], bed rest [63], and intensive care-associated critical illness myopathy [40]. While some have observed disuse-induced alterations in nNOSμ synthesis and/or degradation, the mislocalization of nNOSμ in postural muscles is a common event associated with atrophy. Suzuki et al. [83] observed a causal link between nNOSμ untethering and proteolytic FoxO3a activation during hindlimb unloading. Our group demonstrated that administration of a superoxide dismutase (SOD)/catalase mimetic (EUK134) reduced the extent of nNOSμ untethering from the sarcolemma and translocation to the sarcoplasm following 54 h of hindlimb unloading [36]. This effect attenuated the activation of FoxO3a, subsequent atrophy, and shift in fiber type in the soleus muscle [36]. These data suggest that disuse-induced translocation of nNOSμ is due in part to a rise in oxidative stress.

Much remains to be learned concerning the many roles of nNOSμ during mechanical unloading. For example, further examination is needed to elucidate whether translocated nNOSμ leads to elevated localized NO within the sarcoplasm of the fibers and which signaling pathways are affected. Does nitrosylation of glutathione play a role in dephosphorylation of FoxO3a? What are the mechanisms of substrate limitation? What are the mechanisms underlying redox regulation of nNOSμ during unloading? A causal link between nNOSμ translocation that is directly associated with depressed protein synthesis is also of interest. In addition, identifying the molecular mechanisms guiding nNOSμ untethering from dystrophin and alpha-syntrophin in the DGC is of significant concern. In addition, are the effects of nNOS translocation fiber type dependent? Does nNOS translocation trigger fiber-type switch from slow to fast twitch with unloading?

11.6 Conclusions

In summary, disuse-induced skeletal muscle atrophy is due to elevation of ROS in the myofiber environment. Oxidative stress is implicated in the regulation of blunted rates of muscle protein synthesis and increased proteolysis (summarized in Fig. 11.2). Various myofiber oxidant sources participate in the production of ROS and are potentially involved in a ROS-induced ROS-release feedback mechanism. In addition, future studies are needed to determine the specific upstream signaling events that trigger the disuse-induced ROS production from each oxidant source. While the data remains unclear concerning NO production during disuse, there is a better understanding of the link between atrophying muscle and nNOSμ translocation. However, future studies are needed to elucidate the connections between oxidative stress, nNOSμ translocation, and the affected atrophic signaling cascades.

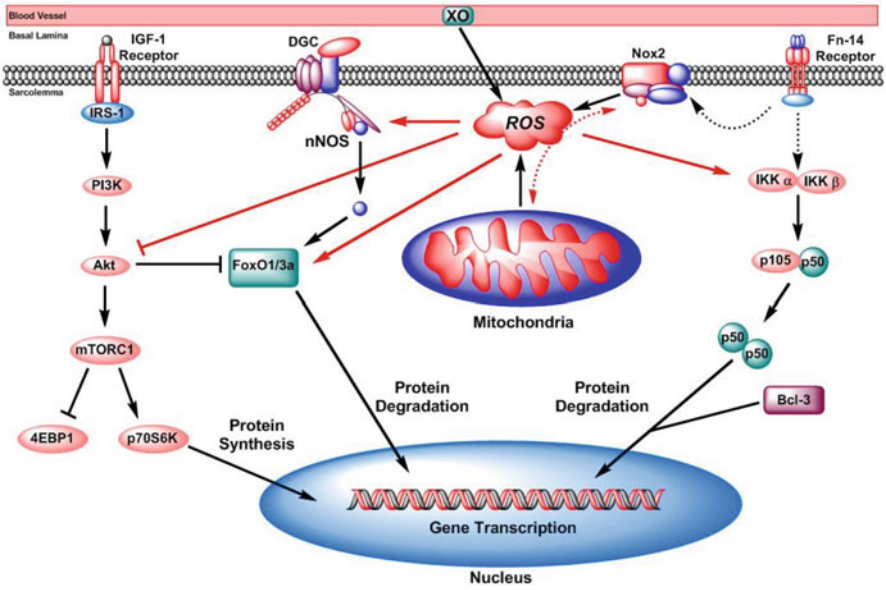

Fig. 11.2 Critical signaling pathways controlling muscle fiber size during disuse-induced atrophy and the regulatory schemes exerted by ROS. The combination of reactive oxygen species (ROS) derived from mitochondria, NADPH oxidase isoform 2 (Nox2), and xanthine oxidase (XO) results in an increased ROS concentration in myofibers during periods of disuse. Elevated ROS can blunt Akt-mTORC1 protein synthesis signaling, induce dislocation of nNOS from the DGC toward the sarcoplasm, enhance dephosphorylation of FoxO1/3a, and promote NFkB signaling. In addition, ROS from Nox2 and/or mitochondria may promote a ROS-induced ROS-release amplification scheme (as shown by the dashed red line with arrowheads). ROS regulatory functions are shown with red lines. Dashed lines indicate hypotheses concerning signaling events made by the authors based on published research findings. This is a simplified figure illustrating ROS regulation of disuse fiber atrophy; refer to text for brief discussions on alternative pro-degradation routes (e.g., calpains, caspase-3, autophagic–lysosomal)

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Chapter 12
Participation of AMPK in the Control of Skeletal Muscle Mass

Tatsuro Egawa

Abstract The skeletal muscle plays crucial roles in whole-body glucose, lipid, and energy metabolism and in locomotive functions. The maintenance of the skeletal muscle mass is regulated by protein turnover: the balance between protein synthesis and protein degradation. The metabolic sensor 5′ AMP-activated protein kinase (AMPK) has important functions in the maintenance of cellular homeostasis and modulates glucose, lipid, and protein metabolism in the skeletal muscle. Recent studies warrant consideration of AMPK as a crucial regulator of muscle mass and suggest that AMPK controls skeletal muscle hypertrophy and atrophy by suppressing protein synthesis and promoting protein degradation via various signaling pathways. In addition, AMPK may stimulate myogenesis and regeneration of the skeletal muscle from injury. Conversely, the lack of AMPK activation probably restricts protein turnover during aging, potentially contributing to muscle loss. Taken together, these data indicate that AMPK triggers accelerated muscle turnover by regulating protein metabolism and/or myogenesis and thereby facilitates muscle mass homeostasis.

Keywords Protein synthesis • Protein degradation • Ubiquitin–proteasome system • Autophagy • Myogenesis

12.1 Introduction

The skeletal muscle is the largest tissue in the body, accounting for approximately 40–50% of total body mass and plays crucial roles in whole-body glucose, lipid, and energy metabolism and in locomotive functions. Hence, it is recognized that decreased skeletal muscle function leads to reduced health status and higher

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mortality. Aging is one of the most important factors that contribute to loss of muscle function and mass. Elderly people lose approximately 1% of muscle mass each year [38] as a natural effect associated with aging. However, muscle mass loss is not unavoidable, warranting the development of strategies for muscle maintenance and studies of the biological mechanisms of muscle mass regulation.

Maintenance of muscle mass is regulated by the balance between protein synthesis and protein degradation, which increase and decrease skeletal muscle mass, respectively, with changes in turnover dynamics. Several signaling cascades are associated with this balance and the control of muscle mass.

5′ AMP-activated protein kinase (AMPK) is a serine/threonine protein kinase that has emerged as a master sensor of cellular energy balance in mammalian cells [45]. AMPK was originally discovered as a kinase associated with critical metabolic enzymes of lipid metabolism, acetyl-CoA carboxylase [12], and 3-hydroxy-3-methylglutaryl CoA reductase [173]. In subsequent studies, AMPK was shown to regulate lipid metabolism and various other metabolic pathways. In general, AMPK is activated in response to energy-depriving stresses such as contraction, hypoxia, hyperosmolarity, decreased oxidative phosphorylation, and decreased electron transport [50]. These conditions lead to the inhibition of anabolic pathways that consume ATP and induction of catabolic pathways that produce ATP. In addition, oxidative stress [156], Ca^{2+} release [49, 171], adipokines such as adiponectin and leptin [46, 64, 105], and myokines such as interleukin-6 and brain-derived neurotrophic factor [86, 118] activate AMPK through energy-independent mechanisms. Subsequently, activated AMPK induces various metabolic adaptations in skeletal muscle by promoting glucose transport [50, 51, 103, 157], glucose transporter 4 expression [53, 106, 177], fatty acid oxidation [57, 160, 167], mitochondrial biogenesis [36, 62], insulin sensitivity [27, 175], and a fiber-type shift toward the slower and more oxidative phenotype [127]. In addition, skeletal muscle AMPK partially mediates the hypoglycemic effect of metformin, which is the most widely used drug for type-2 diabetes [64]. Given these diverse metabolic effects, skeletal muscle AMPK is widely accepted as a signaling intermediate that strongly promotes health (Fig. 12.1).

According to these varied and central roles of AMPK in muscle metabolism, we hypothesized that AMPK regulates muscle mass. The association between AMPK and muscle mass regulation was first suggested in 2002 by Bolster et al. [7], who showed novel evidence that AMPK modulates protein synthesis in rat skeletal muscle. Moreover, Mu et al. [102] showed that the soleus and extensor digitorum longus muscles of muscle-specific kinase-dead AMPKα2 transgenic mice tended to be larger than those of their wild-type littermates. Several subsequent studies have demonstrated crucial regulatory roles of AMPK in the control of skeletal muscle mass. In this chapter, we provide an overview of the role of AMPK in the regulation of skeletal muscle mass.

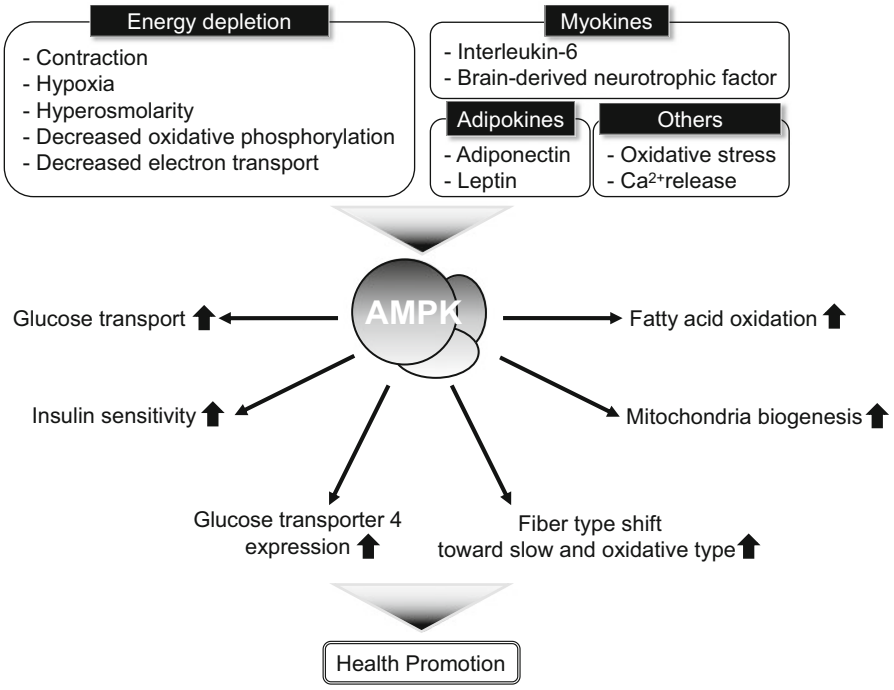

Fig. 12.1 Accepted metabolic effects of AMPK in skeletal muscle. AMPK is activated by several metabolic stresses such as energy depletion, myokines, adipokines, oxidative stress, and Ca^{2+} release from sarcoplasmic reticulum. Subsequently, activated AMPK induces diverse metabolic adaptations in skeletal muscle and thereby contributes to promote health

12.2 AMPK: Subunit Structure and Activation Mechanism

AMPK comprises a catalytic α-subunit and the regulatory subunits β and γ [45] in a total of 12 possible heterotrimeric combinations of two α-, two β-, and three γ-subunits [46]. In the skeletal muscle, the predominant heterotrimeric complexes include α1/β2/γ1, α2/β2/γ1, and α2/β2/γ3 [170]. In these complexes, the α-subunit has a catalytic domain that contains the activating phosphorylation site (Thr^{172}) at the N-terminus, an auto-inhibitory domain, and a conserved C-terminal domain that interacts with β- and γ-subunits [17, 60, 61, 112, 169]. The two distinct α isoforms have different localization patterns in mammalian cells, and whereas α1 is expressed widely, α2 is dominant in the skeletal muscle, the heart, and the liver [142]. The regulatory β-subunit contains a C-terminal region that interacts with α- and γ-subunits and a central region that binds glycogen [87]. Moreover, the regulatory

γ-subunit contains a number of repeating domains that are involved in the activation of the AMPK complex, including Bateman domains that bind adenine nucleotides (AMP, ADP, or ATP) [46].

Muscle AMPK classically acts as a signaling molecule and monitor of cellular energy levels and is sensitive to AMP/ATP ratios, creatine/creatine phosphate ratios, and AMP levels [45]. Binding of AMP to Bateman domains of the γ-subunit of AMPK causes allosteric activation of AMPK and phosphorylation of the Thr^{172} residue of the α-subunit, which increases AMPK activity by tenfold. In contrast, phosphorylation of Thr^{172} by upstream kinases including liver kinase B1 (LKB1) and Ca^{2+}/calmodulin-dependent protein kinase kinase (CaMKK) increases the AMPK activity by 100-fold [48]. Although the LKB1 complex is constitutively active and is not activated directly by AMP, the binding of AMP to AMPK leads to a structural change that facilitates phosphorylation of AMPK by the LKB1 complex [47, 130]. On the other hand, CaMKK can activate AMPK in response to increased intracellular Ca^{2+} levels independently of energy status [49, 56, 171].

12.3 AMPK and Skeletal Muscle Hypertrophy

Skeletal muscle hypertrophy is defined as a gain of muscle mass due to increases in sizes rather than numbers of pre-existing muscle fibers and occurs in response to increased loading such as resistance training [111], mechanical stretching [37], heat stress [40], and anabolic hormonal stimulation [93].

Although numerous AMPK transgenic and knockout (KO) animals have been produced, few studies show direct roles of AMPK under hypertrophic conditions (Table 12.1). Functional overload is commonly used in studies of muscle hypertrophy, and AMPKα1 activity is stimulated in these models [99]. Moreover, diminished AMPKα1 activity during functional overload reportedly accelerated muscle hypertrophy [99]. Functional overload-induced hypertrophy in plantaris muscles has also been shown to be diminished in aged (30 month) rats [149] and in the soleus muscles of obese rats [66]. Besides, both of these rat models exhibited hyper-phosphorylation of AMPKα Thr^{172} during overload, with a significant negative relationship ($r = -0.82$) between AMPK phosphorylation status and percent hypertrophy [66, 149]. In addition, hypertrophic consequences of constitutive activation of Akt, which is induced by infection with NH(2)-terminal myristoylation signal-attached Akt (MyrAkt), were reportedly elevated in the tibialis anterior muscles of AMPKα1-deficient mice in vivo [99] and in AMPKα1/α2-deficient muscle cells in vitro [73]. In our previous in vitro study [23], the pharmacological AMPK activator 5-aminoimidazole-4-carboxamide-1-β-D-ribonucleoside (AICAR) inhibited myotube hypertrophy in skeletal muscle cells, and this response was not induced in AMPKα1/α2 knockdown cells. Taken together, these data indicate that AMPK is a negative regulator of muscle mass under hypertrophic conditions.

Table 12.1 Muscle mass in genetic animal models of AMPK

Animal model	Type of muscle	Treatment	Change	References
Normal condition				
AMPKα2 DN	EDL, SOL	–	Mass↑	[102]
AMPKγ1R70Q	–	–	Mass→	[3]
AMPKα1$^{-/-}$	PLA	–	Mass↓	[99]
AMPKα1$^{-/-}$α2$^{-/-}$	SOL	–	Mass↑, CSA↑	[73]
AMPKγ3R225Q	GAS	–	Mass→	[176]
AMPKβ1$^{-/-}$β2$^{-/-}$	SOL, EDL	–	CSA→	[109]
AMPKα1$^{-/-}$	TA	–	Mass↓, CSA↓	[30]
AMPKα2$^{-/-}$	TA	–	CSA↑	
AMPKα2DN	QC	–	Mass→, CSA→	[110]
AMPKβ1$^{-/-}$β2$^{-/-}$	TA	–	CSA↓	[148]
LKB1$^{-/-}$	GAS	–	Mass↓	[139]
	TA, EDL	–	Mass→	
AMPKα2$^{-/-}$	TA, GAS, SOL, EDL	–	Mass↓	[13]
AMPKα1DN	SOL, EDL, GAS/PLA	–	Mass→, CSA→	[22]
AMPKγ3R225Q	PLA	–	Mass↑	[125]
AMPKγ3$^{-/-}$	PLA	–	Mass→	
Hypertrophic condition				
LKB1$^{-/-}$	PLA	Overload	Mass→	[89]
AMPKα1$^{-/-}$	TA	MyrAkt, transfection	CSA↑	[99]
AMPKα1$^{-/-}$	PLA	Overload	Mass↑	[99]
AMPKγ3R225Q	PLA	Overload	Mass→	[125]
Atrophic condition				
AMPKα1 DN	SOL	Hindlimb suspension	Mass↑, CSA↑	[22]

↑ increase or preventing decrease; ↓ decrease; → no change
EDL extensor digitorum longus, *SOL* soleus, *PLA* plantaris, *TA* tibialis anterior, *GAS* gastrocnemius, *QC* quadriceps, *CSA* muscle fiber cross-sectional area, *DN* dominant negative

12.4 AMPK and Skeletal Muscle Atrophy

Skeletal muscle atrophy is defined as a decrease in muscle mass due to disuse [108], aging [44, 78], malnutrition [54], and disease such as diabetes [15], cancer cachexia [153], sepsis [67], chronic renal failure [164], and chronic obstructive pulmonary disease (COPD) [4]. In particular, disuse muscle atrophy is a serious health care issue during aging and is closely related to the bedridden state.

Observational studies have demonstrated associations between AMPK and disuse muscle atrophy. In human studies, 7 [126] or 24 days [8] of bed rest in young men did not affect the phosphorylation status of AMPKα Thr^{172} in vastus lateralis muscles. In addition, 14 days of immobilization in young adult men and women had no effect on the phosphorylation status of AMPKα Thr^{172} or the expression

of AMPKα2 and AMPKβ2 subunits in vastus lateralis muscles [26]. In contrast, 14 days of immobilization in young and older men led to increased expression of AMPKα1, AMPKβ1, and AMPKγ3 subunits, but did not affect the other subunits or the phosphorylation status of AMPKα Thr^{172} [161].

In animal studies, 14 days of hindlimb unloading in young male rats decreased the AMPKα expression and the phosphorylation status of AMPKα Thr^{172} in soleus muscles [43]. Moreover, 28 days of hindlimb unloading in young male mice reduced the phosphorylation status of AMPKα Thr^{172} in gastrocnemius muscles [80]. In contrast, long-duration (4–13 weeks) hindlimb unloading in male rats enhanced the phosphorylation status of AMPKα Thr^{172} in soleus muscles [52, 174]. Moreover, short-duration (3 days) hindlimb unloading in male mice increased the phosphorylation status of ACC Ser^{79} [11], which is an endogenous indicator of AMPK activity [20, 114].

Although the dynamics of AMPK activity following muscle disuse remains controversial, we demonstrated direct evidence of AMPK-mediated progress of skeletal muscle atrophy in transgenic mice (AMPK-DN) expressing a dominant negative mutant of AMPKα1 in the skeletal muscle [22], in which predominant reduction of AMPKα2 activity rather than AMPKα1 activity has been observed [32, 65, 91, 145]. In the experiments, 14 days of hindlimb unloading led to a 30% decrease in soleus muscle mass and muscle fiber CSA in wild-type littermate mice, but only a 15% decrease in AMPK-DN mice [22], indicating that the deficiency of skeletal muscle AMPK activity (mainly AMPKα2) hinders the progress of skeletal muscle atrophy during disuse.

Taken together, these data suggest that AMPK is required for proper adaptation of muscle mass during disuse-induced skeletal muscle atrophy. Skeletal muscle atrophy due to disuse, including bed rest or immobilization, occurs especially during the early phase (∼10 days) [5, 96, 162]. Hence, because AMPK activation is enhanced during the early phase (∼3 days) of muscle disuse [11] and returns to basal or lower levels in the following phase [26, 43, 126, 161], AMPK may regulate muscle atrophy during the early phase of disuse. However, further evidence is required to clarify the precise roles of AMPK in the regulation of skeletal muscle atrophy.

12.5 Molecular Mechanisms of AMPK-Mediated Regulation of Muscle Mass

AMPK is a master regulator of metabolism and multiple signaling molecules are implicated in AMPK-mediated regulation of muscle mass (Fig. 12.2).

12.5.1 Protein Synthesis Pathway

The mammalian target of rapamycin (mTOR) plays crucial roles in the regulation of protein synthesis [6] as a serine/threonine protein kinase of phosphoinositol-

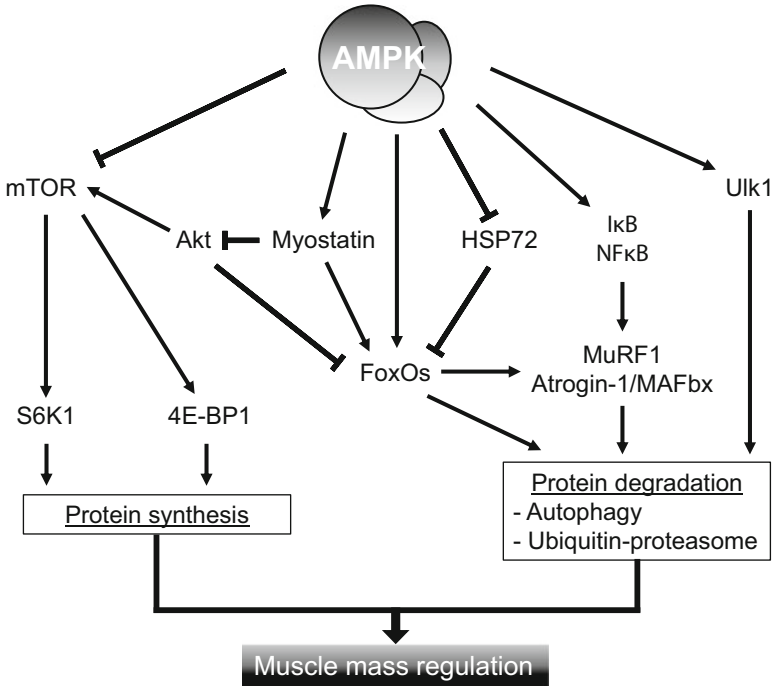

Fig. 12.2 Molecular mechanisms of AMPK-mediated regulation of muscle mass. AMPK down-regulates mTOR signaling and inhibits protein synthesis. In contrast, AMPK stimulates protein degradation systems including ubiquitin–proteasome and autophagy. These regulations are mediated by multiple signaling molecules such as myostatin, HSP72, FoxOs, NFkB, MuRF1, Atrogin-1/MAFbx, and Ulk1. The *arrow* indicates positive regulation whereas the blocked line indicates negative regulation

3-kinase (PI3K)-related kinase family. Specifically, mTOR interacts with several proteins to form the distinct mTOR complexes 1 (mTORC1) and 2 (mTORC2), which are known to differ in subunit composition, sensitivity to rapamycin, and cell signaling activity [74]. It is accepted that mTORC1 signaling is more closely related to the control of protein synthesis than mTORC2 and is negatively regulated by the tumor suppressor tuberous sclerosis complex 1/2 (TSC 1/2) [146]. Increased activity of mTORC1 stimulates protein translation and synthesis following phosphorylation of key direct downstream effectors, including the 70-kDa ribosomal protein S6 kinase (S6 K1) and eukaryotic initiation factor 4E-binding protein 1 (4E-BP1) [33, 74]. S6 K1 activation also enhances translation elongation via signals that eventually lead to decreased phosphorylation of eukaryotic elongation factor 2 (eEF2) [163]. Moreover, AMPK has been shown to interact with mTOR signaling, and activation by AICAR injection inhibits mTOR signaling and protein synthesis in rat skeletal muscle [7]. AMPK phosphorylates and activates TSC2 [59] to inhibit mTORC1 and S6 K1 activity [58] and directly phosphorylates the mTOR binding partner

raptor, leading to binding of 14-3-3 to raptor and allosteric inhibition of mTORC1 [42]. Thus, mTORC1 is a potent intermediate of AMPK-mediated muscle mass regulation.

In a previous study, impairment of muscle hypertrophy during functional overload in aged rats was accompanied by reduced activation of mTOR/S6 K1/eEF2 and mTOR/4E-BP1 signaling, and these responses were negatively correlated with the phosphorylation status of AMPKα Thr^{172} [150]. Furthermore, Viollet and colleagues showed that S6 K1 and 4E-BP1 signaling are promoted in AMPKα1 KO mice during functional overload-induced muscle hypertrophy [99]. In addition, increased cell size in AMPKα1/α2-deficient myotubes was prevented by treatments with the mTOR inhibitor rapamycin [73]. Hence, crosstalk between AMPK and mTOR/S6 K1 signaling is likely important for muscle mass control under conditions of hypertrophy.

In contrast with these studies, we found little association of mTOR signaling with skeletal muscle atrophy [22]. Specifically, protein synthesis and phosphorylation status of S6K1 Thr^{389} were decreased during disuse-induced soleus muscle atrophy. However, suppression of AMPK activity did not rescue these responses [22], indicating that AMPK/mTOR/S6K1 signaling may not be a crucial axis in skeletal muscle atrophy.

Distinct roles of α isoforms (α1 and α2) of AMPK may influence the regulation of muscle mass via mTOR/S6K1 signaling. Accordingly, Viollet and colleagues indicated that AMPKα1-mTOR/S6K1 interactions are important in skeletal muscle hypertrophy. However, another study showed that both skeletal muscle hypertrophy and activation of mTOR/S6K1 signaling remained unchanged in response to functional overload in LKB1 KO mice [89], in which AMPKα2 but not AMPKα1 activity is completely abolished. Consistent with these findings, Viollet and colleagues showed that MyrAkt-induced hypertrophy was similar in muscle fibers lacking AMPKα2 and in control fibers [98]. These results suggest that AMPKα1 is more closely associated with mTOR/S6 K1 activation and skeletal muscle hypertrophy than AMPKα2.

In contrast with these studies, we demonstrated attenuation of unloading-induced skeletal muscle atrophy in AMPK-DN mice, in which AMPKα2 activity was almost completely inhibited but AMPKα1 activity was reduced by only 20% [22]. AMPK is activated in response to energy-depleting stresses [50], and AMPKα2 is more sensitive to energy depletion than AMPKα1 [131, 143]. Mitochondrial dysfunction-mediated impairment of ATP production has been suggested to activate AMPK under conditions of disuse and thereby contributes to muscle atrophy through protein degradation rather than protein synthesis [123]. Taken together, these observations indicate that AMPKα2 regulates skeletal muscle atrophy via mechanisms that are independent of protein synthesis pathways. These findings imply that there are isoform-dependent regulation of muscle mass, although multiple roles of AMPK α, β, and γ isoforms in muscle mass regulation remain unresolved.

12.5.2 Ubiquitin–Proteasome System

The ATP-dependent ubiquitin–proteasome system is the primary protein degradation pathway and intrinsically induces degradation of myofibrillar proteins in skeletal muscle [104]. The ubiquitin ligase enzyme E3 binds its protein substrate and catalyzes the movement of ubiquitin from the ubiquitin-conjugating enzyme (E2) to the substrate. This rate-limiting step of the ubiquitination influences subsequent proteasome-dependent degradation. The human genome contains more than 650 ubiquitin ligases [75], and important skeletal muscle-specific ubiquitin E3 ligases include muscle-specific RING finger protein 1 (MuRF1) and atrogin-1/muscle atrophy F-box (MAFbx). MuRF1 and atrogin-1/MAFbx are both primarily expressed in skeletal muscle [5]. Whereas MuRF1 ubiquitinates several myofibrillar proteins, such as myosin heavy chains [16] and actin [122], atrogin-1/MAFbx targets growth-related proteins such as MyoD [152] and eIF3f [18]. Moreover, MuRF1 and atrogin-1/MAFbx mRNA levels are rapidly upregulated in numerous models of muscle atrophy and play accepted roles in the initiation of atrophy [28].

In the past decade, AMPK has been shown to interact with several E3 ubiquitin ligases. In skeletal muscle, MuRF1 and atrogin-1/MAFbx are potent targets of AMPK-mediated ubiquitination, and MuRF1 and atrogin-1/MAFbx mRNAs were upregulated following treatment with the AMPK activators AICAR [23, 68, 107, 155] and metformin [68] in skeletal muscle cells in vitro. Our recent in vivo studies demonstrated that suppression of AMPK activity attenuates increases in ubiquitination and MuRF1 mRNA expression, thus inhibiting the progress of disuse-induced muscle atrophy in mice soleus muscles [22]. Other studies have also suggested associations between AMPK and E3 ligases during muscle atrophy. Specifically, TNF receptor adaptor protein 6 (TRAF6) was associated with the dimeric ubiquitin-conjugating enzyme 13/ubiquitin-conjugating enzyme variant 1A and promoted the formation of Lys63-linked poly-ubiquitin chains rather than conventional Lys48-linked poly-ubiquitin chains, which are target proteins for degradation [71, 121]. Muscle-specific TRAF6 KO mice were resistant to muscle atrophy following denervation, cancer, or starvation, and TRAF6-mediated ubiquitination was shown to be mediated partly by activation of AMPK [116, 117]. Thus, various relationships between AMPK and the ubiquitin–proteasome system are important in the regulation of skeletal muscle mass.

12.5.3 Autophagy

Autophagy is an important cell proteolytic system that controls protein turnover in skeletal muscle [85] and is regulated by multiple proteins. Among these, Unc-51-like kinase 1 (Ulk1, also known as Atg1) is considered an important serine/threonine protein kinase during the initial stage of autophagosome formation [168]. In

subsequent stages, microtubule-associated protein light chain 3 (LC3) is converted to the active form of LC3 (named LC3II) through lipidation and then participates in the formation and elongation of autophagosomes. The presence of LC3 in autophagosomes and the conversion of LC3 to the lower migrating form LC3II have often been used as indicators of autophagic activity [63]. In the final step of autophagy, autophagosomes fuse with lysosomes and their cargoes are degraded [94]. The ubiquitin-binding protein p62, which binds to LC3, is preferentially degraded during autophagy [113], and thus breakdown of p62 is generally used as a marker of autophagy flux [95].

Recently, AICAR-induced AMPK activation was reported to stimulate autophagosome formation in skeletal muscle cells [133]. Accordingly, interactions of AMPK, mTOR, and Ulk1 appear to play crucial roles in the modulation of autophagy [1, 76]. Specifically, mTOR directly binds and negatively regulates Ulk1 activity by phosphorylating the protein at Ser^{637} and Ser^{757} [35, 140]. In contrast, AMPK binds and phosphorylates Ulk1 at Ser^{317}, Ser^{467}, Ser^{555}, Thr^{575}, Ser^{637}, and Ser^{777} and thereby promotes Ulk1 activity and disrupts the Ulk1–mTOR interaction [1]. AMPK also indirectly induces autophagy by inhibiting the mTORC1 complex [35, 76].

Roles of AMPK-mediated autophagy in muscle mass regulation have been reported, and in vitro treatments with the synthetic glucocorticoid dexamethasone have been shown to increase the ratios of LC3II/LC3I dependently of AMPK in skeletal muscle cells [159]. In addition, chronic dexamethasone treatment led to muscle atrophy and AMPK activation in mice, and the muscle atrophy was attenuated by blocking AMPK activation [81]. These results indicate associations of AMPK-mediated autophagy and dexamethasone-induced muscle atrophy.

In a previous study, we demonstrated that increased LC3II/LC3I ratios in mouse soleus muscles during unloading-induced muscle atrophy were attenuated following suppression of AMPK activity [22]. However, unloading was previously shown to result in p62 protein accumulation in atrophic soleus muscles, indicating impairment of autophagic flux [95]. Accordingly, p62 protein expression was increased by unloading in mouse soleus [22], tibialis anterior, and gastrocnemius muscles [80], and no changes in LC3 expression or decreases in other autophagy-related proteins (Atg7 and Beclin-1) were reported after unloading. Taken together, these data warrant further research to precisely determine the roles of AMPK in autophagy activation during disuse-induced muscle atrophy.

AMPK-mediated autophagy has been shown to play critical roles in the regulation of skeletal muscle mass during fasting and/or aging [10]. These investigators showed that myofiber CSA in tibialis anterior muscles was reduced by fasting in wild-type mice but not in muscle-specific AMPK-KO mice and that LC3II/LC3I ratios and phosphorylation states of Ulk1 Ser^{555} after fasting were higher in wild-type than muscle-specific AMPK-KO mice. In addition, AMPK deficiency resulted in increased activation of mTOR and inhibitory phosphorylation of Ulk1 Ser^{757}. These findings suggest that induction of muscle atrophy during fasting follows AMPK-mediated activation of autophagy. In addition, they also showed that AMPK deficiency led to greater aging-associated impairments of muscle quality, with increased central nuclei, large round fibers, small angular fibers, and necrotic

fibers, and decreased muscle force production from that in wild-type mice. Besides, low muscle quality was reportedly accompanied by p62 accumulation, implying that aging-induced myopathy follows impairments of AMPK-mediated autophagic activity. Collectively, these findings indicate that both activation and inactivation of AMPK-autophagy systems result in muscle loss, and hence activation of autophagy systems by AMPK might be essential for muscle mass homeostasis.

12.5.4 Forkhead Box O (FoxOs)

The FoxOs family of transcription factors has been associated with ubiquitin–proteasome and autophagy systems [132, 144]. Among these, FoxO1, FoxO3 (also known as FoxO3a), FoxO4, and FoxO6 have been identified in skeletal muscle, and FoxO1 and FoxO3a are key factors in muscle homeostasis. Akt inhibits FoxO3a by phosphorylating residues Thr^{32}, Ser^{253}, and Ser^{315} [9], and phosphorylation of FoxO3a at Ser^{253} results in exclusion from the nucleus and inhibition of transcription [9]. In contrast, AMPK phosphorylates FoxO3a at six regulatory sites (Thr^{179}, Ser^{399}, Ser^{413}, Ser^{355}, Ser^{588}, and Ser^{626}) and promotes FoxO3a transcriptional activity without affecting subcellular localization [41].

Previous studies suggest that AMPK-mediated upregulation and/or nuclear translocation of FoxO1 and FoxO3a contributes to activation of the ubiquitin–proteasome system in skeletal muscle cells [107, 133, 155]. Furthermore, phosphorylation of FoxO3a Ser^{588} by AMPK leads to increased expression of autophagy-related proteins such as beclin and LC3II [133]. In our experiments, decreased phosphorylation of FoxO3a Ser^{253} in soleus muscles was accompanied by Akt inactivation during disuse-induced muscle atrophy, but suppression of AMPK activity maintained phosphorylation of FoxO3a Ser^{253} at the basal level despite Akt remained inactivated [22]. These data indicate that AMPK affects the phosphorylation status of FoxO3a Ser^{253} through an Akt-independent mechanism during muscle atrophy. Thus, although the mechanisms behind the relationship between AMPK and FoxOs activities in muscle mass regulation have not been characterized, AMPK appears to directly or indirectly regulate FoxOs expression and/or nuclear translocation and thereby stimulates ubiquitin–proteasome and/or autophagy systems.

12.5.5 Nuclear Factor κB (NF-κB)

The transcription factor NF-κB is sequestered in the cytoplasm by a family of inhibitory proteins known as IκBα [101]. In this mechanism, the IκB kinase complex phosphorylates IκBα, resulting in its degradation, and thus facilitates nuclear translocation and activation of NF-κB. NF-κB activation has been detected under physiological and pathological atrophic conditions, including unloading, denervation, aging, cancer, sepsis, and diabetes, and pharmacological or genetic inhibition of NF-κB can protect from skeletal muscle atrophy [79, 165]. Moreover,

NF-κB signaling was reportedly more important than FoxOs signaling in disuse muscle atrophy, since NF-κB sites, but not FoxOs sites, for transcription of MuRF1 during hindlimb unloading [172]. In our study, the expression of IκBα tended to decrease with muscle atrophy in mice and was higher in AMPK-DN mice than in wild-type mice after hindlimb unloading [22]. Hence, AMPK may regulate NF-κB signaling via the expression of IκBα during disuse-induced muscle atrophy. However, no other reports show associations of AMPK with NF-κB signaling during muscle mass regulation, warranting further research to clarify the precise roles of NF-κB signaling in AMPK-mediated muscle mass regulation.

12.5.6 Heat Shock Proteins (HSPs)

HSPs are stress-induced molecular chaperones that play crucial roles in maintaining correct the folding and intracellular transport of proteins. Accordingly, as regulators of cell signaling, HSPs have various reported cytoprotective functions [69, 82, 115]. Multiple HSPs have been identified in skeletal muscle, including HSP27, HSP47, HSP60, HSP70, HSP90, and HSP110. Among these, inducible HSP70 (so-called HSP72) is the most widely studied in skeletal muscle, and profound impacts on muscle adaptation have been shown [82]. HSP72 expression is increased in skeletal muscle following exercise [97] and muscle injury [138], and during muscle regrowth and regeneration [135, 138], but is decreased by muscle disuse [22, 137]. Accordingly, overexpression of HSP72 in skeletal muscle prevented immobilization-induced atrophy in rats [137] and improved structural and functional recovery from atrophy of mouse muscles [92]. Moreover, inhibition of FoxOs and NF-κB signaling is considered a potent mechanism by which HSPs regulate skeletal muscle mass [21, 70, 136, 137]. Therefore, HSP72 may be a positive regulator of skeletal muscle mass that downregulates protein degradation pathways.

In our studies, AMPK was implicated in the regulation of HSP72 expression during skeletal muscle hypertrophy and atrophy. Specifically, AICAR-induced AMPK activation decreased HSP72 protein expression in skeletal muscle cells and was not induced following suppression of AMPKα1/α2 [23]. This was the first report to show that AMPK modulates HSP72 expression in skeletal muscle. Furthermore, we recently demonstrated that metformin-induced AMPK activation also downregulated HSP72 mRNA and protein expression in skeletal muscle cells [24], suggesting transcriptional regulation of HSP72 by AMPK. In addition, in vivo experiments showed higher HSP72 expression in intact muscle from AMPK-DN mice than from wild-type mice, and decreased HSP72 expression during unloading-induced muscle atrophy was attenuated in AMPK-DN mice [22]. Taken together, these data suggest an inverse relationship between AMPK and HSP72 in skeletal muscle and indicate that AMPK-mediated regulation of muscle mass may be mediated by HSP72.

The transcription of HSPs is mediated by HSF1 binding to the corresponding regulatory elements [82]. Hence, if AMPK affects the HSF1-mediated transcription

of HSPs, several HSPs may be induced by AMPK activation. However, our experiments showed that activation of AMPK by AICAR treatment affected HSP72, but not HSP25 or constitutively expressed HSC70 [23]. Furthermore, HSF1 expression was downregulated following disuse-induced muscle atrophy independently of AMPK activity [22]. These observations indicate that AMPK modulates the expression of HSPs through HSF1-independent mechanisms.

12.5.7 Myostatin

Myostatin is known as growth differentiation factor 8 (GDF-8) and is a member of the transforming growth factor-β (TGFβ) superfamily, which has emerged as central to the regulation of skeletal muscle mass [90]. Myostatin binding to activin type IIB receptor (ActRIIB) leads to the recruitment of activin-like kinase-4 (ALK-4) or ALK-5, and phosphorylation and activation of Smad2 and Smad3, which form complexes with Smad4 [124, 128]. Subsequent nuclear translocation of the Smad2/Smad3/Smad4 complex promotes the transcription of genes involved in proliferation and differentiation and the protein metabolism in skeletal muscle [72, 134, 147]. Activation of Smad2 and Smad3 also impairs Akt activity, leading to inhibition of mTOR-dependent protein synthesis pathways [158] and stimulation of FoxOs-dependent protein degradation pathways [88]. Consequently, myostatin acts as a negative regulator of muscle mass.

Myostatin was shown to be associated with AMPK in skeletal muscle during diabetic muscle atrophy, with concomitant AMPK Thr^{172} phosphorylation and increased myostatin expression [55]. Accordingly, some studies have shown that AMPK activation by AICAR stimulates myostatin expression in skeletal muscle cells [19, 77]. These findings suggest that AMPK stimulates myostatin expression and thereby negatively regulates skeletal muscle mass. This hypothesis is supported by our unpublished findings showing that increases in myostatin mRNA expression in atrophic soleus muscles are abolished by suppression of AMPK. We also observed lower plasma myostatin levels in AMPK-DN mice than in wild-type mice (unpublished data). Hence, AMPK may regulate muscle mass by modulating skeletal muscle and/or whole-body myostatin expression. In addition, treatment of skeletal muscle cells with recombinant myostatin reportedly stimulated AMPK by depleting energy stores [14], suggesting that a feed-forward loop between AMPK and myostatin contributes to the control of muscle mass.

12.6 AMPK and Myogenesis

Myogenesis is defined as the formation of muscular tissue and includes growth, differentiation, and repair of muscles. Myogenic regulatory factors (MRFs) include basic helix-loop-helix (bHLH) transcription factors that are essential for determi-

nations of muscle lineage. Among these, myogenic factor 5 (Myf5), myogenic differentiation (MyoD), myogenin, and MRF4 are characterized by the capacity to convert various cell lines into myocytes and to activate muscle-specific gene expression [141]. Myf5 is the first MRF to be expressed during embryonic development and integrates multiple developmental signals to initiate myogenesis [29]. MyoD is also an important transcriptional regulator of myogenesis and is considered a master switch gene for muscle formation [120]. Myogenin and MRF4 are involved in later stages of myogenesis and are directly associated with differentiation processes that lead to myotube formation and maturation [2, 84].

As shown in Table 12.1, AMPK transgenic and KO mice exhibit higher or lower muscle mass and muscle fiber CSA under normal growth conditions, further indicating that AMPK is involved in myogenesis. Indeed, several studies have provided evidence that AMPK mediates myogenic processes by regulating MRFs, especially myogenin. Accordingly, low myogenin, but not MyoD and MRF4, levels were observed in primary myoblasts from AMPKα1 KO mice, which were incapable of forming myotubes [31]. Furthermore, AICAR-induced activation of AMPK in skeletal muscle cells increased myogenin expression and promoted myotube formation [31]. Epigenetic histone and DNA modifications are considered important for cell differentiation and tissue development [119, 129], and histone deacetylase 5 (HDAC5), which belongs to the class IIa HDAC family, was reported to act as a conserved transcriptional repressor of myogenin [83]. In agreement, AMPK-mediated myogenin expression and myogenesis have been shown to be mediated by HDAC5 expression in skeletal muscle cells of the tibialis anterior [30]. Taken together, these studies suggest that AMPK positively regulates myogenesis through a HDAC5–myogenin mechanism.

Previous studies have demonstrated that AMPK is an important player during muscle development. Specifically, deletion of LKB1, a crucial upstream kinase of AMPK, led to defects in muscle development and growth through AMPK-mediated myoblast proliferation and differentiation [139, 151]. It has also been demonstrated that impairments of AMPK activity in fetal muscles of obese sheep were associated with decreased densities of muscle fibers per area [178]. In another study, metformin-induced AMPK activation attenuated developmental impairments of skeletal muscle in the offspring of obese mice [154]. In addition to muscle development, the importance of AMPK in muscle regeneration from injury has been reported. The postinjury regeneration process induced by cardiotoxin in tibialis anterior muscle followed the growth of new muscle fibers, which were characterized by central locations of nuclei, but deficiencies of AMPKα1 or LKB1 retarded these regeneration processes [100, 139].

In contrast, AMPK was shown to act as a negative regulator of myogenesis. Glucose restriction-mediated inhibition of myoblast differentiation was shown to be AMPK dependent [34]. Moreover, treatment with high doses of AICAR (0.5–1.0 mM), which were sufficient to fully activate AMPK, inhibited myoblast fusion to myotubes and decreased levels of myogenin [34, 139] and MyoD [166]. However, low doses of AICAR (0.125 mM), which induces moderate AMPK activation, were shown to promote myogenesis [31]. Taken together, these data suggest that whereas AMPK is basally required for myogenesis, excessive stresses such as glucose

restriction or high doses of pharmacological stimulation that induce robust AMPK activation may inhibit myogenesis through undetermined mechanisms.

12.7 Summary and Perspectives

Skeletal muscle is highly adaptable to various stimuli, and AMPK appears to mediate the adaptations as a metabolic sensor. Accordingly, AMPK probably controls hypertrophy and atrophy by suppressing protein synthesis and promoting protein degradation systems and thereby regulates muscle mass. In addition, AMPK may stimulate myogenesis and regeneration of skeletal muscle following injury. Collectively, AMPK triggers accelerated muscle turnover by mediating degradation of unnecessary proteins and concomitant stimulation of protein synthesis and thereby contributes to the maintenance of muscle mass homeostasis. Indeed, previous studies indicate the necessity of proper AMPK activation for preventing muscle loss during aging [10, 39] or myopathy [148]. However, both excessive and impaired AMPK activation may cause muscle loss by disrupting protein turnover and/or myogenic processes (Fig. 12.3). Therefore, moderate activation of AMPK in daily life using optimal stresses such as voluntary exercise, non-exercise activity, phytochemicals [25], and medicines may facilitate the maintenance of skeletal muscle mass.

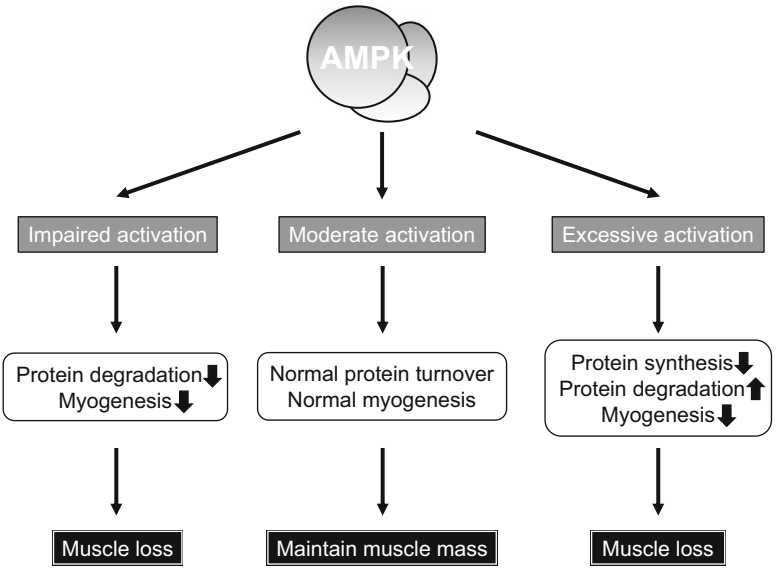

Fig. 12.3 Proposed role of AMPK in muscle mass maintenance. Moderate activation of AMPK properly modulates protein turnover and/or myogenesis and thereby contributes to maintain muscle mass. On the other hand, both impaired activation and excessive activation of AMPK disrupt the protein turnover and/or myogenesis and result in muscle loss

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Chapter 13
Therapeutic Potential of Skeletal Muscle Plasticity and Slow Muscle Programming for Muscular Dystrophy and Related Muscle Conditions

Gordon S. Lynch

Abstract Duchenne muscular dystrophy (DMD) is a devastating life-limiting disease causing progressive and severe muscle wasting in boys and young men. It is simply unacceptable that ~30 years after the discovery of the culprit protein, dystrophin, there is still no cure or effective treatment. Dystrophic muscles are fragile, injury prone and compromised in their regenerative capacity. Interestingly, in DMD and in two well-characterised murine models of the disease (*mdx* and *dko* mice), fast muscle fibres are more susceptible to damage and pathological progression than slow muscle fibres, which are resistant to damage and relatively spared. Therefore, therapies that promote a slower, more oxidative phenotype could protect muscles from damage, ameliorate the dystrophic pathology and improve patient quality of life. Muscle plasticity can be achieved through exercise and/or well-described pharmacologic approaches, including activation of AMP-activated protein kinase (AMPK). Exercise has beneficial effects on muscle health, but unfortunately many patients cannot exercise, especially DMD patients confined to wheelchairs. Modulating muscle activity through low-frequency stimulation (LFS) protocols could mimic exercise to promote a slow phenotype, protect muscles from damage and enhance muscle repair. Enhancing these adaptations by combining LFS with pharmacologic modifiers of muscle phenotype potentially represents a novel therapy that could find immediate application to improve the pathology and enhance patient quality of life. Alternative approaches like anabolic agents or myostatin inhibition also have therapeutic potential, but their efficacy occurs through different mechanisms. Better understanding of the mechanisms underlying skeletal muscle

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adaptations to different interventions and stimuli will help optimise novel strategies to address the pathophysiology of DMD and related muscle conditions.

Keywords Muscular dystrophy • Dystrophin • DMD • Skeletal muscle diseases • Muscle wasting • Electrical stimulation • Exercise • Muscle injury • Muscle regeneration • Fibre type • Fast-to-slow • Slow-to-fast • Muscle phenotype • Anabolic agents • AMPK • Muscle plasticity • Muscle adaptation • Neuromuscular • Muscle function • Contractile properties • Muscle contraction

13.1 Introduction

Modifying muscle phenotype to confer protection from injury or pathology has its origins in the study of muscle plasticity. Skeletal muscles are highly plastic and capable of adapting to different perturbing stimuli. Muscle fibre composition can be altered through pharmacologic manipulation of biochemical pathways that regulate contractile and regulatory protein isoform composition as well as the muscle's metabolic machinery. Altering the pattern of neural stimulation to skeletal muscles can similarly alter muscle phenotype. It is theoretically possible to completely alter a muscle's phenotype, from fast-to-slow or vice versa, depending on the nature (frequency, intensity and duration) of the intervening stimuli. In most cases, the nature of interventions like physical activity (exercise) or functional neuromuscular electrical stimulation, like that applied to humans for therapeutic or rehabilitative purposes, means that they are not sufficient to elicit extreme changes in muscle phenotype. Regardless, the therapeutic potential of electrical stimulation for muscle diseases has been identified and represents an exciting field of research. Despite some promising outcomes from early studies on patients with Duchenne muscular dystrophy (DMD), electrical stimulation as a therapy has not found wide application for this condition.

This chapter describes the underlying basis of skeletal muscle programming and its therapeutic potential for DMD and related conditions. It describes how muscle phenotype can be altered by different stimuli, with potentially opposing effects on parameters such as muscle fibre size and fibre composition. It highlights how pharmacologic and electrical stimuli can alter muscle phenotype, to confer beneficial outcomes that could improve muscle structure and function and ultimately enhance quality of life for patients.

13.2 Duchenne Muscular Dystrophy

DMD is the most common of the muscular dystrophies, caused by mutations and deletions in the dystrophin (*dmd*) gene on chromosome Xp21, leading to a lack of expression or a non-functional corresponding protein in muscle. It is a

devastating, life-limiting disease affecting ~1:3500–6000 live male births, resulting in progressive and severe muscle wasting and weakness in boys and young men [10]. Patients become wheelchair dependent before their teens and have only 25% of the muscle mass of healthy children. Eventually all muscles are affected and patients eventually succumb to respiratory or cardiac muscle failure.

Sadly, there is still no cure or effective treatment for DMD.

Although a cure may eventually come from stem cell or corrective gene therapies, limitations of delivery systems, gene carrying capacity, dissemination efficiency, expression persistence and immunological tolerance all pose significant obstacles for clinical application [92]. Until these techniques are perfected, DMD patients will continue to die prematurely. The current mainstays in treating DMD are glucocorticoids (prednisolone or deflazacort) which despite slowing the disease progression have many deleterious side effects [30]. DMD patients also need regular corrective surgeries to relieve stiff joints, correct scoliosis and similar muscle-related interventions [18], which can aggravate the dystrophic pathology and compromise an already defective regenerative process. Clearly, there is a profound, urgent and unmet clinical need for therapies that can ameliorate the pathology, preserve and protect muscles from damage and enhance muscle fibre regeneration.

The most widely used animal model of DMD is the *mdx* dystrophic mouse which has a point mutation in the dystrophin gene and an absence of dystrophin protein expression in muscle [85]. Although sharing the same genetic deficit as DMD, the muscle phenotype of *mdx* mice differs in that the hindlimb muscles undergo severe degeneration at 3–4 weeks of age, but an enhanced regenerative capacity ensures almost complete functional recovery. In *mdx* mice there is compensatory upregulation of the dystrophin-like protein, utrophin, which may account for its more benign phenotype. Unlike *mdx* mice, dystrophin-utrophin double knockout (*dko*) mice exhibit severe wasting and weakness, spinal deformities (kyphosis) from an early age and a shortened lifespan more phenotypically representative of DMD. These models are fundamental for understanding the dystrophic pathophysiology since functional roles for dystrophin and utrophin remain unclear, and much information can be gained from knockout phenotypes [75, 76]. These murine models are essential for understanding how dystrophic skeletal muscles adapt to different interventions or stimuli that could ameliorate the pathophysiology of DMD and related conditions [28].

13.3 Skeletal Muscle Diversity and Adaptability

Skeletal muscle is comprised of functionally diverse fibres ranging in size, metabolism and contractility [4, 9, 37, 81, 82]. Based on myosin heavy chain (MyHC) protein isoforms, which largely dictate the rate of force development, shortening velocity and rate of cross-bridge cycling, mammalian muscle fibres are

broadly classified as slow-twitch (type I) or fast-twitch (type IIa, IId/x and IIb). Type I and IIa fibres primarily generate ATP via oxidative metabolism, whereas type IId/x and IIb fibres generate energy mostly through glycolysis [59, 80].

Muscle fibres are highly plastic and can alter their structural, functional, metabolic and molecular properties in response to altered contractile demands or pharmacologic interventions that manipulate signalling pathways that regulate isoform composition. Altered motor neuron activity can dramatically change muscle fibre composition, a phenomenon first demonstrated through a series of elegant nerve cross-reinnervation studies by Sir John Eccles and colleagues [8]. Together, these studies revealed that when fast muscles were innervated by a slow nerve, the muscle transformed from a fast (glycolytic) to a slower, more oxidative phenotype and contracted more slowly. When slow muscles were innervated by a fast nerve, the muscle transformed from an oxidative to a more glycolytic phenotype and contracted more quickly. Such phenotypic changes were attributed to the specific impulse patterns delivered to the muscle via the motor neuron [7]. Chronic low-frequency (10–15 Hz) stimulation induces transcription of slow oxidative genes in fast muscles through sustained elevations in low-amplitude intracellular [Ca^{2+}] transients, which stimulate downstream signalling pathways and key proteins regulating muscle phenotype, specifically promoting fast to slow changes [52, 68, 94]. Through studies using transgenic mouse lines and specific drug targeting, these key proteins have been identified and include: calcineurin, peroxisome proliferator-activated receptor (PPAR) γ coactivator 1α (PGC-1α), PPARβ/δ, silent mating type information regulator 2 homologue 1 (SIRT1) and AMP-activated protein kinase (AMPK) [14, 42–44, 89]. These studies have contributed significantly to our understanding of the signalling pathways regulating skeletal muscle adaptation and plasticity and have been reviewed elegantly in detail elsewhere [44].

13.4 Promoting a Slower, More Oxidative Muscle Phenotype – A Therapeutic Target for DMD

The lack of dystrophin in the muscles of DMD patients and *mdx* and *dko* mice renders muscle fibres fragile and prone to injury. Interestingly, muscles composed of fast fibres are more susceptible to damage and pathological progression than predominantly slow muscles, both in DMD patients and in *mdx* and *dko* mice. Thus, dystrophin deficiency in fast muscle fibres of DMD patients is associated with degenerative changes, while slower muscle fibres are more resistant to damage and relatively spared [99]. Therefore, therapies that can promote a slower, more oxidative muscle phenotype could ameliorate the dystrophic pathology and improve patient quality of life.

That slower, more oxidative muscle fibres express significantly more utrophin-A protein compared with their faster, more glycolytic counterparts, has been suggested as one factor protecting slower fibres against damage. Studies in transgenic*mdx* mice

(overexpressing utrophin) established that utrophin can functionally substitute for dystrophin and ameliorate the dystrophic pathology [5, 20, 34, 95]. Activation of signalling pathways that promote a slower, more oxidative phenotype also promote increases in utrophin-A expression, highlighting the therapeutic relevance of manipulating muscle plasticity to mitigate the dystrophic pathology. For example, calcineurin-NFAT (nuclear factor of activated T cells) signalling plays an important role in regulating fast-to-slow muscle phenotypic adaptations [17]. Calcineurin is a Ca^{2+}/calmodulin-dependent phosphatase that dephosphorylates NFAT, resulting in its nuclear translocation and binding to specific sequences on the promoters of target genes that induce slow oxidative fibre programming [1, 23, 64, 65] with potential benefits for the dystrophic phenotype [13, 14, 87, 88]. For example, muscles of transgenic *mdx* mice expressing an active form of calcineurin exhibited a shift to a slower, more oxidative phenotype, increased utrophin-A expression and an attenuated dystrophic pathology [89, 90]. Inhibition of this signalling exacerbated the dystrophic pathology in skeletal muscles of *mdx* mice [15].

Conversely, promoting a slower, more oxidative muscle phenotype may have beneficial effects on DMD that extend beyond simply increasing utrophin-A expression [93]. Muscles of DMD patients and *mdx* and *dko* mice have impaired oxidative phosphorylation and mitochondrial function, which contributes to the disease aetiology [56]. Therefore, promoting a slower, more oxidative muscle phenotype may rescue normal mitochondrial function and help alleviate the dystrophic pathology.

13.5 Exercise, Low-Frequency Stimulation and DMD

Physical activity, especially endurance training, has many beneficial effects on muscle health including the potential to promote a slow, oxidative phenotype [11, 44, 70]. Whether exercise has beneficial effects for DMD patients remains contentious, with some activities like low-intensity, low-weight bearing exercise shown to have therapeutic effects in some studies, while other exercises involving potentially injurious lengthening (eccentric) contractions can aggravate the pathology [50, 96]. For a comprehensive review on the effects of exercise on dystrophic skeletal muscle, see Markert et al. [49].

Unfortunately many patients are simply unable to exercise, especially boys with DMD who are confined to a wheelchair usually before their teens. Devising contraction/activity protocols that mimic the benefits of exercise to attenuate loss of muscle mass and improve function in these situations could provide a significant improvement in patient quality of life. Low-frequency stimulation (LFS) is a well-established model of muscle training that can promote a slower, more oxidative muscle phenotype [51, 79]. A multitude of studies have shown that LFS mimics the electrical discharge pattern of slow motor neurons innervating slow muscles and

induces downstream signalling pathways that promote transcription of slower, more oxidative, fibre-specific genes [54, 67]. The resultant faster-to-slower adaptations include increased oxidative metabolism and mitochondrial biogenesis concurrent with fibre transitions in the type IIb→type IId/x→type IIa→type I direction, even within 14 d of daily LFS [42, 43]. While LFS challenges a muscle to its full adaptive potential, it does so efficiently and in the absence of injury and regeneration (Pette & Vrbova [53, 54, 71, 72]). Collectively, LFS is an ideal model for investigating the therapeutic potential of promoting a slower, more oxidative muscle phenotype to ameliorate the dystrophic pathology.

Electrical stimulation (especially LFS) to enhance muscle function in health and disease has been studied for nearly a century and remains an intervention with broad therapeutic relevance, for patients in the intensive care unit [86], with spinal cord injury [12, 46], cerebral palsy [73], sarcopenia [2] and as a supplement for sports training [41, 57].

From a clinical perspective, there was significant interest in LFS as a therapy for DMD during the late 1970s to early 1990s, but this waned as the field shifted to tackle the dystrophic pathophysiology through molecular biochemistry, especially after the discovery of dystrophin in 1987 [31]. These early studies conducted on DMD patients led by Vrbová, Dubowitz, Salmons, Zupan and colleagues were highly encouraging (including conferring a preservation of strength in some studies), but generally they were preliminary in nature (consisting of relatively few patients and usually of limited duration) with a resulting lack of scientific and statistical clarity [22, 83, 84, 101, 102]. There remains a dearth of information regarding the application of such a well-described and utilised intervention like LFS (with its current broad applications in rehabilitation medicine and physical therapy) for ameliorating the dystrophic pathology. Until recently, the only previous studies of LFS performed on dystrophic mice had not been conducted on mouse models of DMD [19, 97]. These studies had shown LFS to have beneficial effects on diseased (laminin-deficient) muscles of C57Bl/6J–dy2J (*dy/dy*) mice (a model of congenital muscular dystrophy). These effects were different from those observed in normal muscle and were not strictly relevant to DMD [97]. Only one study [41] has looked at short-term (2 week), 20 min/day LFS in dystrophic *mdx* mice, and this very preliminary proof-of-concept study was of too short a duration to have any therapeutic relevance for the dystrophic pathophysiology [41]. Longer-term studies evaluating the therapeutic merit of LFS have yet to be performed on the accepted mouse models of DMD nor have studies been undertaken to determine whether muscle wasting can be attenuated or reversed using different protocols of electrical stimulation. As to the therapeutic merit of LFS for DMD, my contention is that the jury is still out! Understanding how dystrophin-deficient muscles (in *mdx* and *dko* mice) adapt to LFS is critical for informing best clinical practice for any strategy that might be applied for DMD and related muscle conditions.

13.6 Pharmacologic Activation to Promote Slow Muscle Programming

There is considerable evidence that upregulating key proteins like calcineurin, PPARγ, PGC-1α, SIRT1 and AMPK (among others) can exert fast to slow changes within muscles, including in *mdx* mice. Ljubicic et al. [44] comprehensively and elegantly evaluated the merits of different pharmacologic and transgenic approaches to exert fast to slow phenotypic changes within dystrophic muscles. AMPK activation is among the best studied and effective approaches, with the AMPK activator, AICAR, conferring significant slow oxidative myogenic programming and improving the dystrophic pathology in *mdx* mice (Ljubicic et al. [42–44]. A critical discovery by Ljubicic et al. [43] revealed that prior pharmacologic conditioning with an AMPK activator was a salient determinant in how dystrophic muscles adapted to complementary, acute physiological stress stimuli, like treadmill running. Therefore, pharmacologic AMPK activation could potentially enhance the LFS-mediated favourable phenotypic adaptations in dystrophic muscles. Since DMD patients cannot exercise, one therapeutic option could be to combine AMPK activation with LFS, ideally to amplify the favourable effects of either intervention alone. LFS could confer 'exercise-like' contraction-mediated benefits on muscle fibres that pharmacological exercise mimetics are simply unable to elicit – producing not only local muscle effects but potentially amplifying systemic benefits. The therapeutic merit of this seemingly straightforward approach should be evaluated as a priority. There are other powerful and effective activators of AMPK signalling that could be employed to promote a slow, oxidative muscle phenotype that could potentially ameliorate the dystrophic pathology, and these are therapeutic targets for muscular dystrophy and related conditions.

It should be noted that adaptations within skeletal muscle fibres might differ between those mediated by contraction or pharmacologic stimuli. For example, physical activity or electrical stimulation may induce release of myokines from activated muscles that regulate mitochondrial biogenesis [21, 24, 91] through different mechanisms than what may be achieved through pharmacologic activation [35]. Load-bearing exercise may confer different cellular adaptations than pharmacologic activation. Although the loading on skeletal muscle fibres would be less during electrical stimulation than with physical activity, even that level of cellular (mechanical) stress could induce different adaptive programming than with pharmacologic activation. This issue remains speculative until hypotheses regarding comparative adaptations and plasticity are tested rigorously.

13.7 Slow Muscle Programming and Protecting Against Muscle Damage

Slow muscle programming may also confer protective effects within muscles that promote better functional outcomes after surgeries, especially where concomitant ischemia-reperfusion damage is unavoidable. This is relevant not just for DMD

patients who must undergo regular surgical procedures, but for millions of otherwise healthy patients worldwide who experience long-term disability and functional deficits after such traumatic surgeries. Muscle injury is a significant public health problem contributing to the large burden of musculoskeletal disability and suffering worldwide [27]. Muscles can be injured in many ways including ischaemia-reperfusion (I-R), contusion, crush, strains, laceration, extremes of temperature, chemical (myotoxic) and metabolic injury. While regeneration usually occurs spontaneously after damage, the process can be slow, incomplete and accompanied by fibrosis (scarring) that compromises the restoration of function. This is especially the case when the muscle blood supply has been occluded or when blood vessels, nerves, basal laminae and other supporting structures have been compromised.

I-R injury concomitant with tourniquet application is common during many surgeries, especially those involving muscle transfers and microsurgical procedures. I-R injury can also occur in muscles that have been crushed, with compartment syndromes, in limbs that have been broken or traumatised and with the replantation of amputated limbs. After injury induction, there is currently no effective treatment [25]. I-R occurs when the blood/oxygen supply to a muscle is occluded (ischemia) but later restored (reperfusion). Muscle fibres can be damaged in two ways during I-R: during the ischemia when blood flow is occluded or during reperfusion where free radical production accompanies a 'second wave' of injury [27]. This damage can impact negatively on the outcome of surgical interventions and so protecting muscles from I-R has the potential to improve tissue repair and enhance functional restoration. Antioxidants to attenuate I-R damage have largely proved unsuccessful [58] or produced only modest beneficial outcomes [6], and so novel and effective approaches that better protect muscles during these surgeries are needed.

The fact that slow muscles are better protected than fast muscles from I-R injury [98] provides the key rationale for advocating slow muscle programming to confer protection from this type of damage. Successful repair is vital for restoring mobility and patient quality of life, and there is an important medical need for therapies that can attenuate muscle damage, promote regeneration, reduce fibrosis and enhance function [27]. There has been renewed interest in LFS, primarily at a cellular and subcellular level, with demonstrated increases in stem cell proliferation, differentiation, matrix formation and migration, important for tissue regeneration [40]. The potential for LFS to stimulate regeneration remains a hypothesis worthy of rigorous testing in appropriate models of muscle health and disease. Furthermore, since it has been argued that AMPK activators could 'prime' muscle for complementary interventions, it is important to determine whether co-treatment of an AMPK activator with LFS might confer greater improvements in regeneration than either intervention alone. If such interventions hasten restoration of muscle function post-trauma, they could be rapidly applied in rehabilitation medicine to optimise recovery in a wide range of affected patients with muscle injuries.

13.8 Muscle Plasticity in the Other Direction – Are Slow-to-Fast Muscle Fibre Modifications Contraindicated in Muscular Dystrophy?

Although conferring fast-to-slow muscle fibre modifications has therapeutic relevance for muscular dystrophy, whether modifications in the opposite (i.e. slow-to-fast) direction exacerbate the dystrophic pathophysiology is not definitive. Another consideration is whether making muscles smaller (an adaptation with an intervention like chronic LFS) has greater protective effects for the dystrophic pathology than making muscles larger, such as with chronic administration of anabolic agents such as β-adrenoceptor agonists (β-agonists). Are there therapeutic interventions that preserve or increase muscle strength while simultaneously conferring fast-to-slow muscle fibre modifications in muscle phenotype?

Slow-to-fast muscle fibre modifications are possible through high-frequency electrical stimulation (HFS) as demonstrated in studies on rat skeletal muscle [29] and in studies using variable (often higher) frequency protocols for potential therapeutic and sports applications [3]. It is also well established that there are transcriptional activators or repressors that control genes that regulate or alter fibre composition (towards the fast, glycolytic myogenic programme) to ultimately affect muscle performance and metabolism [74]. These include RIP140, NCoR1, Ets-2 repressor factor (ERF), E2F1 and Baf60c and their roles in myogenic programming and skeletal muscle metabolism and phenotype have been reviewed in detail elsewhere [44].

Pharmacologic stimuli can also affect muscle fibre composition and muscle metabolism. Chronic administration of β-agonists to rats and mice can exert significant anabolic effects (increasing muscle mass through increases in muscle fibre cross-sectional area) and shift muscles from an oxidative to a more glycolytic phenotype, depending on the type of β-agonist, dose, mode of administration and duration of treatment [47, 69, 77, 100]. In some studies, chronic β-agonist (clenbuterol or formoterol) administration to rats transformed muscle fibre composition in the soleus muscle from predominantly slow-twitch to a more mixed fast and slow fibre composition, as well as increasing cross-sectional area of both of the main fibre types [78]. The implications of a shift in muscle phenotype from slow to fast, as well as an increase in muscle fibre size, are potentially significant for the aetiology of muscular dystrophy. Therapeutic strategies in DMD to increase muscle mass may well produce larger and stronger muscle fibres, but are they contraindicated by increasing muscle susceptibility to contraction-induced injury and so aggravating the dystrophic pathology? Large, fast type II fibres produce higher forces than smaller, slow type I fibres and can be more susceptible to damage after lengthening contractions [45, 48]. Fast muscle fibres are preferentially affected in DMD [16, 66, 99], whereas smaller calibre fibres are relatively spared in DMD and in animal models of muscular dystrophy [32, 33]. But the relationship between muscle fibre size and susceptibility to damage in muscular dystrophy is not always

clear. In one study, tibialis anterior muscles of *mdx* mice were not more susceptible to contraction-induced injury if the mice had been treated with formoterol (100 μM for 4 weeks). In fact, despite formoterol treatment increasing muscle mass and force production, the cumulative force deficit was actually lower in TA muscles of treated than untreated *mdx* mice [26]. This relatively low dose of formoterol did not change fibre type or oxidative capacity (i.e. no slow-to-fast fibre changes) but was sufficient to elicit a hypertrophic response in type IIb fibres that conferred protection from contraction-mediated injury [26]. These findings support the contention that anabolic agents also have therapeutic potential for DMD and related conditions.

13.9 Inhibiting Myostatin Signalling

Are there therapeutic interventions that preserve or increase muscle strength while simultaneously conferring fast-to-slow muscle fibre modifications in muscle phenotype? Such an attractive combination of phenotypic changes has therapeutic relevance for muscle wasting disorders including DMD. One approach that can confer these effects is myostatin inhibition. Myostatin, originally termed growth and differentiation factor-8 (GDF-8), is a member of the transforming growth factor-β (TGF-β) superfamily. Described as a negative regulator of skeletal muscle mass because it inhibits myoblast proliferation and differentiation [38, 55], inhibiting myostatin through genetic deletion or pharmacologic inactivation increases muscle mass and strength [39]. In a series of studies examining the therapeutic applications of myostatin inhibition, our laboratory showed that the myostatin inhibitory antibody PF-354 (developed by Pfizer Inc.) conferred favourable effects in mouse models of cancer cachexia, muscular dystrophy (*mdx*), aging (sarcopenia) and disuse atrophy with plaster casting (Murphy et al. [60–63]). In the Lewis lung carcinoma (LLC) mouse model of cancer cachexia, PF-354 attenuated muscle atrophy and loss of force production with improvements in muscle mass and fatigue (force during repeated stimulation of tibialis anterior muscles in situ), accompanied by increases in succinate dehydrogenase (SDH) activity and the proportion of oxidative muscle fibres [62]. PF-354 conferred similar improvements in these parameters in aged mice [61] and improved diaphragm structure-function in young *mdx* mice [60] and in mice with unilateral plaster caster casting PF-354 attenuated muscle atrophy and loss of force [63]. There is still much to be learned regarding the therapeutic potential of this and similar approaches for manipulating myostatin/activin signalling in skeletal muscle. Conferring changes in muscle phenotype (fast-to-slow) to improve muscle fatigue resistance while increasing muscle mass and strength is an attractive combination of effects relevant to multiple muscle wasting conditions, especially DMD. There is still considerable interest in developing novel strategies to manipulate TGF-β signalling for therapeutic application in skeletal muscle conditions [36].

13.10 Conclusion

Altering muscle phenotype can have dramatic effects on skeletal muscle structure, function and metabolism. Muscle plasticity can be achieved through various means including physical activity, electrical stimulation and pharmacologic activation, and each approach has potential therapeutic merit for muscular dystrophy. Better understanding the mechanisms underlying skeletal muscle adaptations to different interventions and stimuli will help optimise novel strategies to address the pathophysiology of DMD and related muscle conditions.

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