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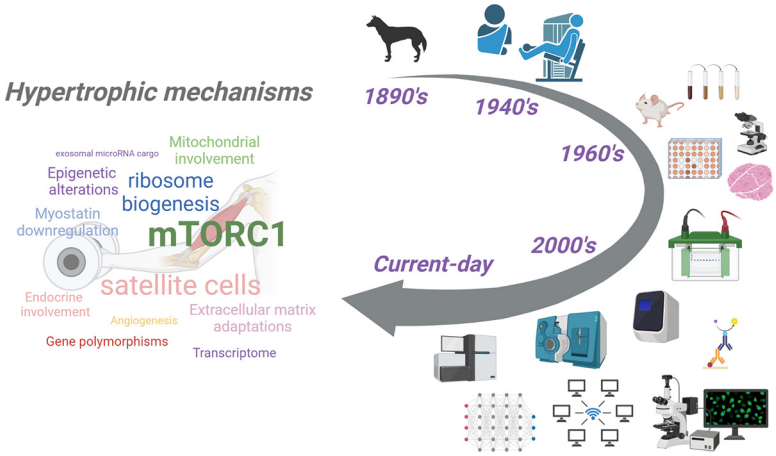
REVIEW ARTICLE

MECHANISMS OF MECHANICAL OVERLOAD-INDUCED SKELETAL MUSCLE HYPERTROPHY: CURRENT UNDERSTANDING AND FUTURE DIRECTIONS



AUTHORS

Michael D. Roberts, John J. McCarthy, Troy A. Hornberger, Stuart M. Phillips, Abigail L. Mackey, Gustavo A. Nader, Marni D. Boppart, Andreas N. Kavazis, Paul T. Reidy, Riki Ogasawara, Cleiton A. Libardi, Carlos Ugrinowitsch, Frank W. Booth, Karyn A. Esser

CORRESPONDENCE

mdr0024@auburn.edu; kaesser@ufl.edu

KEY WORDS

hypertrophy; mechanical overload; myofiber; resistance training; skeletal muscle

CLINICAL HIGHLIGHTS

- Loss of muscle mass with aging and certain noncommunicable diseases (e.g., cancer, COPD, and others) is associated with increased mortality. Thus, understanding the mechanisms controlling skeletal muscle hypertrophy can help determine the most effective interventions to preserve or enhance muscle mass.
- Studies in animals and humans suggest that mechanical overload (e.g., resistance training) best achieves skeletal muscle hypertrophy. Bouts of mechanical overload induce transient increases in mammalian/mechanistic target of rapamycin complex 1 (mTORC1) signaling leading to elevations in muscle protein synthesis rates. With repeated bouts of mechanical overload, these events contribute to skeletal muscle hypertrophy.
- An expansion in translational capacity through ribosome biogenesis and increases in satellite cell abundance and myonuclear accretion also contribute to skeletal muscle hypertrophy following days to weeks of repeated mechanical overload bouts.
- Aside from these three aforementioned mechanisms, several lines of past, current, and emerging research suggest that other mechanisms may also contribute to mechanical overload-induced skeletal muscle hypertrophy (e.g., mTORC1-independent signaling, microRNAs, genetic polymorphisms, and enhanced angiogenesis among others).
- There are also potential manners in which epigenetic alterations in myonuclear and mitochondrial DNA, extracellular matrix remodeling, cytoskeletal remodeling, mitochondrial biogenesis, bioenergetic adaptations, and other mechanisms can contribute to mechanical overload-induced skeletal muscle hypertrophy.
- The current and rapidly emerging molecular tools available to researchers as well as rodent and human studies being performed in tandem will continue to provide insight into novel mechanisms that are needed for mechanical overload-induced skeletal muscle hypertrophy to occur.

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REVIEW ARTICLE

MECHANISMS OF MECHANICAL OVERLOAD-INDUCED SKELETAL MUSCLE HYPERTROPHY: CURRENT UNDERSTANDING AND FUTURE DIRECTIONS

Michael D. Roberts,[1] John J. McCarthy,[2] Troy A. Hornberger,[3] Stuart M. Phillips,[4] Abigail L. Mackey,[5] Gustavo A. Nader,[6] Marni D. Boppart,[7] Andreas N. Kavazis,[1] Paul T. Reidy,[8] Riki Ogasawara,[9] Cleiton A. Libardi,[10] Carlos Ugrinowitsch,[11] Frank W. Booth,[12] and Karyn A. Esser[13]

[1]School of Kinesiology, Auburn University, Auburn, Alabama, United States; [2]Department of Physiology, College of Medicine, University of Kentucky, Lexington, Kentucky, United States; [3]Department of Comparative Biosciences, University of Wisconsin-Madison, Madison, Wisconsin, United States; [4]Department of Kinesiology, McMaster University, Hamilton, Ontario, Canada; [5]Institute of Sports Medicine Copenhagen, Department of Orthopedic Surgery, Copenhagen University Hospital-Bispebjerg and Frederiksberg, and Department of Clinical Medicine, University of Copenhagen, Copenhagen, Denmark; [6]Department of Kinesiology and Huck Institutes of the Life Sciences, The Pennsylvania State University, University Park, Pennsylvania, United States; [7]Department of Kinesiology and Community Health, University of Illinois at Urbana-Champaign, Urbana, Illinois, United States; [8]Department of Kinesiology, Nutrition and Health, Miami University, Oxford, Ohio, United States; [9]Healthy Food Science Research Group, Cellular and Molecular Biotechnology Research Institute, National Institute of Advanced Industrial Science and Technology (AIST), Tsukuba, Japan; [10]MUSCULAB-Laboratory of Neuromuscular Adaptations to Resistance Training, Department of Physical Education, Federal University of São Carlos, São Carlos, Brazil; [11]School of Physical Education and Sport, University of São Paulo, São Paulo, Brazil; [12]Department of Biomedical Sciences, University of Missouri, Columbia, Missouri, United States; and [13]Department of Physiology and Aging, College of Medicine, University of Florida, Gainesville, Florida, United States

Abstract

Mechanisms underlying mechanical overload-induced skeletal muscle hypertrophy have been extensively researched since the landmark report by Morpurgo (1897) of "work-induced hypertrophy" in dogs that were treadmill trained. Much of the preclinical rodent and human resistance training research to date supports that involved mechanisms include enhanced mammalian/mechanistic target of rapamycin complex 1 (mTORC1) signaling, an expansion in translational capacity through ribosome biogenesis, increased satellite cell abundance and myonuclear accretion, and postexercise elevations in muscle protein synthesis rates. However, several lines of past and emerging evidence suggest that additional mechanisms that feed into or are independent of these processes are also involved. This review first provides a historical account of how mechanistic research into skeletal muscle hypertrophy has progressed. A comprehensive list of mechanisms associated with skeletal muscle hypertrophy is then outlined, and areas of disagreement involving these mechanisms are presented. Finally, future research directions involving many of the discussed mechanisms are proposed.

hypertrophy; mechanical overload; myofiber; resistance training; skeletal muscle

1. INTRODUCTION

Hypertrophy (hy·per·tro·phy)

/hāˈpərtrəfē/

Noun PHYSIOLOGY

Definition: Increase in the size of a tissue or organ as a result of an increase in cell size rather than increased numbers of cells (hyperplasia).

Source: *Oxford Dictionary of Sports Science and Medicine*

Skeletal muscle hypertrophy occurs in response to various loading paradigms over prolonged periods, and these stimuli have been deemed as providing "mechanical overload" to the involved musculature. There are various methods to achieve mechanical overload in animals including the surgical removal of synergist muscles (i.e., synergist ablation), simulated resistance training through electrical hindlimb stimulation, loaded wheel running,

CLINICAL HIGHLIGHTS

- Loss of muscle mass with aging and certain noncommunicable diseases (e.g., cancer, COPD, and others) is associated with increased mortality. Thus, understanding the mechanisms controlling skeletal muscle hypertrophy can help determine the most effective interventions to preserve or enhance muscle mass.
- Studies in animals and humans suggest that mechanical overload (e.g., resistance training) best achieves skeletal muscle hypertrophy. Bouts of mechanical overload induce transient increases in mammalian/mechanistic target of rapamycin complex 1 (mTORC1) signaling leading to elevations in muscle protein synthesis rates. With repeated bouts of mechanical overload, these events contribute to skeletal muscle hypertrophy.
- An expansion in translational capacity through ribosome biogenesis and increases in satellite cell abundance and myonuclear accretion also contribute to skeletal muscle hypertrophy following days to weeks of repeated mechanical overload bouts.
- Aside from these three aforementioned mechanisms, several lines of past, current, and emerging research suggest that other mechanisms may also contribute to mechanical overload-induced skeletal muscle hypertrophy (e.g., mTORC1-independent signaling, microRNAs, genetic polymorphisms, and enhanced angiogenesis among others).
- There are also potential manners in which epigenetic alterations in myonuclear and mitochondrial DNA, extracellular matrix remodeling, cytoskeletal remodeling, mitochondrial biogenesis, bioenergetic adaptations, and other mechanisms can contribute to mechanical overload-induced skeletal muscle hypertrophy.
- The current and rapidly emerging molecular tools available to researchers as well as rodent and human studies being performed in tandem will continue to provide insight into novel mechanisms that are needed for mechanical overload-induced skeletal muscle hypertrophy to occur.

weighted ladder climbing, weighted sled pulling, weighted limb stretches, and resistance-loaded devices to challenge animals as they obtain food. Mechanical overload in humans is most adequately achieved through progressive resistance training. When performed consistently, resistance training over an 8- to 16-wk period can lead to a 5–20% increase in skeletal muscle volume or mass in younger to middle-aged adults (1).

Over the past 40 years researchers have sought to identify the mechanisms that are associated with mechanical overload-induced skeletal muscle hypertrophy. These investigations have led to a plethora of comprehensive reviews on this topic (2–52), and the word cloud sizing in the graphical abstract is representative of these viewpoints.

Although sections of the present review rearticulate excellent perspectives from these reviews, the broader aims here are to *1*) provide a historical perspective of the early discoveries in the field (~late 1800s to 1940s), discuss animal and human studies from the 1960s to 2000s that were the first to mechanistically interrogate mechanical overload-induced skeletal muscle hypertrophy, and highlight how these studies have guided current-day research efforts; *2*) discuss highly investigated mechanisms that are thought to promote skeletal muscle hypertrophy, as well as opposing evidence when applicable; and *3*) posit mechanisms that may be involved in promoting skeletal muscle hypertrophy but have little to no evidence and warrant further investigation. Other sections of the present review include discussions on skeletal muscle architecture, methodological considerations with skeletal muscle hypertrophy research, and an abbreviated discussion focused on how sex, race, and aging affect hypertrophic outcomes.

2. BRIEF OVERVIEW: MUSCLE AS TISSUE, MYOFIBERS, THE EXTRACELLULAR MATRIX, AND OTHER RESIDENT CELLS

Mammalian muscle cells (a.k.a. myofibers) are typically considered postmitotic (or nondividing) and possess a unique structure in that they are multinucleated and the largest mammalian cells with a tubular morphology (53). It has been posited that myofibers require multiple nuclei to regulate cellular homeostasis (i.e., the myonuclear domain theory) (51, 54). Most of the intracellular area in myofibers is occupied by myofibrils (~70–85%) (55–57), and these specialized organelles consist of thick filaments, thin filaments, and other associated proteins. These proteins contribute to active and passive force generation as well as sarcomere structure for muscle contraction. A cytoskeletal network is also present within myofibers and consists of actin, microtubules, microfilaments, and other associated proteins that anchor nuclei and myofibrils within the cell, while also serving as a scaffold for force transmission (58). Proteins enriched in myofibers have been subclassified into different categories including (59) *1*) contractile proteins (e.g., myosin, actin, tropomyosin, troponins), *2*) sarcomeric-associated proteins (e.g., titin, myosin binding protein C, α-actinin, myomesin, and M protein), *3*) cytoskeletal proteins (e.g., tubulin, desmin, and actin), and *4*) membrane-associated proteins (e.g., dystrophin, spectrin, talin, vinculin, and ankyrin). Although the ultrastructural characteristics of myofibers have been largely limited to two-dimensional analyses, the Glancy laboratory (60) has recently used three-dimensional ion beam scanning electron microscopy (FIB-SEM) to show that myofibers contain interconnected myofibrils whereby branching is higher in slow-twitch versus fast-twitch myofibers in adult mice.

Some of the intracellular space within myofibers (~5–10%) is also occupied by the mitochondrial reticulum and sarcoplasmic reticulum, and these organelles are primarily responsible for supporting muscle contraction through adenosine triphosphate (ATP) replenishment

and calcium handling, respectively (61). Again, the Glancy laboratory (62) has also provided excellent insight into muscle mitochondrial structure, using FIB-SEM to develop the hypothesis that membrane potential conduction is the prominent pathway for skeletal muscle energy distribution. The cytoplasm (a.k.a. sarcoplasm) is an aqueous medium that facilitates the exchange of ions and metabolites to and from different organelles (8). Several enzymes, proteins, and protein complexes that facilitate anabolic and catabolic reactions also reside in the sarcoplasm. The cell membrane of myofibers is termed the sarcolemma, and this structure contains transmembrane proteins that aid in ion transport, nutrient transport, ligand-receptor signaling, and the anchoring of intracellular cytoskeletal proteins to the extracellular matrix (8). The latter of these functions is responsible for force transmission during muscle contraction from the Z disk to the basal lamina via the dystrophin-glycoprotein and integrin adhesion complexes (63).

The basal lamina is a thin layer of connective tissue that sheaths myofibers, is linked to the sarcolemma through protein-protein interactions, and is considered part of the extracellular matrix (64). Proteins enriched in the basal lamina include fibronectin, laminin, α-dystroglycan, and other proteins that participate in the sarcolemmal protein-protein interactions discussed above (65). The thick fibrillar extracellular matrix (a.k.a. the interstitial matrix) is mainly comprised of collagen proteins and various extracellular growth factors (65). In muscle tissue, a variety of cell types reside in the extracellular matrix and include resident immune cells, fibro-adipogenic progenitor cells, fibroblasts, adipocytes, endothelial cells, and pericytes (66). At the interface between the sarcolemma of myofibers and the basal lamina are satellite cells. Microscopic evidence supports that myofibers, rather than stromal cells of the extracellular matrix, spatially occupy ∼85–90% of muscle tissue (67). This is largely due to myofiber cross-sectional areas in adults typically averaging between 5,000 and 6,000 μm^2 and the cell bodies of stromal cells only being slightly larger than their nuclei, which (although not commonly measured) average to be $<100\ \mu m^2$ (68, 69). However, it should be noted that the extracellular matrix contains numerous stromal cell types that outnumber the presence of myofibers. In this regard, histological examinations of muscles from young healthy adults suggest that per 100 myofibers there are ∼10 satellite cells (70), ∼2 lymphocytes and ∼20 macrophages (71, 72), ∼30 fibro-adipogenic progenitor cells (73), ∼13 fibroblasts (74), ∼35 pericytes (75), and ∼200 capillaries, which are comprised of endothelial cells (76, 77). Thus, cells residing in the extracellular matrix potentially outnumber myofibers at a ratio of ∼3:1, and this estimate does not consider age-related changes or the influx of cells into muscle tissue following exercise bouts or injury (e.g., neutrophils and macrophages) (71, 78).

Single-nucleus sequencing studies in rodents have recently provided estimates of the cell types contained in muscle tissue (66, 79). These investigations suggest that of the total nuclear pool in skeletal muscle tissue ∼50–70% are associated with myofibers, 20% are from fibro-adipogenic progenitor cells, 17% are from endothelial cells, 4% are from pericytes, 3% are neuronal, 3% are from macrophages, 2% are from satellite cells, and 1% are from neutrophils. Notably, these estimates are not constant, since some reports suggest that slow-twitch myofibers contain more nuclei per fiber than fast-twitch fibers (80, 81), and immune cell abundance is also higher in slow-twitch versus fast-twitch muscles (82). **FIGURE 1** provides a summary diagram of content discussed in this section of the review, and readers are referred to other recent reviews providing related schematics (1, 48, 58, 65, 83–85).

A final topic of discussion in this section is myofiber type classification and some of the characteristic differences that exist between myofiber types. As described by Schiaffino and Reggiani (86), mammalian skeletal muscle contains different myofiber types that can be differentiated by either myosin isoforms and contraction speed or metabolic characteristics such as oxidative capacity. Whereas the authors explained the history of past methods used for fiber type classification (e.g., red vs. white appearance and histological classifications using succinate dehydrogenase and myosin ATPase staining), monoclonal antibodies against different myosin heavy chain isoforms developed in the 1980s have been widely used via immunohistochemistry to report myofiber type adaptations to mechanical overload (87). The four predominant mammalian myosin heavy chain isoforms include the slow-twitch type I isoform (encoded by the *MYH7* gene) and fast-twitch isoforms including IIA (encoded by the *MYH2* gene), IIX (encoded by the *MYH1* gene), and IIB (encoded by the *MYH4* gene) (86). Although most fibers express a prominent myosin heavy chain isoform, hybrid myofibers coexpressing multiple isoforms in humans have been reported with histochemical and electrophoresis-based techniques (88–93). Notwithstanding, several studies suggest that the commonly biopsied vastus lateralis (VL) muscle in men and women contains a high percentage of type I myofibers (∼30–50%) and type IIA + IIA/X hybrid fibers (∼40–50%) and ∼5% of I/II-coexpressing hybrid myofibers and ∼2% of type IIX myofibers (88, 91, 94, 95). Moreover, a common adaptation to resistance training (and endurance training) in humans is the rapid downregulation of IIX gene expression and shift of IIX + IIA/X to IIA myofibers (96, 97).

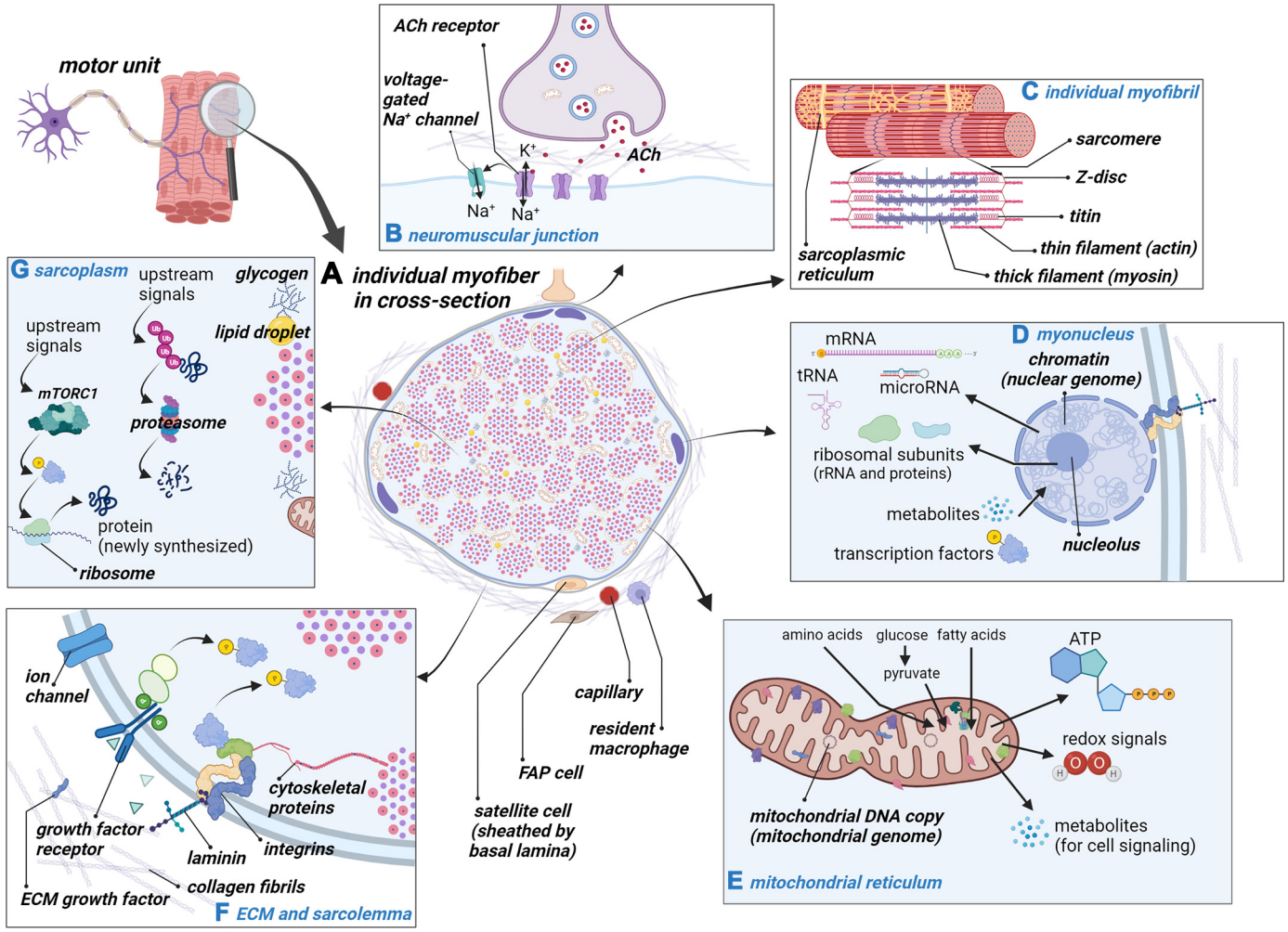

FIGURE 1. Skeletal muscle fiber components and biological processes. This schematic (constructed with BioRender.com, with permission) illustrates the molecular attributes and processes that occur in a myofiber. *A* represents an individual myofiber in cross section as well as some of the stromal cells that exist in the extracellular matrix. *B* depicts the neuromuscular junction and how the ligand binding of acetylcholine (ACh) can lead to myofiber activation through voltage-gated sodium (Na^+) channels. *C* shows an individual myofibril and some of the prominent proteins that make up the structure of the sarcomere. *D* shows a single myonucleus, some of its key structures (e.g., chromatin), and some of its functions (e.g., RNA transcription and output). *E* depicts a portion of the mitochondrial reticulum, some of its key structures (e.g., mitochondrial DNA), and some of its key functions (e.g., producing ATP and metabolites). *F* shows the interface of the extracellular matrix (ECM) and muscle cell membrane (or sarcolemma), and signaling through growth factor receptors and laminin-integrin complexes are also summarized. *G* shows a portion of the sarcoplasm (which makes up <10% of the myofiber spatially), some of the features between myofibrils (e.g., glycogen granules and lipid droplets), and some of the many reactions that can occur in this region (note that protein synthesis can also occur at ribosomes localized in close proximity to myofibrils). FAP, fibro-adipogenic progenitor cell; K^+, potassium; mTORC1, mammalian target of rapamycin complex 1.

Intracellular morphology may differ between type I and IIA/X myofibers, albeit this may depend on species. Beyond myosin typing, the characterization of metabolic features within muscle fibers gets more complex (93), so again it is important to consider the muscle analyzed and species. In rats, Schiaffino et al. (98) used transmission electron microscopy (TEM) to report that slow-twitch soleus myofibers (which possess >90% type I fibers) contain more mitochondria in cross section relative to extensor digitorum longus (EDL) myofibers (which possess >90% type II fibers). These authors also reported that myofibril diameters are larger in fast-twitch EDL myofibers, although Z disks are slightly thicker, and sarcomeres are slightly longer in slow-twitch soleus myofibers. These myofiber type morphology differences in rats are not as dramatic in humans. For instance, Alway and colleagues (57) reported that type I and II myofibers from gastrocnemius and soleus biopsies in men spatially possess ~5% and ~3% mitochondria, respectively, whereas myofibril area occupies similar intracellular spacing in both fiber types (~80%). Ruple et al. (55) more recently used immunohistochemistry to report that type I and II myofibers from the VL muscle in men spatially possess ~5–6% mitochondria, whereas myofibril area occupied similar intracellular spacing in both fiber types (~80%), and this largely agrees with a

prior study by Wang et al. (99), who used TEM to interrogate type I and II myofiber characteristics from VL muscle tissue in 12 women. Also notable are the data from Wang and colleagues suggesting that myofibril size (~0.70 μm^2) and density (1.06 myofibrils per μm^2) are similar in type I and IIA myofibers in humans. Interestingly, recent protein expression profiling between type I and II myofibers in humans indicates that, of the ~3,800 proteins detected, ~400 (or 10%) show significant fiber type-specific differences. Hence, these proteome profiles between type I and II myofibers, rather than robust morphology differences, likely drive the divergence in cellular phenotypes (i.e., oxidative potential, force generation, and excitation-contraction coupling characteristics) (100).

3. A HISTORICAL ACCOUNT OF RESISTANCE TRAINING RESEARCH, MECHANICAL OVERLOAD STUDIES IN RODENTS, AND THE MOLECULAR INTERROGATION OF MUSCLE HYPERTROPHY

Research into skeletal muscle hypertrophy has flourished over the past 50 years. However, a general interest in this topic has existed for several millennia. Milo of Croton (~6th century B.C.) is considered by most to be the first documented practitioner of progressive resistance training. According to anecdote and written history, Milo hauled a newborn calf (which developed into a full-grown bull) over his shoulders daily for nearly 4 years, leading to enhancements in muscle mass and strength (101). Despite the clear implications of progressive overload, scientists would not intensively research resistance training for another two and a half millennia. Much of the current mainstream interest in resistance training and skeletal muscle hypertrophy was largely driven by Eugen Sandow (102), a Prussian bodybuilder and showman (~1890s), and Canadian Louis Cyr, who was an avid weightlifter and strongman (~1880s to 1890s) (103).

The notion that tissue could grow via cellular hypertrophy can be traced back to classic work of the German pathologist Rudolf Virchow. In 1858, Virchow (104) published a study detailing the morphology of lymph nodes through the use of microscopy. Virchow reported greater cell counts in enlarged versus normal-appearing lymph nodes and reported that other organs could grow without increasing cell number. These observations led to the current-day definitions of "hyperplasia" and "hypertrophy" and inspired Morpurgo's landmark study published in 1897 showing that skeletal muscle hypertrophy occurs in response to exercise training (105). Interestingly, Morpurgo reported that 2 mo of treadmill training increased sartorius myofiber diameters by ~50% without increasing myofiber number or length, and he termed this phenomenon "work-induced hypertrophy." This study was the first scientific documentation of skeletal muscle hypertrophy in response to exercise training. Nevertheless, research from the early 1900s to 1930s that examined work-induced hypertrophy neglected skeletal muscle in favor of cardiac muscle adaptations (reviewed in Ref. 106), with these studies utilizing regimens that were endurance training by today's standards.

Scientific writings on progressive resistance training surfaced around the time of Morpurgo's work. Wilhelm Roux and Willi Lange authored perspectives between 1895–1917 suggesting that skeletal muscle hypertrophy occurred when bouts of work intensity routinely exceeded that which was performed during normal daily activities (107). Dr. Theodor Hettinger, a research fellow at the Max Planck Institute from 1950 to 1960, attributed the beginnings of resistance training research to studies published by Petow, Siebert, and Eyster between 1925 and 1927 that documented the strength adaptations to weightlifting (107). Viewpoints on the hypertrophic and strength adaptations to resistance training were also provided in a 1933 commentary by Steinhaus (108) and in MacFadden's 1940 *Encyclopedia of Health and Physical Education*. In the mid-1930s through the 1940s entrepreneurs Bob Hoffman (founder of York Barbell Company) and Joe Weider (founder of the International Federation of Bodybuilding as well as several mainstream fitness magazines) largely stoked public interest in resistance training (109). Skeletal muscle hypertrophy research was also published in the late 1940s and early 1950s (110–112), albeit these observational human studies examined masseter muscle hypertrophy due to excessive chewing or clinching of the jaws under stressful conditions. As written by Bompa and Haff (113), theories regarding recovery and adaptation from exercise training were also published in the 1940s by Folbrot. The triphasic stress response termed the "general adaptation syndrome" (or "GAS") by Hans Selye (114) was published in this same era, and the exercise physiology field has largely adopted this model to explain the stress response to mechanical overload.

In 1945, US Army Captain Dr. Thomas Delorme (115) reported that progressive resistance training promoted skeletal muscle hypertrophy and a restoration of muscle function in rehabilitating soldiers. This publication led to a flurry of human research into how resistance training affected strength and local muscle endurance in diseased and rehabilitating patients (116–119). Around the same time (circa 1949), Novikov and Ozolin published papers detailing the implementation of complex training methods (i.e., strength and endurance training) (120). Although these papers were informative, there would be a 25-year lapse between these reports and mechanistic

human investigations since skeletal muscle biopsies were not widely adopted in the research setting until the late 1960s.

Although sparse research in the 1950s and early 1960s utilized exercise paradigms in livestock to examine meat quality outcomes (121, 122), Geoffrey Goldspink (1964) (123) published the first mechanistic interrogation of mechanical overload-induced skeletal muscle hypertrophy. The author indicated that myofiber and myofibril diameters are generally larger in mice trained with a resistance-loaded pulley apparatus versus untrained mice, albeit it is unclear how this interpretation was formulated given that the light microscopy that was utilized did not provide clear resolution of myofibrils. Notwithstanding, this publication, in part, led to a widely adopted mindset in the field that resistance training increases myofiber hypertrophy through increasing myofibril size rather than number. Three years after Goldspink's report, Goldberg (124) used tenotomy to elicit hindlimb skeletal muscle hypertrophy in rats (i.e., functional overload). This technique was later refined in mice (125), and these seminal investigations led to the widespread utilization of the synergist ablation model to study hypertrophic mechanisms. The synergist ablation model surgically excises a portion of the gastrocnemius muscle, which imposes continuous mechanical overload on the remaining muscle(s). Numerous rodent studies have utilized various forms of synergist ablation (126), and although it is generally viewed as a nonphysiological form of rapid hypertrophy, these studies were foundational for many of the anabolic signaling mechanisms that are discussed here. Interestingly, a 1975 review by Goldberg and colleagues (127) summarizing findings of studies that utilized these models indicated that overload-induced skeletal muscle hypertrophy was largely independent of endocrine factors (e.g., growth hormone, insulin, testosterone, and thyroid hormones). This viewpoint has since been supported by research suggesting that intrinsic signaling mechanisms, such as mechanotransduction-based signaling, are prominently responsible for mechanical overload-induced skeletal muscle hypertrophy (128, 129).

In the 1960s, researchers utilized biochemical assays to determine how postnatal muscle growth affects tissue protein, deoxyribonucleic acid (DNA), and ribonucleic acid (RNA) concentrations in animals (130, 131). First-generation tracer studies were performed using "hot" radioisotopes in isolated muscles and cell-free preparations in the late 1950s and early 1960s to determine the fate of amino acids exposed to cellular environments (132, 133). These studies inspired subsequent research in the late 1960s and early 1970s by Goldberg, Millward, and others, who administered radioisotope tracers to live rodents to determine muscle protein, DNA, and RNA synthesis rates during different loading paradigms (134–137). The collective evidence from these studies supported that mechanical overload increased the synthesis rates of these macromolecules. Human research examining skeletal muscle tissue adaptations to resistance training also surfaced in this same decade because of skeletal muscle biopsy sampling, pioneered for research purposes by Jonas Bergstrom in 1962 (138). Penman (1969) (139) provided the first report in humans that used TEM to describe the ultrastructural myofiber adaptations in response to 8 wk of knee extensor resistance training. In 1970, Penman (140) published a similar report with three college-aged male participants who underwent 10 wk of resistance training. Although Penman's investigations were limited in scope, his scientific approach of obtaining skeletal muscle tissue from humans who performed resistance training was soon adopted by other scientists in the field.

In the late 1960s and early 1970s, histological staining methods were used to characterize the metabolic phenotypes of slow- and fast-twitch myofibers. Animal work by Ogata and Mori (141), Edgerton et al. (142), Barnard et al. (143), and Brooke and Kaiser (144) and human work by Edström and Nyström (145) were foundational in establishing several of these techniques. A 1972 paper by Gollnick et al. (146) built upon Penman's previous work given that it was adequately powered from a statistical perspective to compare fiber type characteristics between weightlifters, endurance-trained athletes, and untrained participants. The investigators subjected muscle sections to specialized reagents to assess glycogen content, myosin ATPase activity (for myofiber type), succinate dehydrogenase and reduced diphosphopyridine nucleotide-diaphorase (DPNH-diaphorase) activity (for oxidative capacity), and phosphofructokinase activity (for glycolytic capacity). Compared with the other participants, weightlifters were reported to possess larger myofibers as well as a lower percentage of oxidative myofibers. In 1973, Schiaffino and Bormioli (147) utilized similar histological techniques to support that synergist ablation in rats elicits myofiber growth accompanied by a shift toward a more oxidative phenotype. Additional work performed by Schiaffino and colleagues (148) in the early 1970s, which involved [^{3}H]thymidine administration and TEM-based autoradiography, indicated that satellite cell proliferation occurs days after mechanical overload induced by synergist ablation in rats. These authors published a separate report supporting the idea that satellite cells become incorporated into the myofibers as myonuclei (149). These studies provided the first evidence that satellite cells have a role in mechanical overload-induced skeletal muscle hypertrophy and inspired work in this area of muscle biology described below in this review.

The first study to formally assess whether myofiber hypertrophy occurred in humans with resistance training was published by Thorstensson and colleagues (150) in 1976. Although it was reported that 8 wk of resistance training increased strength outcomes, slow- and fast-twitch myofiber cross-sectional area (fCSA) values were not significantly altered. The authors hypothesized that the duration of training was not long enough to observe the myofiber hypertrophy reported in the weightlifters that Gollnick and colleagues examined (146). Contrary to this report, 1979 reports by Dons et al. (151) and Costill et al. (152) indicated that myofiber hypertrophy occurred after 7 wk of resistance training. That same year, Moritani and deVries (153) published a landmark electromyography paper indicating that neural factors accounted for the initial strength gains during the first few weeks of resistance training. The authors also posited that muscle hypertrophy (as assessed through limb circumference measurements) became a more influential factor for continued strength gains thereafter. MacDougall et al. (154) published a paper in 1980 showing that 6 mo of resistance training increased slow- and fast-twitch fCSA values, and the authors utilized TEM to support a mechanism of myofiber growth primarily occurring through the expansion of the sarcoplasmic space. Although these findings were provocative, several studies published in later years would challenge this mode of hypertrophy (1, 155), and this area of the literature remains controversial. In the 1980s, several research groups continued to detail the histological, biochemical, and ultrastructural differences of biopsied muscle between weightlifters and nonweightlifting participants (56, 57, 156–162). Research by Staron, Hikida, Dudley, Kraemer, Gonyea, and others in the 1990s documented how weeks to months of resistance training in previously untrained participants affected fCSA as assessed by the myosin ATPase staining technique (99, 163–166). Research by Tesch, Costill, and associated colleagues, which employed biochemical assays and other staining techniques, was also published in this same era detailing metabolic adaptations in muscle tissue following months of resistance training (152, 167, 168). Although hyperplasia has been largely dismissed as a significant contributor to mammalian skeletal muscle hypertrophy (1), it is notable that the Gonyea laboratory performed experiments on this topic during the 1980s and 1990s by chronically stretching and loading the anterior latissimus dorsi muscle in quails (169–172) or performing various forms of resistance training in cats (173, 174). Indeed, much of the resistance training research performed during the 1970s and 1980s provided information on myofiber size and metabolic adaptations. However, aside from sparse TEM reports by MacDougall and colleagues and others through the early 1990s (56, 67, 99, 175), researchers have since largely neglected examination of the ultrastructural adaptations that occur in myofibers in response to mechanical overload.

Seminal molecular work in the late 1980s, the 1990s, and the early 2000s led to a research breakthrough focused on mechanisms associated with skeletal muscle hypertrophy. One line of research spanning from the early to late 1990s involved infusing stable isotope tracers into human participants to assess the muscle protein synthesis and breakdown kinetics to single or multiple bouts of resistance exercise (176–180). The collective evidence from these studies indicated that changes in muscle protein synthetic and breakdown rates were significantly elevated for hours to days after resistance exercise bouts. These findings led to the widely adopted hypothesis that resistance training facilitates myofiber hypertrophy through intracellular protein accretion and that this process is largely driven by pulsatile postexercise increases in muscle protein synthesis that eventually supersede muscle protein breakdown rates as individuals become more trained (181). This initial human tracer research was innovative and foundational in establishing the more accessible and less invasive methodology of administering deuterium oxide (D_2O) to humans and rodents through drinking water to assess longer-term (or integrated) mixed-muscle or myofibrillar protein synthesis rates (182, 183); notably, these reports provide cumulative protein synthetic responses ranging from days to months into training interventions. What is less appreciated, however, is that the rodent radioisotope tracer work discussed above in this section largely inspired this line of research in humans.

Another line of innovative molecular research during this era involved the elucidation of skeletal muscle mRNA and phosphosignaling responses to mechanical loading, and again studies in rodents predated human investigations. In 1990, the Booth laboratory was the first to document skeletal muscle RNA expression responses to acute and chronic mechanical overload. In short, these authors reported that one bout of concentric exercise via hindlimb muscle stimulation against load in rats did not affect relative expression levels of α-actin mRNA, cytochrome *c* mRNA, 18S rRNA, or 28S rRNA in the gastrocnemius muscle (184). However, 10 wk of training increased the overall abundance of these RNAs when considering training-induced increases in gastrocnemius masses. These authors published a second paper detailing the RNA responses that occurred in rat tibialis muscles that were eccentrically targeted during the acute and chronic stimulation models (185). This work largely guided subsequent research examining the acute and chronic RNA responses to mechanical overload and resistance training in rodents and humans, respectively. Additionally, this work inspired research

into how concentric- versus eccentric-only training affects the molecular milieu in skeletal muscle (186).

In 1999, Baar and Esser (187) published a landmark paper on muscle signaling responses to mechanical overload. In short, the authors used Western blotting to demonstrate that the phosphorylation status of the 70-kDa S6 protein kinase (p70S6K) protein 6 h after stimulated lengthening contractions was associated with the degree of muscle hypertrophy in various rat hindlimb muscles after 6 wk of training using the same stimulation protocol. This finding was confirmed by Nader and Esser (2001) (188), who reported that the prolonged increase in p70S6K and a transient increase in protein kinase B phosphorylation occur in response to a hypertrophy-inducing stimulus but not low-frequency stimulation or running exercise. Notably, studies in humans published 5–7 years later indicated that similar signaling responses occur in response to a resistance exercise bout (189, 190). In 2001, Bodine and colleagues (191) published a landmark study that built upon Baar and Esser's work showing that rapamycin, a mammalian/mechanistic target of rapamycin (mTOR) inhibitor, blunted plantaris hypertrophy following 14 days of mechanical overload induced by synergist ablation in rats. This response was also reported to coincide with the diminished phosphorylation of p70S6K, which is now appreciated as being a downstream kinase that is phosphorylated and activated by mammalian/mechanistic target of rapamycin complex 1 (mTORC1) (192). These findings were, in part, validated in humans 5 years later by the Rasmussen laboratory, who reported that rapamycin administration blocks the early (1–2 h after exercise) increases in muscle protein synthesis and mTORC1 signaling after a resistance exercise bout (190). Another notable milestone publication around this time (2000) was the first human muscle mRNA-omics dataset published by the Peterson laboratory (193). These authors isolated muscle RNA from 12 older and 11 younger participants before and 24 h after a resistance exercise bout and used [^{32}P]ATP labeling during cDNA construction (which preceded the currently used fluorometric technology) before chip hybridization reactions. Of the 588 annotated targets the array provided probes for, the authors reported that vascular endothelial growth factor (VEGF) mRNA, inflammatory mRNAs (IL-1β and RANTES), and immediate-early response mRNAs (c-jun, EGR-1) were dynamically altered at the postexercise time point in both cohorts. Indeed, this publication led the way to current high-density microarray and RNA-sequencing (RNA-seq) investigations whereby load-induced changes in all annotated muscle mRNAs and several annotated miRNAs and small RNAs can be interrogated (194–196). A timeline of studies discussed in this section is summarized in **FIGURE 2**.

From the early 2000s to the present day, independent research groups around the world have utilized assays to determine the transient RNA, phosphosignaling, and protein synthetic responses to mechanical overload in animals and resistance exercise bouts in humans (197–217). The widespread availability of antibody-conjugated chromagens and fluorophores to label proteins in myofibers, or cells in the extracellular matrix, has also led to a greater understanding of the cellular and molecular signaling responses to resistance training (218–230). The advancement of genetic mouse models has enabled the determination of genes that may be critically involved in promoting load-induced skeletal muscle hypertrophy (231–233). Also notable is the advent and utilization of various -omics-based techniques in human and rodent resistance training and mechanical overload studies. These investigations have included chip-based genomics (234), chip- and sequencing-based transcriptomics (194, 195, 235–252), DNA methylomics (195, 253–258), and mass spectrometry-based proteomics (100, 195, 259–265), phosphoproteomics (217, 264, 266–268), and metabolomics (269–271). The democratization of these techniques has led to a rapid expansion of molecular data in the field, and current-day research now involves analyzing large-scale multi-omics-based datasets. Examples of such efforts include *1*) the implementation of the MetaMEx interactive database by the Zierath laboratory to elucidate changes in mRNA expression across 66 exercise studies that contained muscle transcriptome information (272); *2*) work from the Phillips laboratory that used a variety of bioinformatics approaches to validate a gene signature responsive to mechanical loading in humans that was associated with hypertrophy and in vitro experiments indicating that this signature is functionally is associated with protein synthesis (250); and *3*) the broader goal of the Molecular Transducers of Physical Activity Consortium (MoTrPAC) to overlay multiple -omics-based datasets and generate a molecular map that is triggered by single and multiple bouts of resistance training (7, 273). These efforts have and will continue to greatly expand the amount of information related to molecular signaling events that are associated with load-induced skeletal muscle hypertrophy. However, the need for research utilizing innovative genetic rodent models will also persist so that novel signaling mechanisms can be validated through loss- and gain-of-function studies. The utilization of higher-throughput in vitro contraction models (see Ref. 274 for example) is also needed to validate or unveil novel contraction-induced signaling mechanisms associated with myofiber hypertrophy. In silico analyses as described by Rupert et al. (275) can also be performed to develop novel hypotheses in this area of muscle physiology by leveraging online rodent muscle phenotype, genotype, and transcriptomic

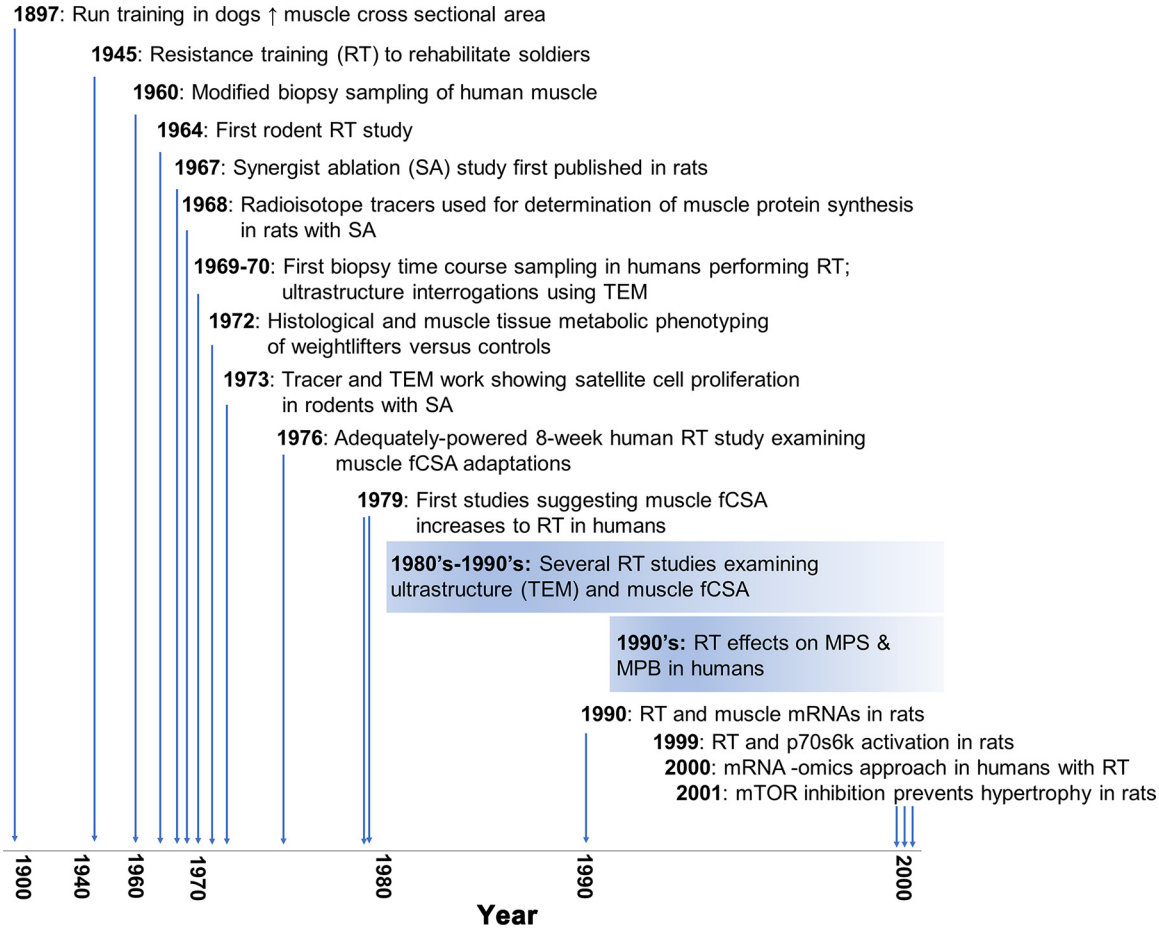

FIGURE 2. An overview of landmark studies. A timeline of landmark studies investigating skeletal muscle adaptations to mechanical overload in rodents and subsequent resistance training studies in humans. fCSA, myofiber cross-sectional area; MPB, muscle protein breakdown; MPS, muscle protein synthesis; mTOR, mammalian/mechanistic target of rapamycin; p70S6K, 70-kDa S6 protein kinase; TEM, transmission electron microscopy.

databases. Finally, refining -omics-based pathway analyses approaches will be instrumental in examining novel mechanisms associated with mechanical overload-induced skeletal muscle hypertrophy. Although this topic is beyond the scope of this review, an excellent review by Stokes et al. (276) provides guidance in performing pathway analyses and modeling of -omics data.

4. METHODOLOGICAL CONSIDERATIONS WITH SKELETAL MUSCLE HYPERTROPHY RESEARCH

To fully appreciate the content of this review, readers should be aware of various methodological aspects involved in skeletal muscle hypertrophy research including tissue processing, limitations to whole tissue lysate analysis, the interpretation of molecular data, time course considerations, the general lack of agreement between surrogate measures of skeletal muscle hypertrophy, and the human translatability of rodent studies.

First, muscle-molecular outcomes can be affected by tissue collection, preservation, and processing methods. Although many studies indicate that tissue is "immediately processed and frozen for future analysis," there is often little to no description of the time taken to preserve tissue for the different analyses. Oftentimes researchers collect animal or human skeletal muscle specimens for multiple assays, which requires more time to triage and preserve samples before freezing. A significant time lapse in tissue processing (e.g., 5 s vs. 10 min) may result in biomarker quality issues (277), and evidence in rodents indicates that postmortem delays in tissue processing cause a linear decay in RNA quality and an exponential decay in phosphoprotein status (278, 279). Tissue retrieval from deep freeze storage and thawing to isolate RNA or protein can also have deleterious effects on RNA and phosphoprotein quality (280, 281). Finally, histological artifacts due to freeze fracture can arise if tissue is not properly mounted and/or is frozen improperly (282). To mitigate some of these issues, researchers are encouraged to preserve tissue after excision as quickly as possible (e.g., snap-freeze in liquid

nitrogen within a minute after extraction). Additionally, tissue can be preserved in specialized reagents to preserve RNA integrity (283). Finally, there are published protocols detailing the process of proper muscle tissue preservation for histology (282, 284). A brief illustrative summary of proper muscle tissue processing is provided in **FIGURE 3**.

Second, much of the protein, RNA, DNA, and tracer work in the field provides information on muscle tissue lysates. There is an appreciable presence of stromal cells in the extracellular matrix, as mentioned above (see **FIGURE 1**). Although it is commonly assumed that information acquired from whole muscle lysates represents phenomena occurring within myofibers, a certain level of non-myofiber-specific signaling exists and must be considered. Researchers are beginning to circumvent this issue by labeling, isolating, and analyzing myonuclei with specialized genetic mouse models (254, 285, 286). The utilization of immunohistochemical techniques is also becoming more common to decipher protein localization responses to mechanical overload paradigms. Notwithstanding, DNA, RNA, and protein data from crude muscle lysates is still largely prevalent in much of the research discussed in this review. Also notable, buffer formulation is critical when working with muscle tissue, and lysates yielded from nonoptimal buffers can contribute to the signal-to-noise issue discussed in this paragraph. Specialized buffers and centrifugation protocols can be used to isolate myofibrils, nonmyofibrillar proteins, mitochondria, nuclear proteins, and extracellular matrix proteins (287–290). However, researchers commonly allocate general cell

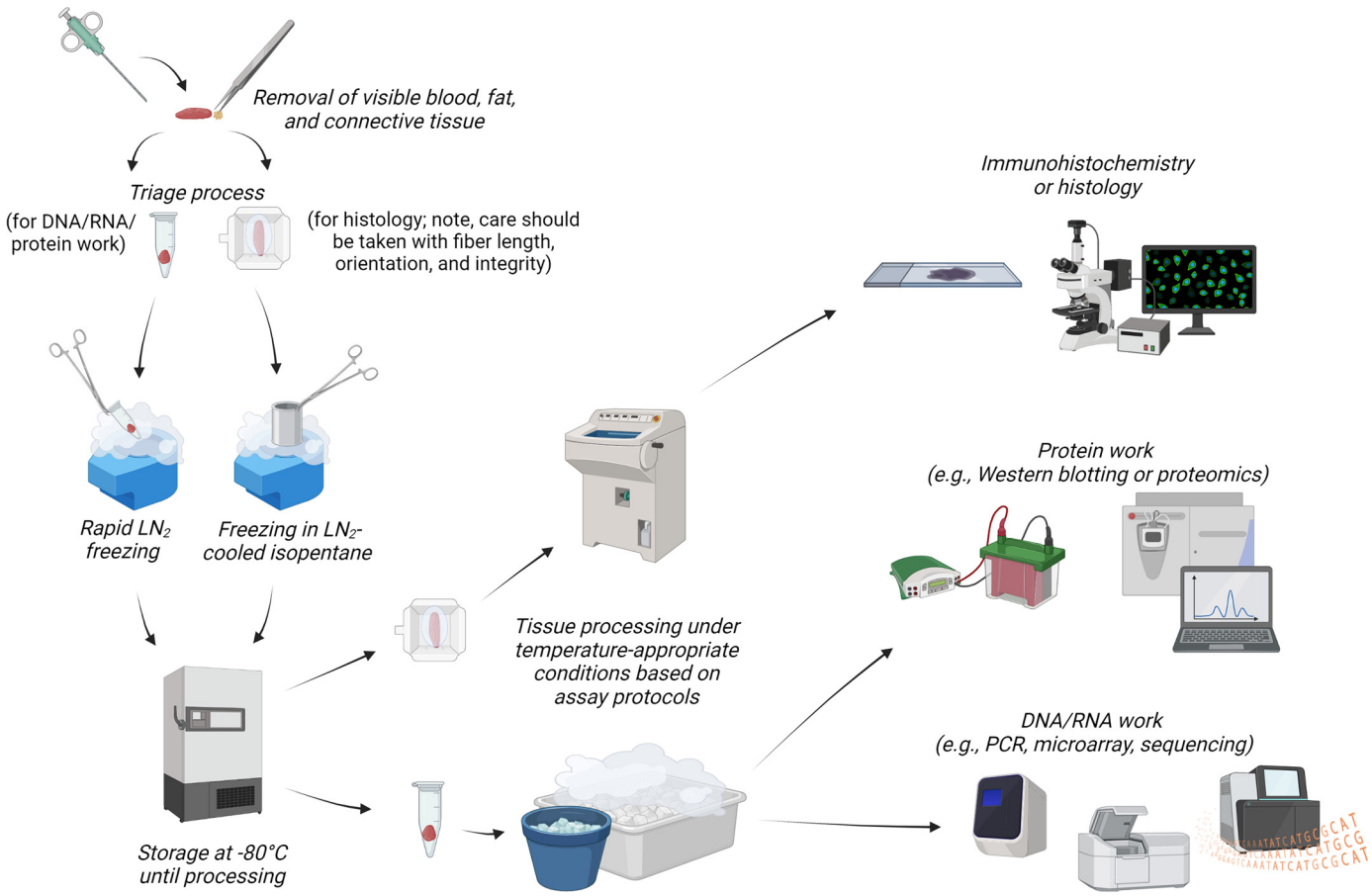

FIGURE 3. General muscle tissue processing steps for histology and molecular analyses. Muscle tissue procurement from human and animal studies involves either a biopsy (humans) or dissections (rodents; not pictured). It is advised that the removal of visible blood, fat, and connective tissue, tissue triage, and liquid nitrogen (LN_2) tissue preservation occur as rapidly as possible (e.g., between 1 and 3 min). Noted in the diagram are different preservation methods when sampling tissue for histology vs. nucleic acid or protein work. Researchers are advised to consult with published literature based on the assays desired to be performed to ensure that tissues are placed in adequate buffers (if needed) before cold storage and/or LN_2 freezing and deep freeze storage. Upon tissue removal from deep freeze storage, care should be taken in most circumstances to ensure that the tissue is kept in a frozen state. As illustrated in the schematic, tissue processing for nucleic acid and protein work involves keeping tissue on dry ice, LN_2-cooled stages, and/or ice throughout several of the processing steps to prevent macromolecule degradation. Tissue processing for immunohistochemistry or histology on nonfixed tissue typically involves sectioning in a cryostat at approximately −20°C. Again, researchers are encouraged to consult with published literature to obtain the desired conditions based on the assay(s) desired to be performed. This schematic was constructed with BioRender.com, with permission.

lysis buffers when analyzing muscle tissue without considering optimal buffer alternatives relative to the research question. Some researchers also use precleared lysates (i.e., removal of insoluble proteins) whereas others use whole muscle lysates, and this methodological difference likely leads to different outcomes being reported. Hence, researchers should attempt to best determine buffer selection according to the research question. Furthermore, although whole muscle lysates are more difficult to work with given the poor solubility of large contractile proteins, working with muscle-specific lysis buffers that solubilize most proteins is ideal in studies that seek to examine how loading paradigms affect certain aspects of the muscle-molecular milieu (e.g., enzyme activities or protein-protein interactions). Finally, whole tissue analysis does not reflect motor unit recruitment changes, which is one of the earliest physiological adaptations to resistance training (291). Whole tissue analysis collectively assays recruited and nonrecruited myofibers together, which has the potential to dilute signals in myofibers that are recruited during training. Single-myofiber analyses provide a more nuanced view of the myofiber size/function and signaling dynamics, albeit there can still be contamination of adherent stromal cells (254). Although single-fiber analysis is cumbersome, this analysis has been performed by several independent laboratories (265, 292–295), and recent work from Murgia et al. (100) indicates that single-fiber preparations from humans are suitable for proteomic analysis.

Third, molecular data interpretation can be challenging in the context of skeletal muscle hypertrophy. Some assays (e.g., qPCR and Western blotting) normalize molecular targets to housekeeping genes or proteins (296, 297), both of which can be altered during or after periods of mechanical overload (298, 299). Western blotting normalization for protein expression can be achieved through Ponceau or stain-free signals, which represents the total solubilized protein pool (296, 300). Phosphorylated proteins are commonly normalized to pan (or total) protein levels for a given target. Omics-based assays have specialized normalization procedures as well. Chip-based RNA and DNA assays are normalized to a global fluorescent intensity (301), mass spectrometry-based proteomic data are normalized as a percentage of total spectra (261), and RNA-seq data are commonly normalized to read counts (302). Finally, there are commonly interrogated variables such as total muscle RNA (a surrogate of ribosome content) and muscle citrate synthase (CS) activity (a surrogate of mitochondrial volume density). These variables can be normalized to wet or dry muscle weights, albeit CS activity can also be normalized to muscle or mitochondrial protein content. It is critical to appreciate that muscle tissue and myofiber hypertrophy is accompanied by an absolute increase in muscle protein and macromolecule content as discussed above. Thus, in models that induce skeletal muscle hypertrophy, researchers should ensure that their normalization variable (e.g., normalizer protein or housekeeping gene mRNA) is not altered. Slight nonsignificant changes in opposing directions from the target and normalization marker could show significant differences in the target-normalized outcome. Likewise, it is important to conceptualize that modest changes, no changes, or even a decrease in the relative abundance or concentration of a target molecule during tissue hypertrophy can indicate an increase in the overall abundance (and thus an upregulation) of the molecule (184). As a contextual example, Roberts et al. (80) reported that rat plantaris total RNA concentrations (μg/g wet tissue) are 19% higher in hindlimbs subjected to 14 days of synergist ablation compared with sham-treated legs. When considering that plantaris masses are also 25% higher in the surgical versus sham-treated legs, plantaris total RNA content in the surgical versus sham-treated legs is estimated to be 47% higher by multiplying RNA concentration in micrograms per gram of wet tissue by wet tissue weights. The Booth laboratory (184, 185) adopted a similar approach when reporting rRNA and mRNA content differences between nonexercised rats and rats that performed hindlimb resistance-like training; specifically, targets were presented in relative (% of total extracted RNA) and absolute (RNA content adjusted for muscle mass) terms. Hence, although not commonly adopted, it is recommended that researchers reporting protein or RNA expression changes during chronic periods of mechanical overload discuss (or even report) how the degree of hypertrophy potentially alters the relative versus total content of assayed biomarkers. Finally, non-steady-state differential equation models have been championed when using D_2O during atrophy models to calculate integrated protein synthetic rates since calculations are contingent on muscle protein pool size (303), and the same could be argued for muscle hypertrophy models that elicit increases in the total muscle protein pool.

Fourth, the timing of tissue sampling after a bout or period of mechanical overload can be critical relative to the research question. For example, the temporal pattern of changes in protein synthesis following exercise does not directly track with intracellular signaling responses (17, 26, 304–306), and this is likely related to the timing of tissue sampling that will require innovative approaches to address. The RNA profile of muscle can vastly differ when sampled minutes, hours, or days after a resistance exercise bout (307, 308). Incongruent findings between muscle protein turnover rates and hypertrophy during the earlier periods of resistance training have also been noted (304, 309, 310), albeit this

relationship is more coupled as subjects become trained (311). These findings indicate that exercise-induced protein synthesis rates may be more of a hypertrophic stimulus as these signals become more "refined" with training. Unfortunately, several studies that have examined the muscle protein synthesis response to naive bouts of resistance exercise likely interrogated a damage-synthesis response versus the hypertrophy-synthesis response that occurs later into training (310). Hence, the timing of tissue sampling is critically important to consider depending on the research question (e.g., examining peak mRNA responses to mechanical overload vs. phosphosignaling responses, etc.).

Fifth, it is often underappreciated that the size and percentage of slow- and fast-twitch myofibers differ depending on depth and proximo-distal location in a muscle group in humans and rodents (312, 313). This issue is difficult to mitigate in humans given the invasiveness of obtaining multiple biopsies. In rodents, however, a common practice is to examine most (if not all) myofibers at the midbelly of excised muscle. Also notable, data from multiple methods used to assess whole muscle versus myofiber size changes to mechanical overload in rodents and humans yield weak-to-moderate correlations. This observation was first noted by Gordon (314) in animals in the 1960s when comparing changes in mean fCSA and muscle weights following a period of treadmill training. Similar findings have since been reported in human resistance training studies that have compared MRI-derived VL muscle volume changes to ultrasound-derived VL muscle thickness changes (315), MRI-derived midthigh muscle CSA changes to dual-energy X-ray absorptiometry (DXA)-derived leg lean mass changes (316), and MRI- and ultrasound-based tissue level changes to mean fCSA changes (317). Why these discrepancies exist is not discussed here, and interested readers are encouraged to refer to Haun et al. (83) for more details. However, readers should be aware that this is still a salient issue that has not been resolved.

Finally, there are strengths and weaknesses with rodent models. Other than the clear discrepancies in myofiber size (81) and oxidative phenotype (93), metabolic and protein turnover rates are appreciably different between humans and rodents (318). Muscle protein synthesis rates have been reported to be 1.3- to 2-fold greater in type I versus type II fibers in rodents (319), and similar evidence exists (295). Conversely, type I versus II fiber differences in muscle protein synthesis rates are less dramatic in the resting and postexercise states in humans (~10–30%) (295, 320). Mechanical overload models in rodents vary in duration and stimulus, and the advantages and disadvantages of these models have been more thoroughly described by Lowe and Alway (321) and Booth and Thomason (322) and more recently by Murach et al. (232). Although studies using this procedure have yielded insightful information, it is a surgical model in which the intact muscle(s) is exposed to persistent load and exhibits rapid hypertrophy. Hence, despite the discussion of several studies using the synergist ablation model, the unfamiliar reader should be aware that this model does not resemble the physiological stimulus provided through progressive resistance training. Finally, it is common for researchers to examine rodents between the ages of 2 and 4 mo, and this can yield incongruent results between studies because this time frame is a formative stage of muscle maturation in the animal (323). There are, however, several strengths with rodent models. For instance, although it has been reported that human and mouse genomes on the whole show ~40% sequence overlap (324), there are similarities between genomes such as genome size (human [GRCh30]: 3,088,269,832; mouse [GRCm38]: 2,725,521,370) and the number of protein-coding genes (human: 19,950; mouse: 22,018), and both species possess ~70% sequence similarities in protein coding gene sequences (325, 326). Genetic mouse models have also been developed to determine loss or gain of function in relation to signaling mechanisms involved with skeletal muscle hypertrophy, and this approach is not possible in humans. Commonly interrogated hindlimb rodent muscles such as the soleus and plantaris predominantly consist of type I or type II fibers (80), whereas human muscles that are commonly biopsied contain a mixture of fibers as discussed in sect. 2. Hence, unless single-fiber approaches are used in humans, examining fiber type-specific mechanisms associated with hypertrophy may be more fruitful in rats given that dual overload via synergist ablation differentially affects muscle protein synthesis, ribosome biogenesis, proteasome activity levels, satellite cell counts, and the magnitude of hypertrophy in the type I fiber-prominent soleus versus type II fiber-prominent plantaris muscle (80). The use of adeno-associated virus (AAV) vectors is becoming more widespread in rodents given the high uptake efficiency across most myofibers within a muscle (275). Genes delivered through AAV-based vectors can also be coupled with a muscle-specific promoter to transduce muscle-specific gene expression across virtually all muscles in the animal. Gene delivery is also possible through the electroporation of plasmids containing genes encoding proteins or shRNAs for gene knockdown (327), and this methodology also has good utility in examining mechanisms. The ability to control environmental factors more stringently in rodents, such as food administration and the light-dark (sleep) cycle, is also advantageous in reducing variability in outcome measures. Finally, a prominent theme in this review is that several mechanistic rodent studies predated (and were confirmed) by human

discoveries (see **FIGURE 3**). Thus, although limitations exist, the discussed strengths and general human translatability of rodent models in skeletal muscle hypertrophy research illustrate their utility in examining associated mechanisms. **FIGURE 4** provides a summary of advantages, limitations, and shared strengths of rodent and human studies in the literature.

4.1. Mechanisms Commonly Associated with Skeletal Muscle Hypertrophy

4.1.1. The involvement of mTORC1 and its upstream activators.

mTOR is a 289-kDa serine/threonine protein kinase in the phosphatidylinositol 3-kinase (PI3K)-related protein kinase (PIKK) family (328). In mammals, mTOR acts as a catalytic subunit of two distinct complexes known as mTOR complex 1 (previously defined as mTORC1) and complex 2 (mTORC2). These complexes differ in their accessory proteins, differential sensitivity to rapamycin, downstream substrates, and functions. Specific to this review, mTORC1 phosphorylates substrates that increase the synthesis of proteins, lipids, nucleotides, and ATP while limiting the autophagic breakdown of cellular components (329). Two and a half decades since the discovery of rapamycin (330, 331) and mTOR (332), mTORC1 has been the most investigated mechanism linked to skeletal muscle hypertrophy. mTORC1 contains six accessory proteins including (333) *1*) mTOR, which possesses kinase activity, *2*) mammalian lethal with sec-13 (mLST8), *3*) DEP-domain containing mTOR-interacting protein (DEPTOR), *4*) the Tti1/Tel2 complex, *5*) regulatory-associated protein of mammalian target of rapamycin (RAPTOR), and *6*) proline-rich Akt substrate 40 kDa (PRAS40). Providing a more expanded discussion of mTORC1 signaling is beyond the scope of this review, and these details are provided elsewhere (10, 13, 334). However, the reader should appreciate that active mTORC1 complexes enhance muscle protein synthesis by regulating the phosphorylation of downstream substrates involved in translation initiation (e.g., p70S6K and 4EBP1) and elongation (e.g., eEF2) (192, 335). Although various cellular conditions are needed to stimulate increases in mTORC1 activity, one that has gained recent notoriety is the interaction of the mTORC1 complex with the lysosome (221, 311, 336–338). Increased translocation of mTORC1 to the periphery of myofibers following mechanical overload has also been reported, and future investigations will likely unveil the relevance of this event (218, 221, 339).

As mentioned in sect. 3, Baar and Esser (187) and Bodine et al. (191) published landmark studies outlining the involvement of p70S6K and mTOR, respectively, in mechanical overload-induced skeletal muscle hypertrophy, and these findings were subsequently validated in other rodent studies (340, 341). Goodman et al. (342) also reported that mTOR within myofibers is the rapamycin-sensitive element that confers the hypertrophic response to mechanical overload in mice, and follow-up mouse studies provided compelling evidence to suggest that mTORC1 is critical in this process (343, 344). This animal work led to other rodent and human investigations reporting enhanced mTORC1 signaling hours to days after a bout (or bouts) of mechanical overload (189, 206, 218, 221, 225, 345–371). Although a large body of research provides strong evidence suggesting that mTORC1 signaling is involved with skeletal muscle hypertrophy, the upstream activators of mTORC1 that are

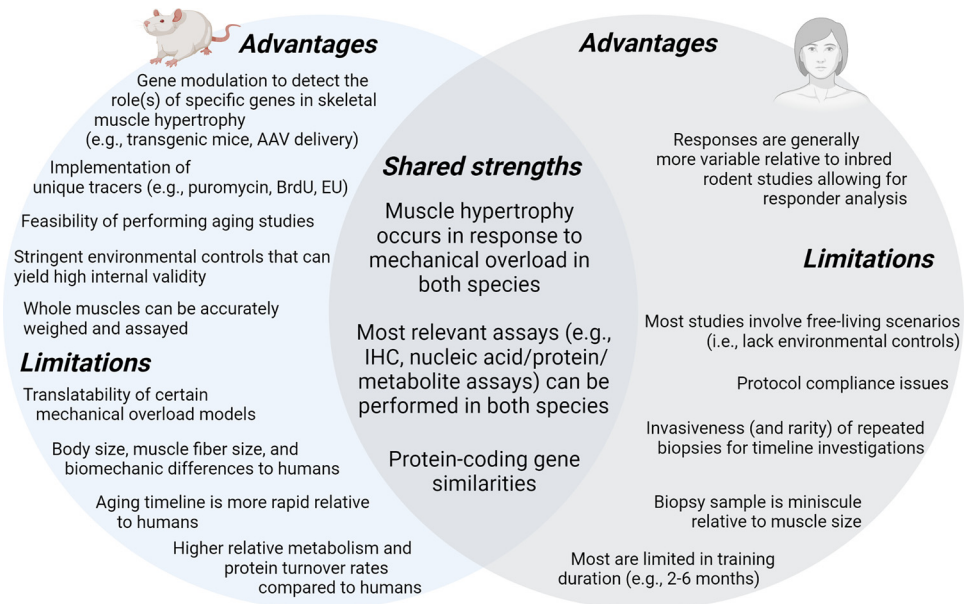

FIGURE 4. Advantages, limitations, and shared strengths of rodent and human studies. This figure (created with BioRender.com, with permission) summarizes the advantages and limitations of using rodent models. Additionally, limitations of human studies are presented. Finally, shared strengths of both models are displayed in the overlap region of the Venn diagram. AAV, adeno-associated virus; EU, 5-ethynyluridine; IHC, immunohistochemistry.

responsive to mechanical loading have not been fully elucidated. Over the years, various signals have been posited to be responsible for mTORC1 activation during overload stimuli including growth factor signaling (namely insulin-like growth factor 1, or IGF1), membrane-associated proteins involved with mechanotransduction, proteins involved with amino acid sensing that converge to activate mTORC1, and other proteins that act as upstream activators and inhibitors of mTOR. These topics are the crux of discussion in this section of the review.

AKT is a protein kinase that acts as an upstream activator of mTORC1 (372). IGF1, and its muscle-specific mechano-growth factor (MGF) variant, are upregulated at the mRNA and protein levels in rodent and human skeletal muscle subjected to mechanical overload (197, 373–382). These observations largely inspired a hypothesis that was pervasive in the literature from the late 1990s through ~2010 suggesting that postloading increases in localized IGF1 isoforms are largely responsible for mTORC1 activation via ligand binding to the IGF1 receptor, IGF1 receptor autophosphorylation, and increased AKT kinase activity (383–387). However, the IGF1 hypothesis has been rigorously challenged. Hornberger et al. (388) reported that the stretch-induced activation of mTOR signaling ex vivo is not abrogated in *Akt1*-knockout mice. Spangenburg et al. (389) reported that synergist ablation-induced mTORC1 signaling and plantaris hypertrophy are not perturbed in dominant-negative IGF1 receptor (*Igf1r*) mice. Maruyama et al. (390) used an AKT inhibitor (MK2206) in rodents to show that mTORC1 activation via hindlimb electrical stimulation occurs independently of AKT1/2 phosphorylation. Miyazaki et al. (391) reported that synergist ablation can still lead to the activation of mTORC1 in mice treated with a PI3K/AKT inhibitor. However, recent reports indicate that *Akt1/2* double-knockout mice present stark impairments in muscle mass and protein synthesis during the rapid growth phase (8–12 wk old) (392), and this extends into adulthood (393). Hence, AKT may be indispensable for muscle maturation and growth, although it does not appear to have a central role in mTORC1 activation in response to mechanical overload.

The conflicting IGF1 and AKT findings presented above have, in part, shifted emphasis to the current-day mechanotransduction hypothesis of mechanical overload-induced skeletal muscle hypertrophy. This hypothesis was pioneered by Goldberg et al. (127) (1975), expanded by Vandenburgh (394) (1987), and further refined by Flück and colleagues (395–397) as well as Hornberger and colleagues (398, 399). Mechanotransduction in myofibers occurs when mechanical perturbations of the basal lamina, sarcolemma, and cytoskeleton catalyze downstream signaling events. It is thought that these biochemical signaling events, in turn, activate mTORC1 and upregulate protein synthesis in an AKT-independent manner. Hornberger and colleagues (400, 401) established that various forms of mechanical overload transiently increase myocellular concentrations of phosphatidic acid (PA) and mTORC1 signaling by stimulating the membrane-associated activity of diacylglycerol (DAG) kinases. This group has also reported that PA can directly bind to and activate mTOR (402, 403). Collectively, these studies established a working model in which muscle contractions upregulate mTORC1 activity by promoting an increase in membrane-associated DAG kinase activity and subsequent increases in intracellular PA. This model is further strengthened by research from Hornberger's laboratory (404) showing that the knockout of the zeta DAG kinase isoform (DGKζ) significantly attenuates increases in fCSA, protein accretion, and plantaris mass after 7 days of synergist ablation. In humans, Thalacker-Mercer et al. (238) reported that human participants who exhibited the greatest muscle hypertrophy after a resistance training program (i.e., termed "extreme responders") exhibited heightened DGKζ mRNA expression before training. Although this latter human report does not provide a cause-and-effect relationship, it further supports the involvement of DGKζ as an anabolic signal during resistance training. However, the upstream mechanisms through which mechanical stimuli increase DGKζ activity have yet to be defined, and this mechanism will likely continue to be investigated.

Another mode of skeletal muscle mechanotransduction potentially involves transmembrane integrins and accessory proteins localized to the internal portion of the sarcolemma that propagate signals to activate mTORC1 (14). This model has been largely inspired by the findings of the Booth laboratory (405) reporting that focal adhesion complex-associated proteins, specifically focal adhesion kinase (FAK), are upregulated in rat soleus muscle 1 day and 8 days after synergist ablation. In this same publication, these authors reported increased FAK autophosphorylation in rooster muscle subjected to chronic loaded stretch. The Flück laboratory (395) later reported that FAK autophosphorylation preceded increases in p70S6K activity during an unloading and reloading paradigm in mice whose hindlimb muscles are transfected with a pCMV-FAK plasmid. Crossland et al. (406) reported in vitro data in this area showing that shRNA-mediated FAK knockdown reduced IGF1-stimulated increases in myotube protein synthesis and hypertrophy. Chaillou et al. (407) reported that mRNAs related to the integrin-linked kinase pathway are upregulated during the earlier phases of synergist ablation in mice, and these authors speculated that transmembrane

integrins may signal an upregulation in other genes that coordinate the anabolic response.

The notion of mechanotransduction operating through integrins or FAK signaling is scientifically grounded given the evidence discussed above. As well, FAK is localized with integrins on the interior portion of the sarcolemma (408), and FAK autophosphorylation associates with mTORC1 signaling in myotubes and other cell types (395, 409–411). However, several studies have challenged whether mechanotransduction operates through integrins or FAK to promote downstream anabolic signaling and skeletal muscle hypertrophy. Relative to wild-type mice, mice overexpressing the α_{7BX2}-integrin subunit in skeletal muscle (α_7Tg mice) exhibit reduced mTORC1 signaling after one bout of downhill running (412) despite these same mice exhibiting rapid myofiber hypertrophy after multiple bouts of the same stimulus (413). Boppart and Mahmassani (14) have also discussed unpublished findings from their laboratory showing that mTORC1 signaling trended downward in α_7Tg mice compared with wild-type mice after 1 day of synergist ablation. Interestingly, Petrosino et al. (414) more recently reported that synergist ablation-induced plantaris hypertrophy is impaired in *Ccn2*-knockout mice and noted that the CCN2 gene (also known as connective tissue growth factor) encodes a matricellular protein that exists in the extracellular matrix. Synergist ablation-induced elevations in muscle protein synthesis are also reduced in *Ccn2*-knockout mice, which presented lower basal levels of pan and phosphorylated FAK concentrations in skeletal muscle. The authors hypothesized that CCN2 might stimulate mechanical overload-induced muscle protein synthesis and hypertrophy through FAK signaling. However, contrary to the authors' own hypotheses, mechanical overload-induced increases in pan and phosphorylated FAK are not impaired in *Ccn2*-knockout mice 3 and 7 days after synergist ablation despite muscle protein synthesis and hypertrophy being dampened. In humans, Glover et al. (415) reported that muscle FAK phosphorylation is not transiently altered after a bout of resistance exercise despite an upregulation in p70S6K phosphorylation being observed, and similar evidence exists in rats subjected to a bout of eccentric contractions (416). Franchi et al. (417) demonstrated that FAK phosphorylation is upregulated with 8 wk of eccentric-only versus concentric-only resistance training in humans. However, both forms of training elicit similar increases in thigh lean mass values, midthigh thickness values, and 8 wk integrated muscle protein synthesis responses. It is also notable that FAK phosphorylation is prevented with mTORC1 inhibition in vitro, which suggests that FAK could be a downstream target of mTORC1 signaling rather than an upstream activator (410). Finally, sarcomere-based mechanotransduction has been shown to contribute to skeletal muscle hypertrophy independently of FAK involvement. Specifically, van der Pijl et al. (418) used a unilateral diaphragm denervation hypertrophy model in genetic mouse models in which titin stiffness is increased ($Ttn^{\Delta IAjxn}$) and decreased ($RBM20^{\Delta RRM}$), respectively, and reported that $RBM20^{\Delta RRM}$ mice (decreased titin stiffness) presented significant impairments in hypertrophy whereas $Ttn^{\Delta IAjxn}$ mice presented exaggerated increases in hypertrophy. Collectively, these conflicting reports make it difficult to determine whether integrin or FAK signaling is involved with load-induced increases in skeletal muscle hypertrophy.

A final mechanotransduction candidate discussed here is stretch-activated channels (SACs), which permit the influx of calcium and sodium ions into myofibers (16). There are various lines of evidence to support that contraction-induced increases in intracellular calcium increase mTORC1 signaling and muscle protein synthesis. For instance, the pharmacological blockade of SACs in rats with streptomycin has been shown to blunt eccentric contraction-induced increases in p70S6K phosphorylation (419). Others have reported that mTORC1 signaling and muscle protein synthesis are attenuated in rats administered a SAC inhibitor after eccentric contractions (420). However, there are also several independent lines of conflicting evidence in this area. The landmark paper by Bodine et al. (191) also reported that the calcium-mediated calcineurin pathway is not affected during periods of mechanical overload and calcineurin inhibition does not impair mechanical overload-induced skeletal muscle hypertrophy. It is also difficult to disentangle how calcium release from organelles in myofibers, rather than the influx of calcium into myofibers via SACs, affects mTORC1 signaling. For instance, Li et al. (421) performed in vitro experiments to show that the inhibition of calcium release from lysosomes reduces mTORC1 activity. Calcium transients from the sarcoplasmic reticulum during muscle contractions also presumably have a role in intracellular calcium signaling (422), and this mechanism operates independently of SAC-mediated calcium influx. Ito et al. (423) reported that synergist ablation-induced hypertrophy is abrogated in *Nnos1*-null mice. These researchers attributed this effect to a mechanism involving neuronal nitric oxide synthase (nNOS)-mediated nitric oxide formation, the subsequent formation of peroxynitrite, sarcoplasmic reticulum Trpv1 channel activation via increased peroxynitrite concentrations, and the increased influx of calcium into sarcoplasm from the sarcoplasmic reticulum to enhance mTORC1 signaling. Subsequent work by this group strengthened this mechanism (424), and a more recent paper suggests that the stimulation of the $P2Y_2$ receptors promotes increased intracellular calcium concentrations to enhance mTORC1 signaling in the type I myofiber-rich soleus muscle (425). Again, although these

data support the role of calcium in propagating anabolic signaling, these calcium-mediated mechanisms do not involve SAC-mediated mechanotransduction. Finally, the manner in which calcium activates mTOR has not been well resolved, and the involvement of calcium-mediated signaling in skeletal muscle hypertrophy has been challenged. A 1999 study supported a mechanism in which calcium-mediated calcineurin activation promotes mechanical overload-induced skeletal muscle hypertrophy in rodents (426). A separate study published the same year suggested that calcineurin acted downstream of IGF1 to elicit the nuclear translocation of the NF-ATc1 transcription factor and drive transcriptional processes that resulted in myofiber hypertrophy (427). However, subsequent research rigorously challenged the notion that calcineurin activation is involved in IGF1-mediated and/or mechanical overload-induced skeletal muscle hypertrophy (428–430). More recently, Ferey et al. (431) demonstrated that the overexpression of calcium/calmodulin-dependent protein kinase kinase-α (CaMKKα/CAMKK1), which is a prominent signaling mediator for intracellular calcium, stimulated mTORC1 signaling and muscle protein synthesis in mice. As with much of the data presented above, this finding supports the notion that calcium signaling (via CaMKKα activation) may act as an upstream activator of mTORC1. What strikingly opposes this paradigm, however, is data in this same paper showing that *Camkk1*-knockout mice exhibited 15% greater muscle hypertrophy and enhanced mTORC1 signaling relative to wild-type mice after synergist ablation. Prior work by Hornberger et al. (402) has also shown that the chelation of intracellular calcium with BAPTA-AM has no effect on the stretch-induced activation of mTORC1. Thus, although various lines of evidence have linked increases in intracellular calcium concentrations to enhanced mTORC1 signaling, the role that SACs (and calcium signaling at large) exhibit during load-induced increases in mTORC1 activity is riddled with conflicting data and needs further clarity.

Aside from the discussed mechanotransduction mechanisms, various upstream activators of mTORC1 signaling may be affected during periods of mechanical overload including amino acid-sensing and amino acid transport proteins. It is generally recognized that dietary proteins and essential amino acids increase mTORC1 signaling and muscle protein synthesis in the basal state (432). It is also recognized that dietary proteins and essential amino acids additively enhance anabolic signaling in skeletal muscle after a resistance exercise bout (433–435). However, preliminary data suggest that skeletal muscle upregulates the activity and content of proteins involved in the transport and sensing of amino acids in a load-dependent and nutrient-independent manner. For instance, electrically simulated hindlimb contractions have been shown to increase the activity of mammalian Vps34 (mVps34), an amino acid-sensing protein, 3 h after contractions (436). Others have reported in humans that chronic resistance training increases the expression and sarcolemmal enrichment of the L-type amino acid transporter 1 (LAT1) protein (219), which is responsible for transporting several essential amino acids into myofibers (437). Although this area is limited, the available data support that load-dependent increases in proteins that promote amino acid transport and sensing may be partially responsible for enhanced mTORC1 activation and skeletal muscle hypertrophy during periods of mechanical overload.

Other upstream activators and inhibitors of mTORC1 signaling are also altered during periods of mechanical overload. TSC2 inhibits mTORC1 by acting as a GTPase-activating protein that converts active GTP-Rheb into inactive GDP-Rheb (438), and TSC2, Rheb, and mTOR are enriched at the lysosome (438). Jacobs et al. (337) demonstrated that eccentric contractions in mice reduced the localization of TSC2 with the lysosome, coinciding with a hyperphosphorylation of TSC2. A subsequent investigation by these same researchers indicated that the inducible and skeletal muscle-specific knockout of *Rheb* led to a reduction in the eccentric contraction-induced activation of mTORC1 signaling (439). These studies lend support for a model in which the load-induced phosphorylation of TSC2 causes it to dissociate from the lysosome and these events enable Rheb to obtain its active GTP-bound state to upregulate mTORC1 signaling. In humans, Song et al. (221) reported that a bout of resistance exercise transiently leads to similar postloading events (e.g., dissociation of TSC2 from Rheb), lending further credibility to this model. The Regulated in DNA damage and development 1 (REDD1) protein, which is an inhibitor of mTORC1 signaling (440), may also be affected during periods of mechanical overload. Gordon et al. (352) reported that skeletal muscle REDD1 protein levels are transiently reduced after an overload stimulus in mice. These authors published a follow-up study showing that load-induced hypertrophy is enhanced in *Redd1*-knockout mice (350), coinciding with heightened mTORC1 activity and a reduction in autophagy. Drummond et al. (441) partially confirmed these findings in humans by showing that REDD1 mRNA is transiently downregulated 3 h after a low-intensity resistance exercise bout with blood flow restriction. However, skeletal muscle *REDD1* mRNA and protein levels have been reported to be elevated 1 and 3 h after a bout of resistance exercise (203). Others have also shown that resistance exercise does not transiently affect REDD1 protein levels (442). Thus, additional research into REDD1 and its role during mechanical overload-induced skeletal muscle hypertrophy is warranted.

Finally, the increased myocellular concentrations of certain substrates linked to enhanced mTORC1 signaling may also be involved with skeletal muscle hypertrophy. For instance, polyamine synthesis enzymes are upregulated in skeletal muscle by synergist ablation in an mTORC1-dependent fashion (443). Polyamines are small compounds containing two or more amino groups (e.g., spermidine, spermine), and various polyamines are required for cellular homeostasis and protein synthesis (444). However, these data are relatively new to the field, and the function(s) that polyamines exhibit during skeletal muscle hypertrophy remains to be determined.

In summary, the collective evidence suggests that enhanced mTORC1 signaling promotes skeletal muscle hypertrophy during various loading paradigms in animals and humans. However, it is critical to note that this signaling likely needs to be pulsatile given that mTOR hyperactivity in TSC1-knockout mice increases oxidative stress, elicits myofiber damage, and causes myofiber loss over the life span (445). It is also apparent that increased mTORC1 activity during these scenarios does not require certain upstream signals such as an upregulation in IGF1 signaling, enhanced AKT activity, or calcineurin activation. Instead, several lines of evidence support that a DAG kinase-mediated increase in PA and a dissociation of TSC2 from the mTOR-lysosome complex are involved with load-induced increases in mTORC1 activity. The other upstream mTORC1 signals discussed (e.g., integrins and FAK signaling, an upregulation in amino acid transport and sensing proteins, a downregulation in REDD1) have limited supporting evidence or are confounded by inconsistent data and require further clarity. **FIGURE 5** summarizes several of the mTORC1-associated mechanisms discussed in this section.

4.1.2. A brief discussion of mTORC1-independent mechanisms.

Although a high level of emphasis has been placed on mTORC1 signaling, several lines of evidence support mTORC1-independent signaling being involved with load-induced anabolic outcomes or skeletal muscle hypertrophy (36). For instance, West et al. (446) demonstrated that mTOR inhibition through rapamycin inhibited 6 h postexercise muscle protein synthesis after sciatic stimulation of the hindlimb muscles in rats. However, rapamycin only partially inhibited protein synthesis 18 h after exercise, and this was attributed to the mTORC1-independent phosphorylation of ERK1/2 (a mitogen-activated protein kinase, or MAPK), eEF2 (which regulates translation elongation), and UBF (a transcriptional regulator of ribosome biogenesis) as well as alterations in the mRNA expression patterns of *Akirin1/Mighty*, *Myc*, and other genes involved in ribosome biogenesis. Ogasawara and Suginohara (447) similarly demonstrated that early hypertrophic signaling (<3 h after exercise) following an electrical stimulation bout in

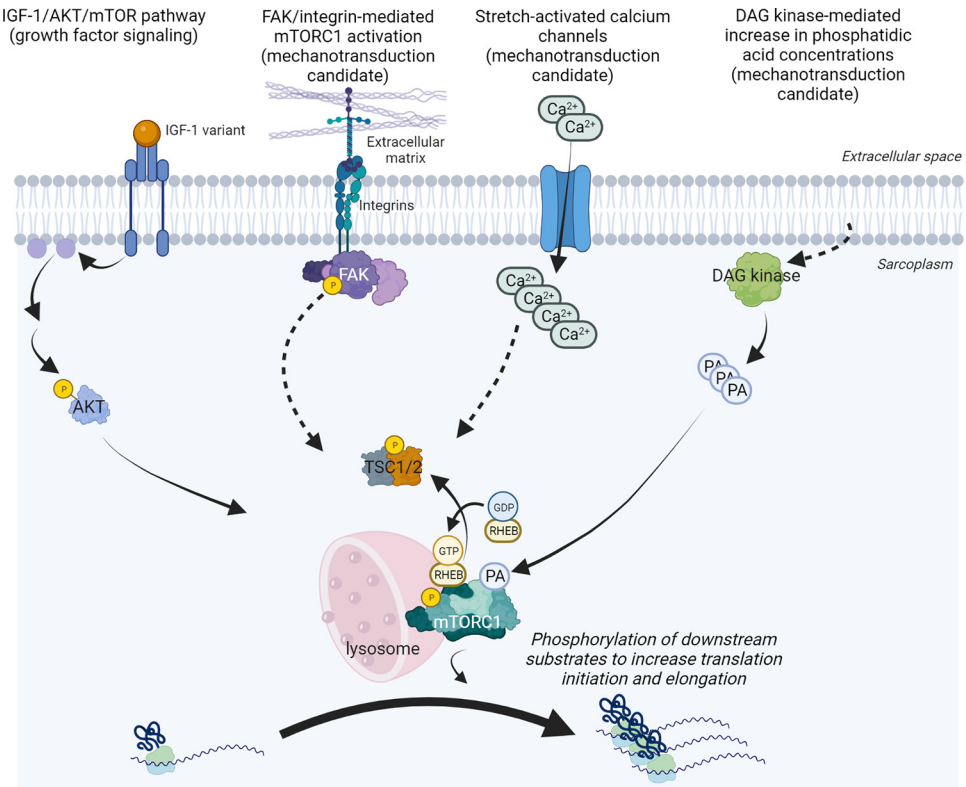

FIGURE 5. Signals associated with mechanical overload posited to upregulate mammalian/mechanistic target of rapamycin complex 1 (mTORC1) activity in skeletal muscle. This schematic (constructed with BioRender.com, with permission) provides an overview of content discussed in the review related to signals associated with mechanical overload that have been posited to upregulate mTORC1 activity in skeletal muscle. Notably, 1 of these signals [insulin-like growth factor 1 (IGF1)] operates through canonical ligand-receptor binding, whereas the other 3 signals are thought to operate through mechanotransduction. Single or multiple solid arrows indicate the pathway (or a portion of the pathway) has been relatively well defined and/or extensively investigated. Dashed arrows indicate that not much is known about how the upstream signal operates in skeletal muscle during periods of mechanical overload. DAG, diacylglycerol; FAK, focal adhesion kinase; PA, phosphatidic acid.

rats is sensitive to rapamycin, whereas later increases in protein synthesis (>6 h after contraction) occurred despite mTOR inhibition. You et al. (343) demonstrated that load-induced increases in protein synthesis are not impaired by the muscle-specific, inducible knockout of *Raptor* or when mice are treated with rapamycin (342). Since contraction-induced muscle protein synthesis was subsequently shown to be completely inhibited by an ATP-competitive mTOR inhibitor (447), it is reasonable to speculate that mTORC2 may act in tandem with mTORC1 to regulate muscle protein synthesis. However, mTORC2 inhibition via muscle-specific *Rictor* knockout in mice does not affect contraction-induced muscle protein synthesis (448). Goodman et al. (449) reported that synergist ablation increases muscle Yes-Associated Protein (YAP) protein concentrations, a transcriptional coactivator of the TEA domain transcription factors and constituent of the Hippo signaling pathway (450, 451). An increase in YAP phosphorylation also occurs, and skeletal muscle YAP overexpression in vivo induces hypertrophy in an mTORC1-independent fashion. Through a series of in vivo transfection experiments, it was shown that increased YAP protein expression enhances the promoter activities of *Myc* and *Myod1* while reducing the promoter activity of *Trim63*/*Murf1*. Hence, the YAP-induced increase in *Myc* expression could drive hypertrophy through enhanced ribosome biogenesis in a mTOR-independent fashion. Contrary to these findings, however, is work by the Wackerhage laboratory (452) showing that the constitutive overexpression of *Yap1* in mice leads to muscle atrophy. Given the novelty of this target, as well as conflicting data, the involvement of YAP in skeletal muscle hypertrophy needs to be further explored. Steinert and colleagues (267) more recently demonstrated that S473 phosphorylation of the Tripartite Motif-Containing 28 (TRIM28) protein is transiently elevated after a bout of maximal hindlimb contractions in mice and this occurs independent of mTORC1 signaling. In addition, TRIM28 phosphorylation confers myofiber hypertrophy in mice transfected with a TRIM28 phosphomimetic plasmid construct. The authors posited that TRIM28 phosphorylation likely occurs through upstream MAPK signaling and, once phosphorylated, the protein could enhance the expression of the muscle specific MYOD and MEF2 transcription factors to promote hypertrophy.

As alluded to in the prior paragraph, MAPK signaling has been commonly cited as an mTORC1-independent signaling mechanism involved with skeletal muscle hypertrophy. Three MAPKs (ERK1/2, JNK1/2, and p38) have been extensively examined with in vitro and in rodent synergist ablation models (354, 453–455). More recent data suggest that activation of MAPKs occurs through mechanotransduction (e.g., the MAP3K ZAKβ localizing to Z disks) (456), and there is evidence that certain aspects of MAPK signaling converge to activate downstream mTORC1 targets in skeletal muscle (351). Several groups have reported that elevated MAPK signaling occurs after one or multiple resistance exercise bouts in humans (363, 457–462), and in some cases these signaling events coincide with elevated mTORC1 signaling and increases in muscle protein synthesis. The Goodyear laboratory (463) reported that mechanical overload-induced increases in fCSA and muscle mass are impaired in inducible and muscle-specific *Mapk8*/*Jnk1*-knockout mice after 14 days of synergist ablation, which again underscores the importance of MAPK signaling in promoting skeletal muscle hypertrophy. There is evidence from a recent human study suggesting that β2-adrenergic signaling operates in an mTORC1-independent fashion to stimulate myofibrillar protein synthesis following resistance exercise (464). And perhaps the most compelling example of non-mTORC1 signaling being involved with overload-induced hypertrophy comes from Ogasawara and colleagues (465), who reported that chronic rapamycin treatments dampened, but did not prevent, increases in hindlimb muscle masses and fCSA in rats following 8 wk of electrically evoked hindlimb contractions. Although the mTORC1-independent mechanisms associated with this response were not determined, this was the first resistance training-like loading paradigm in rodents to show such an effect. This is very important to note because all prior evidence of rapamycin preventing skeletal muscle hypertrophy came from surgical models of chronic mechanical overload.

Indeed, several lines of evidence support that mTORC1 inhibition inhibits skeletal muscle hypertrophy during chronic mechanical overload (191, 340, 341, 343, 466). Notwithstanding, several studies have also suggested that non-mTORC1 signals are involved (e.g., MAPKs, YAP, TRIM28, UBF, MYC, and others), and the importance of these mechanisms during physiologically relevant forms of mechanical overload-induced hypertrophy warrants further consideration.

4.1.3. The involvement of ribosome biogenesis in mechanical overload-induced skeletal muscle hypertrophy.

Millward and colleagues (467) published a report in 1973 indicating that muscle protein synthesis in rat skeletal muscle scaled linearly with changes in ribosomal (r) muscle RNA content. They noted that

> "More than 80% of muscle RNA is ribosomal and this proportion appears to be maintained during protein depletion, so that a change in RNA also reflects a change in ribosome content. If alteration in ribosomal content affects control, then this alteration may be termed a change in the ribosomal capacity for protein

> synthesis. If, however, a change in synthesis is brought about by alterations in the other factors modulating each phase of translation, then it is a change in the ribosomal efficiency."

This study provided the basis for the current-day definitions of translational capacity, the concentration of ribosomes in myofibers, and translational efficiency, the ability of existing ribosomes to catalyze protein synthesis per unit of RNA. Additionally, rRNA accounts for ~80% of the total RNA content of cells (468); this work implied that the determination of total RNA concentrations reliably reflects alterations in skeletal muscle ribosome content. Increases in translational or ribosomal capacity occur through a process termed ribosome biogenesis. Describing the process of ribosome biogenesis is beyond the scope of this review and has been detailed elsewhere (20, 469–472). Subsequent rodent studies have shown that mechanical overload-induced increases in tissue total RNA and rRNA concentrations are associated with skeletal muscle hypertrophy, as reviewed by Goldberg et al. (127). Using an intermittent loading paradigm, Wong and Booth (473) confirmed the increase in RNA content after 10 wk of hindlimb loading. Several studies have since indicated that loading paradigms increase rRNA or total RNA concentrations after one bout or with chronic loading to amplify the ribosomal capacity of the muscle (80, 380, 382, 474–480).

A series of in vitro studies from 1989–2005 suggested that an increase in translational capacity through rRNA synthesis is involved in cardiomyocyte and myotube hypertrophy (481–483). In skeletal myotubes specifically, Nader et al. (481) reported that serum/growth factor stimulation increased rRNA concentrations and that this effect is abrogated by rapamycin, which implicated that ribosome biogenesis in myotubes is largely stimulated through mTORC1 signaling. Although mTORC1 converges at the ribosome to promote increased translational efficiency, this was the first evidence suggesting that mTORC1 signaling also promotes ribosome biogenesis in skeletal muscle. More recently, these same researchers reported that mTORC1 can undergo nuclear localization to bind to rRNA gene promoters, and this process can be inhibited by rapamycin (484). Furthermore, the release of mTOR from ribosomal gene promoters with rapamycin treatment correlated with chromatin marks indicative of transcriptional silencing.

To understand the mechanisms responsible for ribosome biogenesis in skeletal muscle, von Walden et al. (478) investigated the transcriptional response of rRNA genes during the initial stages of mechanical overload. rRNA transcription peaked at 3 days and preceded rRNA accumulation and hypertrophy. A transcriptional burst involved the enrichment of specific transcription factors at the rDNA promoter including the Upstream Binding Factor (UBF), c-Myc (Myc), and the Williams Syndrome Transcription Factor (WSTF), a component of the B-WHICH chromatin remodeling complex. This was consistent with the increase in 45S pre-rRNA and suggests both transcriptional and epigenetic regulation of ribosome biogenesis in skeletal muscle hypertrophy. A recent study by Murach et al. (485) expanded upon the role that Myc transcription factor has in driving ribosome biogenesis. Specifically, in silico analysis of several datasets suggested that Myc gene regulation is evident at the onset of mechanical overload and that certain genes related to ribosome biogenesis (e.g., *Bop1*, *Polr3g*, and *Rps19*) are likely driven by Myc.

Studies in humans have shown results consistent with these data. For example, a bout of resistance exercise stimulates rRNA gene transcription as early as 4 h after exercise (486, 487), and this response can persist for at least 48–72 h after exercise (472, 488). Figueiredo (17) also authored a recent review describing the process of ribosome biogenesis, and a summary table discusses several studies that have reported an increase in muscle total RNA or rRNA concentrations days to weeks into resistance training (353, 489–495). Since myofiber hypertrophy is typically detected after 15–20 training sessions in humans (223, 310, 496), the increase in ribosomal production appears to precede hypertrophy (see **FIGURE 6**). A recent human study by Figueiredo et al. (497) indicates that ribosomal DNA (rDNA) copy number, which can range between hundreds and thousands of copies on an individual-to-individual basis (498), is positively associated with ribosome biogenesis markers in response to an acute bout of resistance exercise. These authors also reported that a mechanical overload stimulus in mice transiently alters the promoter methylation status of genes associated with ribosome biogenesis, confirming earlier findings that load-dependent epigenetic mechanisms, in part, modulate ribosome biogenesis.

Strengthening the case for ribosome biogenesis being a critical mechanism for load-induced skeletal muscle hypertrophy, other research has indicated that the magnitude of ribosome biogenesis in response to different loading paradigms is associated with hypertrophic outcomes. For instance, Kirby and colleagues (477) reported that older mice present impairments in load-induced skeletal muscle hypertrophy compared with younger mice and this corresponded with a diminished ribosomal response in the old mouse cohort. Nakada et al. (476) utilized various forms of synergist ablation in rats to produce four different levels of plantaris hypertrophy. Fourteen days after overload, plantaris masses increased by 8% in the first cohort, 22% in the second, 32% in the third, and 45% in the fourth. rRNA content increased by 80%, 120%, and 150% in the latter three groups 5 days after overload,

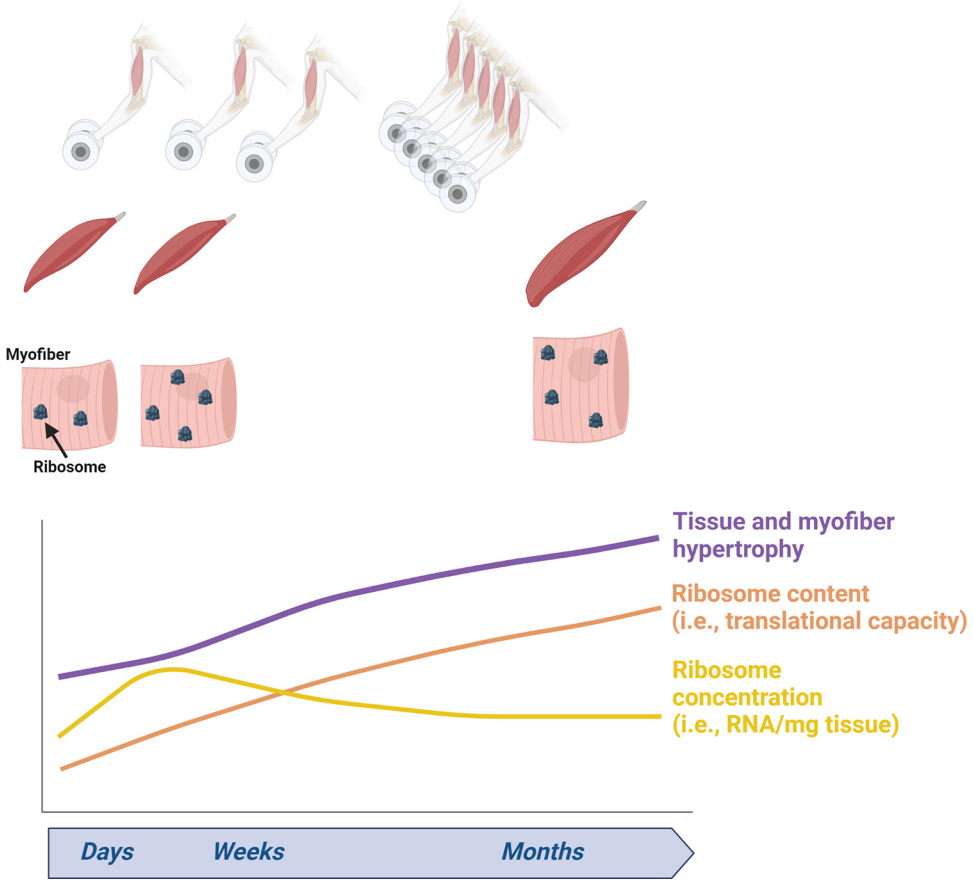

FIGURE 6. Timeline of ribosome biogenesis during load-induced skeletal muscle hypertrophy. This schematic (constructed with Biorender.com, with permission) provides a general timeline of ribosome biogenesis during periods of mechanical overload. Importantly, researchers have shown that ribosome biogenesis precedes skeletal muscle (and myofiber) hypertrophy, and this can result in increases in both ribosome content as well as ribosome concentrations. However, ribosome concentrations renormalize after longer-term training periods where myofiber hypertrophy is evident.

and the 5 day increase in translational capacity was strongly correlated to 14 day muscle weight data. These rodent findings have been validated in humans, in part, by Hammarström et al. (499), who reported that the degree of muscle hypertrophy following 12 wk of resistance training is associated with increased rRNA concentrations. A more recent study by Hammarström et al. (500) in humans also expanded these findings through a time course examination of resistance training-induced changes in rRNA levels. The authors reported that muscle rRNA concentrations increased in response to the first four training sessions, and this was followed by a plateau and peak in concentrations after eight sessions. Furthermore, the increases in muscle total RNA concentrations correlated with the magnitude of resistance training-induced skeletal muscle hypertrophy. This latter finding was recently confirmed by Figueiredo et al. (501), who reported that 2 wk of ambulatory recovery from cast immobilization followed by 2 wk of resistance training increases midthigh muscle CSA and rRNA concentrations.

Despite the strong evidence suggesting that an increase in ribosomal capacity plays a central role in muscle hypertrophy, a few reports are inconsistent with this notion. For instance, Goodman et al. (342) reported that ribosome biogenesis occurs in the absence of myofiber hypertrophy after 7 days of synergist ablation in mice administered rapamycin, but whether this occurs during longer-term mechanical overload was not reported. Others have also shown that AAV-mediated skeletal muscle *Myc* overexpression during a 2-wk period does not stimulate muscle hypertrophy despite upregulating ribosome biogenesis markers and muscle protein synthesis (502). However, this may have been due to the persistent expression of *Myc*, and as discussed with mTORC1 signaling, pulsatile *Myc* responses to mechanical overload may be needed to contribute to the hypertrophic response. Although these limited data challenge the importance of ribosome biogenesis, most of the studies discussed here support that increases in translational capacity through ribosome biogenesis are associated with the magnitude of skeletal muscle hypertrophy in response to mechanical loading.

An additional theme gaining traction, despite limited evidence to date, is the notion of ribosome specialization being involved with load-induced skeletal muscle hypertrophy. A review by Chaillou (18) suggested that ribosome heterogeneity exists within myofibers in that each ribosome likely contains a unique profile of ribosomal proteins and rRNA spliced variants that act to modulate ribosome function. Ribosomal proteins can also be subjected to posttranslational modifications

(e.g., phosphorylation, methylation, and acetylation), which may increase ribosome heterogeneity within myofibers (18). Perhaps most intriguing is the notion put forth by Chaillou suggesting that ribosome specialization during skeletal muscle hypertrophy may lead to enhanced translational fidelity and selection events whereby certain mRNAs are prioritized for translation. Potential evidence of ribosome specialization comes from the McCarthy laboratory (503) showing that the mRNA expression of *Rpl3* and *Rpl3l* is differentially affected in skeletal muscle after synergist ablation (+400% and −82%, respectively), whereas the mRNA levels of all other genes encoding ribosomal proteins are modestly affected or not affected at all. These authors performed additional experiments demonstrating that the induction of *Rpl3l* expression in vitro impaired myotube growth and protein accretion by −23% and −14%, respectively, compared with a control cell line. Beyond these data, no published research to date has determined whether ribosome specialization occurs during periods of resistance training in humans and/or plays an appreciable role in skeletal muscle hypertrophy as implicated in vitro. Thus, like other mechanisms discussed in this review with limited or incongruent data, the research potential in this area is high.

4.1.4. The involvement of satellite cells in myofiber hypertrophy.

Satellite cells were first observed in frog muscle by Mauro (504) and Katz (505) in 1961 through TEM. As noted above, rodent work by Schiaffino et al. (148) indicated that satellite cell proliferation occurs in rodents after mechanical overload and that the fate of some of these satellite cells is to become incorporated into overloaded myofibers (149). Since these landmark studies, human investigations have provided evidence to support a mechanism in which satellite cell-derived myoblasts fuse to myofibers in response to resistance training (70, 223, 480, 493, 506–516). A meta-analysis by Conceição et al. (23) examined 27 resistance training studies totaling 903 participants. The authors reported that myofiber hypertrophy of ≤10% induces a modest increase in myonuclear content and that a significantly higher increase is observed when muscle hypertrophy is ∼22%; notably, these effects are independent of age, sex, and myofiber type composition. Increased satellite cell abundance following either a resistance exercise bout or longer-term resistance training has also been shown to be correlated with the magnitude of skeletal muscle hypertrophy (70, 222, 517, 518). Moreover, although some evidence to the contrary exists (519), increased satellite cell activation during periods of resistance training has been reported to coincide with skeletal muscle hypertrophy in older participants (520, 521).

Genetic mouse models have yielded tremendous insight in this area as well. The Pax7-DTA mouse uses the Cre-loxP system to kill satellite cells by driving the expression of a diphtheria toxin A fragment in a cell-specific fashion through tamoxifen administration. In 2011, McCarthy et al. (522) were the first to use the Pax7-DTA mouse model to remove ∼90% of all satellite cells in adult mice (4 mo of age). In short, the authors reported that plantaris growth in response to 14 days of synergist ablation is not impaired relative to control mice, thus providing the first evidence that satellite cell-mediated myonuclear accretion is not obligatory for load-induced skeletal muscle hypertrophy during this shorter time frame. However, Egner et al. (523) replicated the experimental approach utilized by McCarthy and colleagues to show that satellite cells are necessary for load-induced plantaris hypertrophy in juvenile Pax7-DTA mice (2–3 mo of age). These studies suggested that earlier maturation phases likely influence the requirement for satellite cells in overload-induced skeletal muscle hypertrophy. Murach et al. (323) confirmed the age-dependent requirement of satellite cells in reporting that the plantaris muscle of 2-mo-old Pax7-DTA did not hypertrophy in response to overload when satellite cells were ablated. Goh and Millay prevented myonuclear accretion in response to mechanical loading by inactivating the Myomaker (*Tmem8c*) gene in satellite cells, this being a gene required for satellite cell fusion (524). In agreement with Egner and colleagues, these authors found that satellite cell fusion is required for increasing myofiber size following 14 days of synergist ablation (525). Similarly, Englund et al. (526) reported that muscle hypertrophy induced by 8 wk of resistance-loaded wheel running is blunted in Pax7-DTA mice in which satellite cells had been depleted. Finally, a recent study by Kobayashi et al. (527) utilized inducible satellite cell-specific *Cdk1*-knockout mice, which show impaired satellite cell proliferation, to demonstrate that myonuclear accretion is blunted and increases in fCSA are limited after 14 days of synergist ablation. Although some conflicting evidence exists, these mouse data form a collective paradigm agreeing with the human data to suggest that satellite cells are needed for optimizing load-induced skeletal muscle hypertrophy in maturing rodents and/or during longer periods of mechanical loading in adult rodents. Furthermore, although several of these studies used the synergist ablation model, the data from Englund and colleagues support that satellite cells are needed to optimize hypertrophy induced by progressive resistance-loaded wheel running, which is a more physiological model.

Although data discussed in the prior paragraphs provide strong support to satellite cells assuming a critical role in resistance training-induced skeletal muscle hypertrophy, hypotheses in this area are being rapidly refined given that this is an intensely studied topic in muscle biology. For instance, Murach et al. (25) authored a recent review citing human resistance training studies that show that myofiber hypertrophy occurs either before or without myonuclear accretion (223, 528, 529), and similar data were published thereafter (512, 530–533). These eight studies reported mixed and type II myofiber radial size increases that averaged ~15–20%; thus it is likely that some participants from these studies did not obtain the ~22% fCSA increase threshold proposed by Conceição et al. (23), thereby explaining the lack of myonuclear accretion. Murach and colleagues also provide evidence to suggest that type II myofibers exhibit the ability to hypertrophy with less myonuclear accretion relative to type I myofibers, which results in larger type II fiber-specific myonuclear domains. Type II myofiber myonuclei have also been shown to compensate for the loss of myonuclear accretion via satellite cell depletion by significantly increasing their transcriptional output in response to mechanical overload induced by synergist ablation (534); alternatively stated, the myonuclei of type II myofibers appear to possess a transcriptional reserve to support myofiber growth in the absence of myonuclear accretion. One of the challenges in understanding myonuclear dynamics is that the relationship between myonuclear content and domain size is not constant for different-sized myofibers. For example, in opposition to the concept of a constant myonuclear domain with changing myofiber size, smaller myofibers have markedly smaller domains, and myofiber perimeter (rather than myofiber CSA) per myonucleus is constant across a fCSA range from 2,000 to 8,000 μm^2 (520, 535). Additionally, there are recent data from the Miller laboratory in mice showing increased DNA synthesis of myonuclei in vivo using D_2O, and this process is enhanced with synergist ablation-induced mechanical overload in the plantaris muscle (536). Other reports have indicated that bone marrow-derived cells and other stromal cells (e.g., Twist2 positive and Hox11 positive) provide additional sources of myonuclei (537–539), but more research is needed to definitely determine whether these cells contribute myonuclei in response to mechanical overload. Hence, although satellite cells are seemingly critical for optimizing load-induced skeletal muscle hypertrophy, these data imply that much remains unknown regarding myonuclear dynamics, and satellite cell fusion may not be the sole source of newly acquired myonuclei. As an interesting aside, a consequence of the satellite cell depletion studies using the Pax7-DTA model is the revelation of nonfusion roles that satellite cells seemingly exhibit during different loading paradigms. Independent lines of evidence support that satellite cells interact with myofibers and fibroblasts to promote extracellular matrix remodeling during tissue repair (74, 540–542). This cell-to-cell communication also appears to be bidirectional, with evidence of muscle fibroblasts stimulating satellite cell fusion (74). Mice depleted of fibroblasts have altered satellite cell dynamics and smaller regenerating myofibers after injury (540). In the absence of muscle damage (i.e., a state of strong extracellular matrix adhesion), satellite cells remain quiescent. However, satellite cells rapidly proliferate after a bout of unaccustomed resistance exercise with associated tissue damage, and this occurs without myonuclear accretion and coincides with an upregulation in genes involved with extracellular matrix remodeling (543). The Pax7-DTA genetic mouse model has been used to demonstrate that longer-term (8 wk) loading blunts skeletal muscle hypertrophy, and this coincides with a significant increase in fibrosis (541, 544). In vitro experiments from these studies have provided evidence showing that primary myogenic progenitor cells communicate with primary fibroblasts via miRNA-containing exosomes to downregulate *Rrbp1*, a master regulator of collagen biosynthesis, as well as collagen-related mRNAs. These studies by the Peterson laboratory support a mechanism in which satellite cells assume a nonfusion role in regulating extracellular matrix remodeling during muscle growth as summarized by Murach et al. (545). The findings of Roberts et al. (80), Moro et al. (546), and Damas et al. (533) showing that different loading paradigms increase type II myofiber hypertrophy and satellite cell number in the absence of myonuclear accretion suggest that this mechanism may also be operative in humans; however, these studies did not examine extracellular matrix markers or miRNAs. Beyond fibroblasts, satellite cells communicate with other stromal cells during the early stages of hypertrophy (e.g., endothelial cells, fibro-adipogenic progenitors, and mesenchymal progenitors) (542, 547, 548), as shown in the context of muscle regeneration (549). Hence, the nonfusion roles that satellite cells assume during mechanical overload may be involved in skeletal muscle hypertrophy, and this area of research is ripe for further investigation. Content in this section review is summarized in **FIGURE 7**.

4.2. Other Mechanisms Involved with Skeletal Muscle Hypertrophy

4.2.1. Genetic variants.

Genetic polymorphisms likely play a role in the hypertrophic response to resistance training in humans.

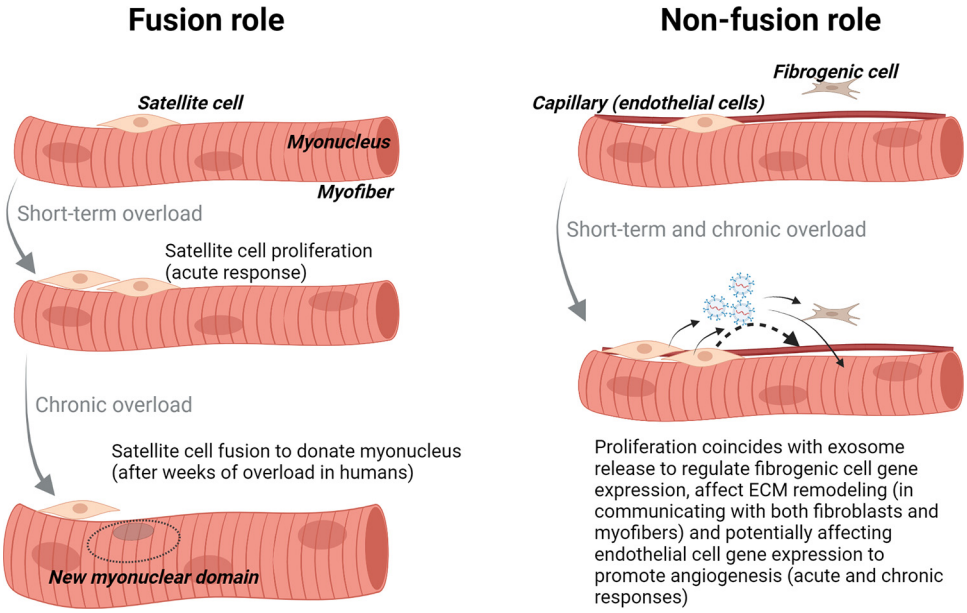

FIGURE 7. Summary of the fusion and nonfusion roles of satellite cells. This schematic (constructed with BioRender.com, with permission) provides a general overview of how satellite cells can respond to mechanical overload. The fusion role has been well defined, and this involves satellite cell proliferation (acute response) followed by the fusion of a subpopulation of satellite cells to increase myonuclear number. The nonfusion role involves satellite cells secreting exosomes containing microRNA (and presumably other cargo). Exosomes can transport this cargo to myofiber and nonmyofiber cell types in the interstitial space to regulate gene expression. In the example pictured, satellite cells are regulating gene expression in myofibers and fibrogenic cells, and this may affect extracellular matrix remodeling during myofiber hypertrophy (as discussed in main text). Satellite cells are likely to communicate with other cell types, and this is also illustrated via cell-cell communication with vascular endothelial cells. ECM, extracellular matrix.

Heterogeneous responses in hypertrophic outcomes exist with weeks to months of training (27), and genetic differences between individuals are commonly touted as being partially responsible for this effect. A recent meta-analysis including 24 heritability studies indicates that strength adaptations to resistance training possess ~50% genetic component (550), and this likely holds true for hypertrophic outcomes. In a larger-scale study, Stokes et al. (250) recently reported that a strong genetic component exists for resistance training and limb immobilization adaptations. Angleri et al. (531) more recently reported that two different unilateral leg training paradigms in 20 resistance-trained subjects led to statistically similar increases in mean fCSA (within-subject r value = 0.89 for this measure, $P < 0.05$). Authors from both studies suggested that intrinsic biological factors (i.e., genetic factors leading to transcriptome-wide responses that promote training adaptations) are likely responsible for these observed effects. Kilikevicius et al. (551) reported that soleus and plantaris hypertrophy elicited by 28 days of synergist ablation differs between eight strains of laboratory mice, and the authors concluded that this is likely mitigated by genetic differences between strains.

Despite these data suggesting that a genetic component exists for skeletal muscle hypertrophy, candidate polymorphisms that affect hypertrophic outcomes have varied and tempered enthusiasm in this area. The Functional Single Nucleotide Polymorphisms Associated with Human Muscle Size and Strength (FAMuSS) multicenter trial was a targeted analysis that provided novel insight into polymorphisms that affect the hypertrophic response to 12 wk of single-arm resistance training (552). In short, the authors examined ~500 gene variants in 1,300 younger adult men and women and reported that polymorphisms in 17 genes (ACE, ACTN3, ANKRD6, BMP2, CCL2, CCR2, CNTF, FST, MSTN, IGF1, IL15, IL15Rα, LEP, LEPR, NOS3, RETN, SPP1) are associated with muscle size changes during resistance training. Contrary to these findings, Vann et al. (234) recently used a DNA microarray to examine whether any of the ~315,000 polymorphism targets are associated with changes in whole body lean mass or mean myofiber fCSA with 12 wk of resistance training in 109 males. In short, none of the assayed polymorphisms, including many of those from the FAMuSS trial, was significantly associated with hypertrophic outcomes. Although the FAMuSS trial and the study published by Vann and colleagues employed similar-length training interventions, discrepancies between studies could have been due to differences in training modality (single-arm vs. full-body training, respectively), methods to quantify hypertrophy (MRI vs. DXA and VL myofiber histology, respectively), and the lower number of participants in the study by Vann and colleagues. However, one insightful finding by Vann and colleagues was that one annotated intronic

gene variant (GLI3; rs10263647) is significantly associated with mean fCSA changes. GLI3 encodes a transcription factor that regulates Sonic hedgehog signaling (553), and Vann and colleagues reported that the GLI3 T/C and C/C genotypes achieved myonuclear addition in response to training, whereas the T/T cohort did not. The *Gli3* gene has been shown to regulate satellite cell differentiation and fusion in mice by affecting the expression of myogenic regulatory factors (i.e., *Myf5*, *Myog*, and *Myod1*) (554). Hence, it is possible that those with the T/T genotype have impairments in satellite cell fusion. However, the significance threshold ($P < 1 \times 10^{-5}$) utilized by Vann and colleagues was adjusted for exploratory purposes and differs from the commonly utilized significance threshold in genome-wide association studies (GWAS) ($P < 1 \times 10^{-8}$). Thus, more research is needed to validate these findings. Additionally, a notable limitation with single-gene candidate studies and GWAS is the lack of resolution in detecting novel polymorphism candidates, and this issue has been illustrated in other research disciplines. For instance, Rivas et al. (555) utilized deep DNA sequencing to interrogate 56 genes and gene regions previously associated with Crohn's disease. These authors identified 70 novel protein-altering variants that likely contribute to the disease phenotype. Novel polymorphisms related to insulin secretion and glucose tolerance have also been recently identified with deep DNA sequencing (556). Findings from both studies imply that unidentified gene variants related to hypertrophy may exist, and future deep DNA sequencing efforts in this area will likely lead to fruitful discoveries. **FIGURE 8** provides a summary of what has been performed to date as well as future directions that could be pursued to increase the knowledge base in this area.

It is important to reiterate that genetic mouse models are useful tools whereby genes can be overexpressed or knocked out to yield a hypertrophic phenotype (231). These models, however, do not support that genetics is the prominent mechanism involved in load-mediated skeletal muscle hypertrophy. As a contextual example, the myostatin (MSTN) gene received considerable attention in the late 1990s and early 2000s as a gene that limits muscle growth. McPherron and Lee (557) reported that Belgian Blue cattle harbored an 11-nucleotide deletion in the MSTN gene that led to a doubling in muscle mass relative to normal cattle. Around the same time McPherron and colleagues developed Mstn-knockout mice, and these mice exhibit robust hypertrophy in the absence of mechanical overload (558). Seven years later, a case report in a child indicated that a rare MSTN mutation led to a hypermuscular phenotype (559). A proliferation of myostatin-related research ensued in the field, and various human studies sought to examine whether MSTN-related polymorphisms are associated

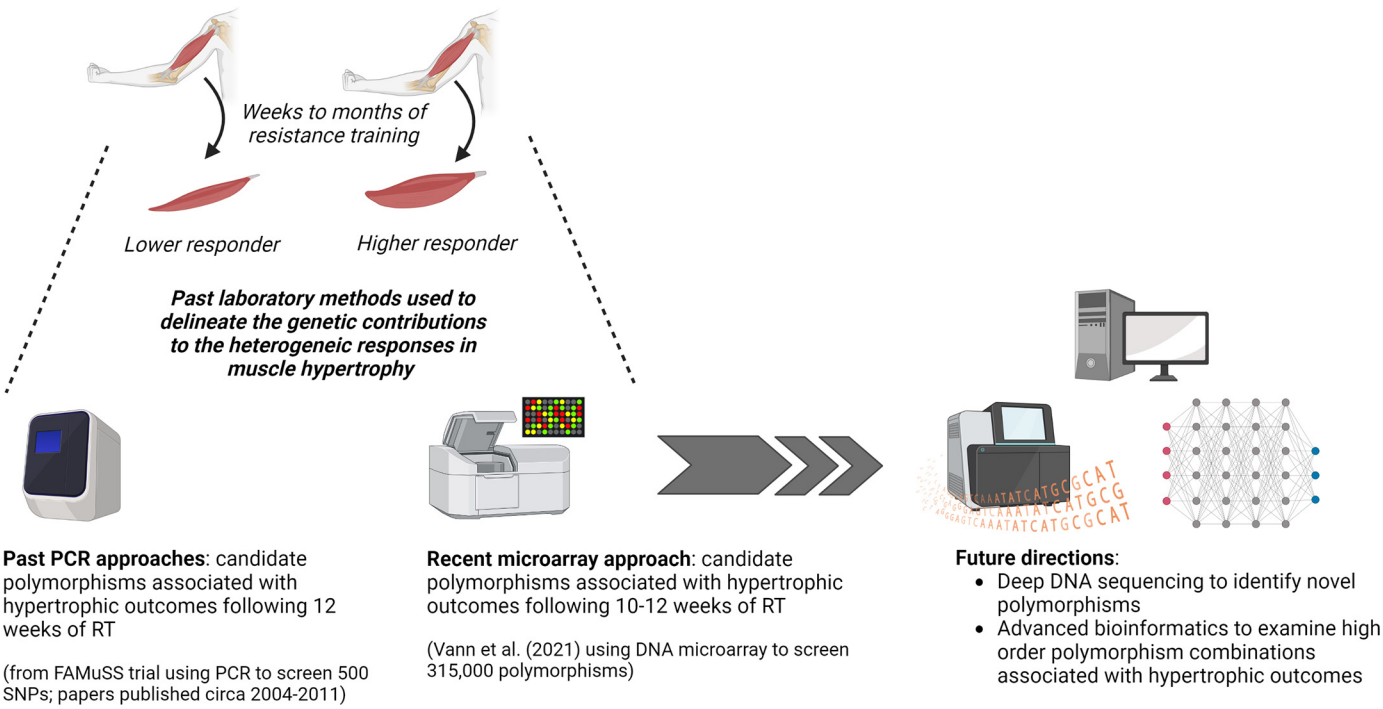

FIGURE 8. Past research and future directions regarding the delineation of gene polymorphisms associated with hypertrophic outcomes. This schematic (constructed with BioRender.com, with permission) provides a summary of past efforts examining genetic polymorphisms associated with the skeletal muscle hypertrophic response to resistance training in humans. As mentioned in main text, future methods using deep DNA sequencing and bioinformatics are needed to garner additional information in this area. FAMuSS, Functional Single Nucleotide Polymorphisms Associated with Human Muscle Size and Strength; RT, resistance training; SNP, single-nucleotide polymorphism.

with muscle mass or skeletal muscle hypertrophy in response to resistance training (234, 560–562). Notably, most of these studies have revealed few to no appreciable effects regarding MSTN genotype-phenotype (hypertrophy) outcomes (234, 561, 562). Hence, readers should appreciate that genetic mouse models (and case reports regarding rare human mutations) can be useful in identifying proteins involved with skeletal muscle hypertrophy, albeit this process is a complex trait that likely involves still-to-be resolved polymorphisms as well as the other mechanisms discussed here.

4.2.2. Epigenetic alterations, with an emphasis on DNA methylation.

Geneticist Adrian Bird was a pioneer in the scientific discipline of epigenetics, or changes in gene activity that do not involve DNA sequence alterations. In a landmark review, Bird (563) discussed research in the 1980s, much of which was from his laboratory, detailing the presence and postulating the significance of DNA methylation in eukaryotes. Through decades of subsequent research, it is now well appreciated that gene expression is regulated, in large part, through DNA methylation. Approximately 98% of DNA methylation occurs on cytosine residues present in cytosine guanine dinucleotide pairing sites (CpG sites), and DNA methylation is catalyzed by DNA methyltransferase (DNMT) enzymes whereas demethylation is catalyzed by ten-eleven translocation (TET) enzymes (564–567). Increased methylation levels in a promoter or enhancer region of a gene generally downregulate RNA transcription by either impairing transcription factor binding or compacting DNA and making it transcriptionally inaccessible (567). Additionally, although data in skeletal muscle are lacking, in vitro work in budding yeast suggests that alterations in genome-wide DNA methylation patterns can cause chromatin remodeling events that may indirectly impact the expression of genes by allowing certain DNA regions access to transcriptional machinery (568).

More recent enthusiasm has surrounded how exercise alters the collective skeletal muscle DNA methylome, and several reviews have been published on this topic (37, 39, 569, 570). Barrès et al. (571) provided the first evidence showing that changes in skeletal muscle DNA methylation transiently occur across various metabolic genes after a single high-intensity aerobic exercise session in humans. These authors also reported that the postexercise DNA demethylation patterns across various metabolic genes correspond with the mRNA expression patterns of these genes. Subsequent genome-wide methylation (or methylome) studies in humans have indicated that resistance training elicits the demethylation and upregulation of genes related to actin/cytoskeletal, extracellular matrix, growth-related, and/or metabolic pathways (255, 257). Moreover, some genes retain a demethylated signature after an earlier period of resistance training and detraining, and the mRNA expression of some of these genes is enhanced during the retraining period. These data have led Sharples and colleagues (572) to hypothesize that skeletal muscle possesses an epigenetic memory following periods of resistance training (or "epi-memory"), which could mechanistically explain why an increase in muscle mass occurs more rapidly during retraining periods following weeks to months of detraining. Also compelling are two recent collaborative studies from the Roberts and Sharples laboratories. The first study by Ruple et al. (258) indicated that 6 wk of resistance training in 65-yr-old men causes a robust demethylation of the mitochondrial genome and these methylation changes correspond with an increased mRNA expression of numerous mitochondrion-specific genes in the presence of skeletal muscle hypertrophy. The second study by Sexton et al. (573) in previously trained college-aged men indicated that global skeletal muscle DNA methylation patterns are more robustly altered 3 h versus 6 h after a resistance exercise bout (239,951 vs. 12,419 CpG site methylation changes, respectively; **FIGURE 9**). Like the aforementioned endurance exercise data discussed by Barrès and colleagues, these data suggest that alterations in skeletal muscle DNA methylation occur rapidly after a loading stimulus. Moreover, these authors used bioinformatics to report that genes related to "focal adhesion," "MAPK signaling," and "PI3K-AKT signaling" are significantly affected at both the DNA methylation and transcriptome-wide levels.

Rodent data also exist supporting dynamic alterations in myonuclear DNA methylation accompanies skeletal muscle hypertrophy. Figueiredo et al. (497) reported that a mechanical overload stimulus in mice alters the promoter methylation status rDNA to favor transcription. von Walden et al. (254) used the HSA-GFP (HSA-rtTA; Rosa26-H2B-GFP) genetic mouse model, as well as myonuclear capture and bisulfite sequencing techniques, to show that 11,210 CpG sites are hypomethylated and 3,491 sites are hypermethylated with plantaris hypertrophy induced by synergist ablation. Several CpG sites in proximity to genes involved in mTORC1 signaling, autophagy, and ribosome biogenesis are also hypomethylated, and an upregulation in several corresponding mRNAs also occurs. Wen et al. (286) used this same mouse model to show that enhanced muscle growth (relative to naive hypertrophy) occurs after detraining-retraining. Notably, detraining-retraining hypertrophy corresponds with a myonuclear methylome "memory" signature, which resonates with the human data from Sharples's group. Murach et al. (285)

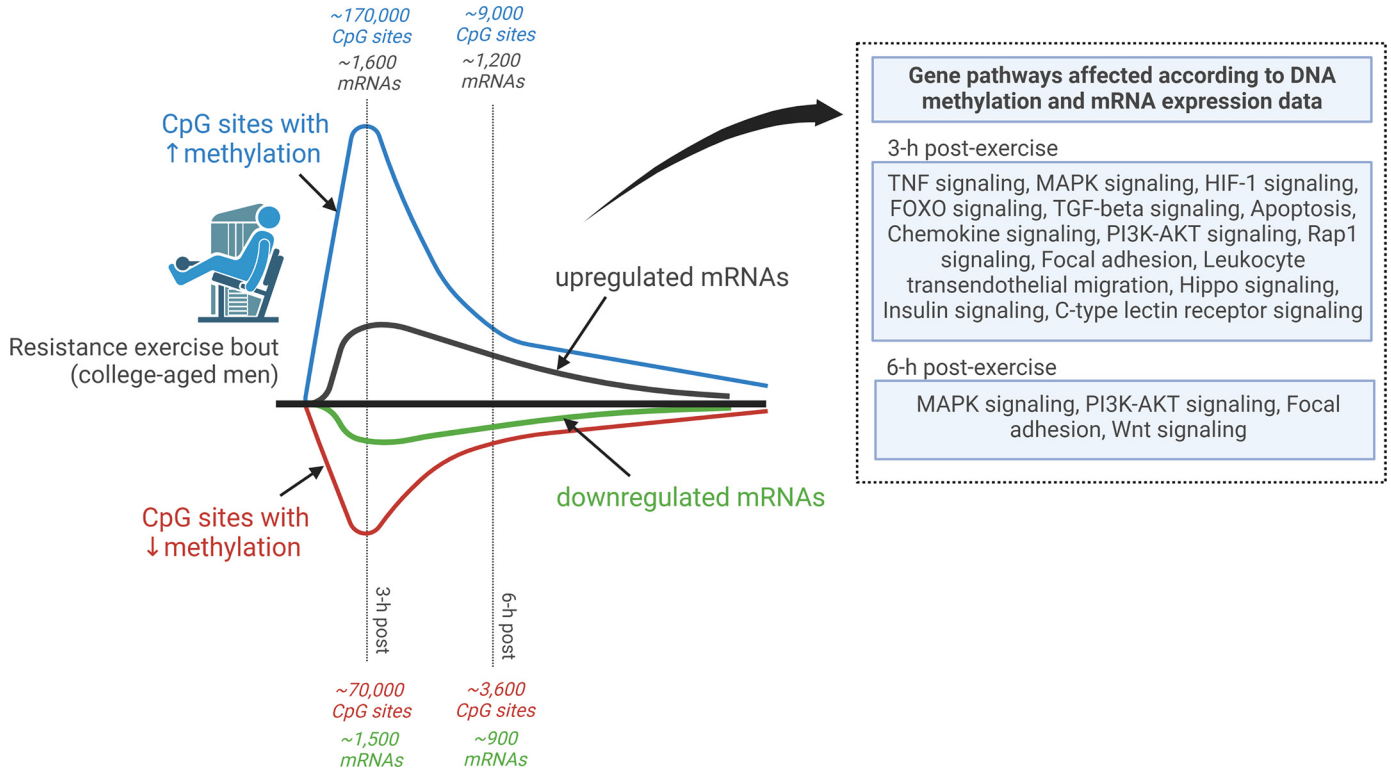

FIGURE 9. Skeletal muscle genome-wide DNA methylation and transcriptome responses to a bout of resistance exercise. This schematic (constructed with BioRender.com, with permission) summarizes recent data [Sexton et al. 2023 (573)] demonstrating alterations in skeletal muscle tissue DNA methylation status after a bout of resistance exercise in humans. The researchers concluded that *1*) alterations in DNA methylation statuses occur very rapidly (i.e., 3 h vs. 6 h after exercise); *2*) contrary to past hypotheses suggesting that exercise generally elicits a reduction in DNA methylation, more hypermethylation events occurred 3 h after exercise relative to hypomethylation events; and *3*) alterations in DNA methylation patterns likely precede and are, in part, responsible for altered mRNA expression patterns. HIF, hypoxia-inducible factor; PI3K, phosphatidylinositol 3-kinase.

subsequently used the HSA-GFP genetic mouse model, as well as myonuclear capture and bisulfite sequencing techniques like von Walden and colleagues, to elucidate epigenetic adaptations that occur after 8 wk of progressive resistance-loaded wheel running in two groups of mice. The first group of mice had resident myonuclei labeled before training to allow for their capture for DNA methylation analysis after training. The second group of mice had both resident myonuclei and newly acquired myonuclei via satellite cell-mediated myonuclear addition analyzed after training. Bioinformatics indicated that genes related to the PI3K-AKT and FOXO signaling pathways favored hypomethylation in the first group of mice. In contrast, genes associated with RNA polymerase II-mediated transcription and cell-to-cell adhesion pathways favored hypomethylation in the second group of mice (285). Additionally, multiple CpG sites near the 28S rDNA transcription termination area and one CpG site in the rDNA enhancer region favored hypomethylation in the second versus the first group of mice. These data suggest that resident myonuclei may utilize DNA hypomethylation to upregulate genes associated with protein turnover in response to mechanical overload, whereas newly acquired nuclei may utilize this process to upregulate genes associated with satellite cell proliferation, fusion, and ribosome biogenesis. The Murach laboratory (574) more recently reported that loaded wheel running in older mice alters muscle DNA methylation suggestive of reduced biological aging (i.e., the Horvath and developmental muscle clocks) and used a skeletal muscle-specific Myc-inducible mouse to suggest that mechanical overload operates through MYC to elicit these changes.

These collective data from independent research groups demonstrate that myonuclear DNA, DNA from cells in the extracellular matrix, and mitochondrial DNA display altered methylation profiles in response to mechanical overload in rodents or resistance training in humans. However, as with other data discussed here, much of the epigenetics work to date has been descriptive and more data are needed to confirm that mechanical overload-induced alterations in DNA methylation result in appreciable mRNA and protein expression changes. It is also difficult to determine the impact that DNA methylation has on downstream processes given that some data in this area indicate a smaller than anticipated overlap of DNA methylation changes and mRNA expression changes. In this regard, Sharples's group

(575) recently compared methylome and transcriptome data in muscle samples collected from humans 30 min and 24 h after various high-intensity running protocols. Only 431 of 879 genes were reported to exhibit exercise-induced mRNA expression patterns corresponding to DNA methylation patterns. More striking are data published by Laker et al. (576) where skeletal muscle transcriptome and methylome data were obtained in humans before and after three resistance exercise bouts over a 9-day period. In short, RNA-sequencing indicated that training alters the expression of 2,616 mRNAs, and methylome analysis using reduced representation bisulfite sequencing (RRBS) indicates that 474 genomic regions are differentially methylated. Surprisingly, only 54 genes exhibited DNA methylation changes that correspond to mRNA expression changes following training, indicating that DNA methylation changes are only associated with only ~2% of the genes whose mRNA abundances are altered. Indeed, this discordance may be due to the timing of tissue acquisition (307). For instance, many of the load-induced alterations in DNA methylation patterns are seemingly transient as discussed above (i.e., within 3 h after a resistance exercise bout), and these events may affect mRNA expression patterns hours to days thereafter. In support of this hypothesis, Telles et al. (577) examined DNA methylation and mRNA expression time course changes for select myogenic regulatory factors (MYOD1, MYF5, and MYF6) immediately after and 4 h and 8 h after a single bout of resistance, high-intensity interval, and concurrent exercise. Although the methylation and mRNA expression responses were reported to be interrelated, the respective profiles were not synchronized at the postexercise time points; specifically, mRNA responses to promoter methylation events seemingly occur after ~8 h. The aforementioned study from Sexton and colleagues (573) also indicates that global DNA methylation patterns 3 h after a resistance exercise bout exhibit a high association with global mRNA expression patterns 6 h after exercise. Thus, there is still much to learn about how dynamic changes in DNA methylation temporally affect gene expression.

Finally, unlike other mechanisms discussed in this review where genetic mouse models have provided cause-and-effect relationships, no study to date has examined how genetic mouse models harboring modified genes that affect skeletal muscle DNA methylation impact overload-induced hypertrophy. Interestingly, Wang et al. (578) reported that *Tet2*-knockout mice do not exhibit impairments in postnatal muscle development, albeit these mice presented fewer newly formed myofibers in response to cardiotoxin-induced injury compared to wild-type mice. These authors also generated tamoxifen-inducible *Tet2*-knockout mice (i.e., Pax7CreERT2:Tet2 flox/flox mice where Tet2 knockout occurs specifically in satellite cells), and similar effects were noted. Given that muscle DNA hypomethylation is commonly reported during periods of overload and TET2 catalyzes DNA demethylation, using similar mouse models with mechanical overload paradigms will provide more compelling evidence as to whether explicit DNA hypomethylation events at certain promoter regions are required for skeletal muscle hypertrophy.

4.2.3. Muscle proteolysis.

As discussed in other sections of this review, many molecular-based studies in the field have focused on examining mTORC1 signaling and/or either mixed or myofibrillar protein synthesis rates. The reasons for such interrogations are twofold. First, data in the field indicate that muscle protein synthesis (rather than muscle proteolysis) is more responsive to mechanical loading during well-fed states (579, 580), and Brook et al. (581) have similarly suggested that "...the measurement of [muscle protein synthesis] remains a cornerstone for understanding the control of hypertrophy—mainly because it is the underlying driving force behind skeletal muscle hypertrophy." Second, there are logistical issues that preclude assessing muscle protein breakdown rates and related mechanisms. Unlike protein synthesis in which mTORC1 acts as a central signaling hub that converges at the ribosome, there are several proteolytic mechanisms operative in skeletal muscle including the ATP-dependent ubiquitin proteasome pathway, calpain-mediated proteolysis, and lysosome-mediated autophagy (582, 583). Given the increased complexity of muscle proteolysis regulation, it is inherently more difficult to ascribe a particular proteolytic mechanism as being involved (rather than coinciding) with mechanical overload-induced skeletal muscle hypertrophy. Examining proteolysis rates in humans can also be technically challenging and invasive (e.g., with tracer infusions or using the invasive arterial-venous balance method requiring arterial and venous cannulations), and other methods require additional analysis and expertise (e.g., assessing isotope dilution in the free muscle amino acid pool for the extrapolation of breakdown rates) (583). Additionally, there are several more assumptions that are applied when calculating muscle protein breakdown versus synthetic rate kinetics. Several studies have assessed how proteolysis-related biomarkers (e.g., poly-ubiquitinated proteins, proteasome activity, and atrogene mRNAs) are transiently and chronically affected during periods of resistance training in humans (308, 369, 492, 493, 584–589) or during periods of mechanical overload in rodents (80, 474, 590). The overactivation of muscle proteolytic pathways in genetic mouse models or during longer-term

fasting, prolonged unloading, or diseased states mechanistically contributes to muscle atrophy (591–594). Under normal physiological circumstances, however, a dysregulation in one or more of the proteolytic systems in skeletal muscle may impair hypertrophy. This theme subtly emerged in human tracer studies in muscle protein synthesis, and breakdown rates were shown to be tightly coupled days after exercise (178, 595). Several human studies have since shown that a bout of resistance exercise transiently upregulates mRNAs, proteins, or protein signaling involved with muscle proteolysis (e.g., *TRIM63* and *FBXO32* mRNAs, calpain mRNAs, and autophagy markers) (308, 345, 369, 584–587, 596–600), and similar data exist after chronic training (589, 600, 601). Baehr et al. (590) demonstrated that proteasome activity, as well as the mRNA expression of *Trim63* and *Fbxo32*, increase after 2 wk of synergist ablation in mice. Abou Sawan et al. (41) also contend that, although muscle lysosomes are generally viewed as cellular "garbage cans" that rid the cell of damaged organelles, research into their emerging roles includes nutrient sensing, regulation of protein synthesis, and cell growth.

Genetic mouse models have also provided additional evidence to suggest that a dysregulation of components in proteolytic mechanisms may impair hypertrophy. For instance, ATG7 is involved in autophagosome formation, and Masiero and Sandri (602) have shown that robust muscle atrophy occurs in *Atg7*-null mice. PSMC4 is a subunit of the 20S proteasome, and a similar atrophy phenotype has been reported in *Psmc4*-knockout mice (603). Visual inspections of myofibers by TEM were provided in both papers, and both reports indicate that these knockout mouse lines possess disorganized myofibrils, abnormal intracellular vacuoles, disruptions to the sarcoplasmic reticulum, and mitochondria with abnormal appearances. Hence, a complete disruption in one or multiple proteolytic mechanisms likely leads to an inability of myofibers to purge damaged macromolecules or organelles, which in turn leads to a broader catabolic (or antianabolic) signaling cascades. Work by Steinert and colleagues (267) further adds to this working hypothesis. As mentioned, a phosphoproteomic approach was used to demonstrate that the S473 phosphorylation of the TRIM28 protein transiently occurs after maximal hindlimb contractions in mice. In addition to showing in mice that myofibers hypertrophy when transfected with a plasmid encoding a phosphomimetic version of TRIM28, the authors reported that tamoxifen-inducible *Trim28*-knockout mice exhibit myofiber atrophy as well as an impairment in hypertrophy in response to mechanical overload induced by synergist ablation. The authors posited that TRIM28 phosphorylation may stimulate the E3 ligase activity of the protein (note that E3 ligases act to transfer ubiquitin from an E2 ubiquitin-conjugating enzyme to targeted proteins) and, subsequently, accelerate proteasome-mediated proteolysis to enhance protein turnover and promote muscle hypertrophy. However, these authors did not extensively pursue this mechanism. Hughes et al. (604) recently demonstrated that the knockdown of an E3 ligase (Ubr5) in murine skeletal muscle leads to myofiber atrophy. Notable in vitro data also exist in this area of research. Osburn et al. (605) demonstrated that the pharmacological blockade of the proteasome and calpains in murine myotubes abrogates leucine-induced increases in muscle protein synthesis. Lewis et al. (606) demonstrated that autophagy inhibition blocks myofibrillar protein synthesis rates in L6 myotubes. Although kinetic interrogations were not performed in either investigation, both groups speculated that proteolysis serves to provide an intracellular pool of free amino acids that is sufficient to support muscle protein synthesis (i.e., intracellular recycling of amino acids).

Although data in the paragraph above support enhanced proteolytic activity being involved with skeletal muscle hypertrophy, there is counterevidence suggesting that these mechanisms are downregulated as well. For instance, Roberts et al. (80) reported a significant reduction in proteasome activity in hypertrophied plantaris and soleus muscles following 14 days of dual overload induced by synergist ablation. Human data from these same researchers indicates that 6 wk of high-volume resistance training reduces muscle polyubiquitinated protein levels (an indirect measure of E3 ligase and proteasome activities) in what were defined as higher and lower hypertrophic responders (492). Human data that have linked changes in protein turnover (i.e., the balance of muscle protein synthesis and muscle protein breakdown) to the hypertrophic response to resistance training are also lacking. Only two studies have utilized tracer techniques to concurrently assess protein turnover in humans after resistance training (607, 608), and one of these studies demonstrated improved muscle net balance in the postabsorptive state and that pre- to posttraining changes in basal muscle protein synthesis rates significantly correlate with changes in VL muscle thickness. Improvements in the intracellular recycling of amino acids may occur in response to resistance training or be a feature of higher responders to promote a more efficient protein turnover and muscle hypertrophy. However, no research has directly measured the intracellular recycling of amino acids from proteolysis to protein synthesis in this context. Further analysis of this hypothesis with utilization of isotope tracers, three-compartment modeling (609), and non-steady-state equations (303) could provide more insight into the role(s) that proteolysis has in intracellular amino

acid recycling for muscle protein synthesis and anabolism during hypertrophy. Depending on the method used and duration and form of tracer labeling, researchers should consider adjusting for possible changes in the protein pool size, use steady-state conditions, or, when this is not possible, adjust with non-steady-state equations appropriate to the chosen tracer model.

These collective data indicate that fully operational proteolytic mechanisms are likely integral for mechanical overload-induced skeletal muscle hypertrophy. What remains less understood, however, is how the coupling between protein synthesis and muscle protein breakdown is modulated during periods of overload to regulate skeletal muscle hypertrophy. Emerging methods that provide insight at the level of the single protein (610), single fiber, and even maintaining the spatial dimensions with emerging technologies such as nanoscale secondary ion mass spectrometry (nanoSIMS) (611) have the potential to greatly advance our understanding of this area of muscle physiology.

4.2.4. A reduction in myostatin mRNA expression and signaling markers.

As mentioned in sect. 4.2.1, myostatin (MSTN) has received considerable attention as a negative regulator of muscle growth since the landmark reports by McPherron and colleagues. MSTN is a putative myokine that is part of the transforming growth factor (TGF)-β superfamily, and signaling pathways associated with MSTN have been described in detail (612–615). Briefly, MSTN mRNA is translated into a propeptide secreted into the extracellular matrix. Once the latent propeptide is cleaved by extracellular matrix proteases, MSTN forms an active dimer that binds to the activin receptor type-2B (ACVRIIB). Ligand binding promotes the recruitment and activation of the activin receptor-like kinase-4 and -5 (ALK4 and ALK5) serine/threonine type 1 receptor kinases, which phosphorylate the SMAD2/3 heterodimer transcription factor (616). The SMAD2/3 complex interacts with SMAD4, and this heterotrimer enters the nucleus to transcriptionally regulate numerous genes that contain SMAD binding sites. It is difficult to determine which MSTN-regulated genes affect hypertrophic outcomes given that in vitro data indicate that 1,787 genes contain SMAD2/3 binding sites, 925 genes contain SMAD4 binding sites, and the TGF-β-mediated activation of SMAD2/3 can either upregulate or downregulate a fraction of these genes (617). However, MSTN signaling can affect the expression of genes without putative SMAD binding elements. For instance, proteolysis-related mRNAs are upregulated in myotubes treated with MSTN in vitro (618), in rat muscles incubated with MSTN (619), and in mice that express a constitutively active mutant of ALK5 (620). Furthermore, rat muscles subjected to MSTN gene electrotransfer display an atrophy phenotype coincident with a downregulation in structural genes (myosin heavy chain IIb, troponin I, and desmin) and myogenic transcription factors (MyoD and myogenin) (621). Data in *Mstn*-knockout mice suggest that MSTN signaling upregulates collagen-related genes (622) and genes that inhibit canonical Wnt signaling (623) in skeletal muscle.

Nongenomic MSTN signaling events have also been documented in skeletal muscle. For instance, MSTN-treated myotubes exhibit a reduction in AKT activity and mTORC1 signaling (624). Sartori et al. (620) reported that SMAD2/3 inhibition through shRNA knockdown promotes muscle hypertrophy in mice through enhanced mTORC1 signaling. Hulmi et al. (625) similarly demonstrated that blocking MSTN signaling in mice through intraperitoneal injections of the soluble activin receptor IIb increases mTORC1 signaling and muscle protein synthesis after 1–2 days and myofiber size after 2 wk of treatment. Also mentioned previously, *Mstn*-knockout mice exhibit a robust hypertrophic phenotype (558), and other genetic mouse models have been used to show that the conditional overexpression of follistatin (Fst), a MSTN inhibitor, and a mutated form of *Acvr2b* increases whole body muscle mass (612). More recent data in mice indicate that the overexpression of Fst increases the protein expression of several mTORC1 signaling-related proteins (e.g., AKT and p70S6k) as well as basal-state and insulin-stimulated muscle protein synthesis rates (626). Recent clinical trials in overweight and insulin-resistant participants have also indicated that 10 or 48 wk of bimagrumab administration, which blocks the activin type II receptor, significantly increases muscle mass and reduces fat mass (627, 628). Although resistance training was not employed in either study, these data reiterate that the blockade of MSTN signaling increases muscle mass in humans. Collectively, these data suggest that MSTN operates through genomic and nongenomic signaling mechanisms to upregulate proteolytic genes, downregulate structural genes and muscle-specific transcription factors, and reduce AKT-mTORC1 signaling in myofibers (**FIGURE 10**). Additionally, independent lines of evidence in mice indicate that longer-term MSTN inhibition does not operate through satellite cells to affect myofiber hypertrophy and, instead, likely acts through the other mechanisms discussed above (629–631).

Mechanical overload can act in various manners to downregulate MSTN signaling. Multiple studies in rats have indicated that one to four bouts of concentric, eccentric, and/or isometric contractions downregulate skeletal muscle *Mstn* mRNA levels (381, 382, 632). In

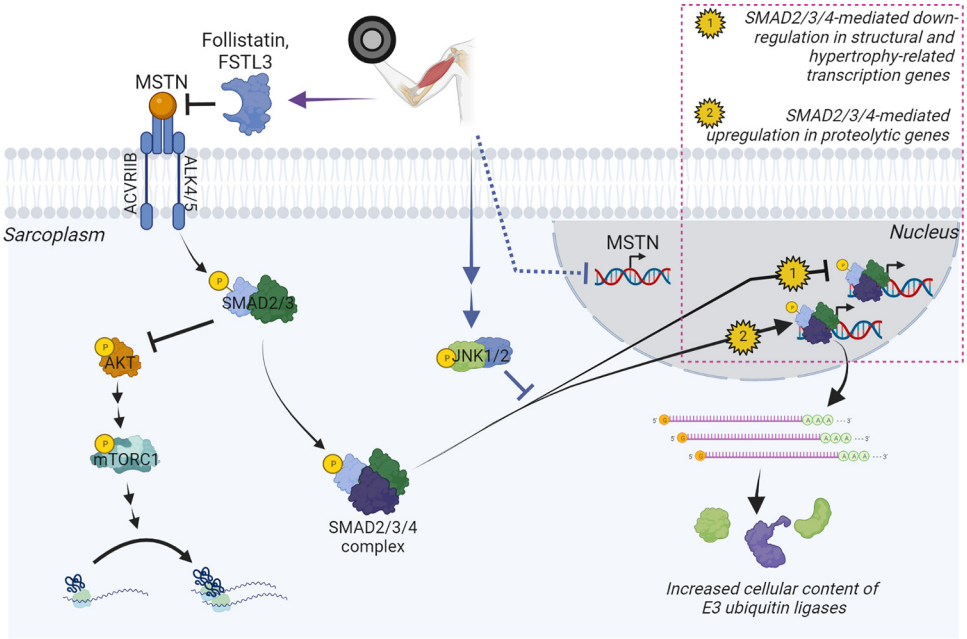

FIGURE 10. Summary of myostatin signaling and how mechanical overload affects signaling outcomes. This schematic (constructed with BioRender.com, with permission) provides a summary of myostatin (MSTN) signaling in skeletal muscle and how mechanical overload in rodents or humans affects signaling outcomes based on the research cited in-text. Upon ligand binding, MSTN leads to the phosphorylation of SMAD2/3, which causes the formation of the SMAD2/3/4 complex. This complex can then enter nuclei and affect the expression of genes that negatively impact muscle size and growth (i.e., genomic effects). A putative nongenomic effect of MSTN signaling includes the inhibition of AKT phosphorylation, and this can lead to diminished protein synthesis rates. Notably, blue arrows and inhibitor indicators illustrate how resistance exercise affects aspects of MSTN signaling. As discussed in main text, acute and/or chronic resistance training has been documented to upregulate myostatin inhibitors (follistatin and FSTL3), reduce SMAD2/3/4 nuclear translocation through JNK1/2 activation, and reduce MSTN mRNA levels through decreasing transcription rates.

mice, Lessard et al. (463) reported that 14 days of synergist ablation-induced mechanical overload enhances JNK/MAPK signaling, which acts to alter SMAD2/3/4 binding activity to gene promoter regions. In humans, Pillon et al. (272) analyzed transcriptome-wide array data from eight resistance exercise studies and reported that MSTN mRNA is downregulated across multiple studies hours to days after exercise. Hulmi and colleagues (633–635) published several reports using qPCR to show that MSTN mRNA is downregulated in response to one bout of resistance exercise, and Dalbo and colleagues (587, 636), Louis et al. (308), and Fernandez-Gonzalo et al. (370) have published similar reports. Hulmi et al. (635) also reported that correlations existed between MSTN mRNA levels 48 h after a naive resistance exercise bout and changes in whole body muscle mass ($r = -0.88$, $P = 0.002$) and VL muscle thickness ($r = -0.51$, $P = 0.12$) after prolonged resistance training. Other reports have also indicated that reductions in serum MSTN concentrations and skeletal muscle MSTN mRNA occur after longer-term resistance training (493, 637–641), and a recent study by McIntosh et al. (642) shows that resistance exercise downregulates MSTN mRNA levels 3 and 6 h after exercise and upregulates muscle follistatin protein levels 6 h after exercise. Collectively, these findings provide the basis for a model in which MSTN signaling is inhibited via mechanical overload through the downregulation of MSTN mRNA and upregulation in muscle follistatin protein, and through other signaling mechanisms such as increased MAPK activation. Additionally, the load-induced reduction in MSTN mRNA appears to be both transient (i.e., after a single exercise bout) and also persisting weeks/months into training.

Although the abovementioned reports are compelling, data are not consistent in this area. Willoughby (643) reported that 12 wk of resistance training in humans upregulates MSTN mRNA and protein expression concomitant with muscle hypertrophy. Kim et al. (644) reported that MSTN mRNA expression is downregulated 24 h after a single bout of resistance exercise and 16 wk after resistance training in humans. However, this pattern is similar in participants classified as hypertrophic nonresponders (i.e., participants who did not experience increases in mean fCSA with training), moderate responders (change in mean fCSA $= +1{,}111\ \mu m^2$ on average), or extreme responders (change in mean fCSA $= +2{,}475\ \mu m^2$ on average). More strikingly, muscle tissue concentrations of the active MSTN peptide were reported to increase by 44%, and, again, this pattern is evident across responder cohorts. Mobley et al. (493) reported that MSTN mRNA is not altered in lower, moderate, and higher hypertrophic responders after 12 wk of resistance training despite serum MSTN

concentrations equally decreasing across responder cohorts. Data from certain rodent studies have not yielded consistent results, either. Minderis et al. (645) reported that Berlin high (BEH) mice, which are homozygous for a mutation causing a dysfunction in *Mstn*, present larger muscle masses versus wild-type mice. However, these authors also reported that soleus mass increases in response to synergist ablation are paradoxically impaired in the BEH line. Aoki et al. (646) reported that muscle *Mstn* mRNA rapidly increases in response to stretch overload in rats, and MacKenzie et al. (647) reported that hindlimb stimulations in rats result in a rapid postbout increase in muscle *Mstn* mRNA. Eight weeks of ladder climbing has been shown to elicit similar increases in flexor hallucis longus muscle masses and fCSA value in rats treated with either a placebo or SB431542, a myostatin inhibitor (648).

Most of the data support that MSTN mRNA is downregulated in skeletal muscle during periods of mechanical overload in rodents and humans, and some human studies suggest that a similar response occurs with circulating MSTN concentrations. There are also various animal models that suggest that *Mstn* gene mutations affect muscular phenotype, and pharmacological studies in rodents indicate that anabolic signaling is enhanced in skeletal muscle with MSTN inhibition. However, whether a downregulation in MSTN gene expression is needed for load-induced skeletal muscle hypertrophy requires further investigation given the conflicting data discussed here. Moreover, although a plethora of human MSTN mRNA data exist, more human resistance training studies are needed to determine how MSTN protein levels, the protein levels of endogenous MSTN inhibitors (e.g., FST and FSTL3), and SMAD signaling are affected, and whether these outcomes are predictive of hypertrophic outcomes.

4.2.5. Extracellular matrix remodeling.

The extracellular matrix has long been recognized as more than a cellular scaffold. Skeletal muscle extracellular matrix components participate in a vast array of molecular processes (649, 650), from acting as a growth factor reservoir to orchestrating fundamental cell behavior in response to loading and injury (651, 652). Given that myofibers are encapsulated by the extracellular matrix, and fCSA increases occur in response to mechanical overload, extracellular matrix adaptations likely coincide with myofiber hypertrophy. In support of this theory, a 1995 review by Millward (653) presented a "bag theory" for intramyofiber protein accretion whereby the extracellular matrix of myofibers acts like a bag and, when filled, the "bag enlargement" (i.e., the extracellular matrix remodeling) is needed for myofiber growth to continue. Several lines of rodent evidence support the idea that extracellular matrix adaptations occur during periods of mechanical overload. For instance, work by the Peterson laboratory (541, 544) supports a fusion-independent role of satellite cells during hypertrophy involving an exosome-mediated downregulation in collagen-related genes in fibroblasts. Mendias et al. (654) reported that the concentrations of a collagen cross-link marker (pyridinoline) increase and concentrations of a collagen structural stability marker (hydroxyproline) decrease in skeletal muscle 3, 7, and 28 days after synergist ablation in rats. Several mRNAs for collagen proteins and matrix metalloproteases (MMPs) were also reported to be higher at one or multiple postsurgical time points. Other rodent synergist ablation studies (414, 485, 654, 655) provide further evidence that extracellular matrix adaptations occur in skeletal muscle subjected to mechanical overload. Likewise, a recent review by Brightwell et al. (48) discusses mouse data showing that several collagen-related mRNAs are upregulated 3–15 days after mechanical overload and collagen I-expressing cells increase in abundance 4–7 days after mechanical overload (541).

Studies in humans also support the notion that extracellular matrix remodeling, via increases in collagen synthesis rates or increased gene/protein expression of extracellular matrix genes (241, 531, 656–659), occurs in response to one bout or weeks of resistance training. A recent study by the Peterson laboratory sought to extensively examine how resistance training mechanistically affects extracellular matrix remodeling mechanisms. In this study, Peck et al. (660) reported that several mRNAs associated with extracellular matrix remodeling are upregulated after 14 wk of resistance training in older adults. These authors also performed elegant in vitro experiments to show that electrically stimulated myotubes secrete leukemia inhibitory factor (LIF) to stimulate the production and secretion of MMP-14 from resident macrophages. Moreover, bone-derived macrophages in vitro treated with media isolated from electrically stimulated myotubes increased type I collagen degradation, which is abrogated with an anti-LIF neutralizing antibody. Given that others have reported that resident macrophages are needed for mechanical overload-induced hypertrophy (661), the data by Peck and colleagues suggest that resident macrophages promote skeletal muscle hypertrophy, in part, through the secretion of MMPs and extracellular matrix remodeling. It should be noted, however, that although the total content of extracellular matrix components likely increases with skeletal muscle hypertrophy via remodeling mechanisms, the relative content is likely not disproportionally altered. In this regard, MacDougall et al. (67) used trichrome staining to show that the proportion of connective tissue is similar (~13% of imaged tissue) in the

biceps brachii muscle of bodybuilders and nontrained control subjects despite bodybuilders demonstrating greater fCSA values. These data were expanded upon by a recent study performed by the Roberts laboratory (73) indicating that 10 wk of resistance training in 38 younger adult men does not affect VL muscle fascial thickness, protein and histology markers of extracellular matrix content, tissue MMP activity, or the protein expression of MMP-2/9 with concomitant VL hypertrophy.

When considering the collective evidence, it seems plausible that mechanical overload initially enhances collagen protein turnover and reduces collagen content to allow for myofiber hypertrophy, and this may occur days after the initial stimulus in rodents or days to weeks into a resistance training program in humans. These events may be promoted, in part, by stromal cells in the extracellular matrix secreting factors (e.g., exosomes from satellite cells) that inhibit the expression of collagen genes from fibroblasts and myofibers and other factors that stimulate collagen breakdown (e.g., MMP14 from macrophages). However, after myofiber and tissue growth, the extracellular matrix may favor collagen synthesis over breakdown to establish a strengthened scaffold. Although data supporting this hypothesis are limited, this notion is partially supported by transcriptome-wide investigations showing that skeletal muscle extracellular matrix-related mRNAs are more dynamically altered in the earlier (rather than later) phases of resistance training (243, 662).

Finally, it is becoming more evident that excessive fibrotic tissue deposition and disorganized collagen orientation in the extracellular matrix can constrain myofiber growth and impair hypertrophic outcomes, respectively. Regarding the former, Fry et al. (541) utilized the genetic Pax7-DTA mouse model to demonstrate that satellite cell depletion before 8 wk of synergist ablation substantially increases extracellular matrix collagen deposition and impairs myofiber hypertrophy. Long et al. (663) more recently reported that pretraining collagen characteristics (e.g., total content and packing density) are negatively associated with hypertrophic outcomes in older participants who performed 14 wk of resistance training. These data, along with the data provided by Peck and colleagues in an older population (660), raise an intriguing possibility that extracellular matrix adaptations in older adults becomes more critical in promoting myofiber hypertrophy.

4.2.6. Involvement of angiogenesis in mechanical overload-induced skeletal muscle hypertrophy.

Angiogenesis, or the formation of new capillaries, is seemingly critical for mechanical overload-induced skeletal muscle hypertrophy to occur. Synergist ablation studies in rodents indicate that angiogenesis occurs in skeletal muscle in response to mechanical overload (664–666), and Degens et al. (667) reported that plantaris hypertrophy (induced via denervation of synergist muscles) is accompanied by an increase in the capillary-to-myofiber ratio. Vascular endothelial growth factor (VEGF) promotes angiogenesis (668), and plantaris hypertrophy has been reported to be impaired in skeletal muscle-specific *Vegf*-knockout mice because of a reduction in the capillary-to-myofiber ratio (669). A recent report by Ato et al. (670) demonstrated that *Heyl*-knockout mice, which show a blunted hypertrophic response to synergist ablation (671), also present impairments in mechanical overload-induced angiogenesis. In humans, VEGF has been reported to be upregulated in skeletal muscle at the mRNA and protein levels 2 and 4 h after a resistance exercise bout (672). Longer-term resistance training studies have also indicated that increased muscle capillarization occurs (673–676). Studies in older adults have indicated that lower pretraining muscle capillary density is associated with limited hypertrophic outcomes (663, 677, 678), and a 12-yr longitudinal study in older men indicates that a reduction in capillary number per fiber accompanies leg extensor muscle atrophy (679). Nederveen et al. (680) reported that satellite cells reside near capillaries in younger men, and the capillary-to-fiber perimeter exchange index is associated with the satellite cell proliferation response 24 h after an eccentric exercise bout. Thomas et al. (77) recently examined how 6 wk of unilateral leg aerobic training affected subsequent 10-wk resistance training outcomes in both legs. These authors reported that aerobic training increased type I and type II myofiber capillary number and augmented subsequent resistance training-induced increases in fCSA and satellite cell abundance. Collectively, these studies support a model in which angiogenesis during mechanical overload affects satellite cell dynamics to potentially enhance skeletal muscle hypertrophy. Furthermore, the age-related loss in muscle capillaries seemingly reduces muscle plasticity to loading paradigms and may be an involved mechanism with muscle atrophy.

Pericytes, or mural cells that encapsulate and support the microvasculature and exist in the extracellular matrix, have also received attention as a cell type that adapts to mechanical overload. Dvoretskiy et al. (681) recently demonstrated that muscle-resident pericytes upregulate genes associated with angiogenesis and extracellular matrix remodeling following an acute bout of resistance exercise in mice. Additionally, pericyte transplantation in combination with exercise training resulted in significant enhancements in the capillary-to-myofiber ratio and collagen turnover. Interestingly, the

Boppart laboratory (682) recently reported that pericyte transplantation in mice recovered fCSA losses and the capillary-to-fiber ratio after 14 days of hindlimb immobilization and remobilization. These findings suggest that pericytes function to promote angiogenesis and, in agreement with the literature cited above, suggest that enhanced capillarization promoted the hypertrophy observed during reloading. In agreement with such a mechanism, Hansen-Smith et al. (665) reported that synergist ablation in rats promoted angiogenesis, an increase in pericyte abundance, and muscle hypertrophy.

Collectively, a large body of evidence suggests that angiogenesis is needed to optimize skeletal muscle hypertrophy induced by mechanical loading. As this is a consistently researched area, more research will undoubtedly examine the stromal cells and signaling mechanisms involved with this process.

4.2.7. Alterations in muscle-specific and circulating microRNAs.

MicroRNAs (miRNAs) are ~20 nucleotides in length, are noncoding, and act to inhibit the translation of mRNAs in a sequence-specific fashion via the miRNA-induced silencing complex (miRISC) (683, 684). In 1993, Lee et al. (685) reported that *Caenorhabditis elegans* express a miRNA (lin-4) during postembryonic development to negatively regulate the LIN-14 protein. Since this foundational discovery, there has been widespread research interest into how miRNA profiles are altered in different tissues during diseased states (686, 687). Alterations in skeletal muscle miRNAs to exercise training have also received considerable attention, and several reviews have been written on the topic (44, 688–691). Various reports have detailed the skeletal muscle miRNA responses to mechanical overload in rodents and humans. McCarthy and Esser (692) reported that 7 days of mechanical overload induced by synergist ablation increased plantaris mass by 45% while reducing muscle-enriched miR-1 and miR-133a levels by ~50%. Several human studies have since provided miRNA targets that are altered acutely or after chronic training interventions (239, 248, 693–696), and recent in vitro and rodent evidence suggests that miR-16 is lower in mechanically overloaded muscle and that this may lead to the derepression of genes involved in myoblast differentiation and ribosome biogenesis (697).

Although these data have been informative, a 2019 study by Vechetti et al. (698) tempers enthusiasm in this area. To determine the role of microRNAs in muscle hypertrophy, the authors generated a skeletal muscle-specific Dicer knockout to globally deplete skeletal muscle miRNAs; notably, Dicer is responsible for producing mature miRNAs from pre-miRNAs (683). Despite an 80% knockdown of Dicer expression, miRNAs levels were only reduced by ~50% and do not appear to affect mechanical overload-induced skeletal muscle hypertrophy, atrophy induced by hindlimb unloading, or age-related muscle loss. In agreement with this finding, Oikawa et al. (699) found that Dicer inactivation only reduced myomiR levels by 30–50% in this same genetic mouse model and did not affect endurance exercise adaptations following 2 wk of voluntary wheel running. Although these studies suggest that the regulation of skeletal muscle miRNA levels is maintained through a yet-to-be described Dicer-independent mechanism, they also question the involvement of miRNAs in mechanical overload-induced skeletal muscle hypertrophy and endurance training adaptations, respectively.

4.2.8. Involvement of sex hormone signaling.

Androgens, which include testosterone and its metabolites, exert downstream effects on skeletal muscle through binding to androgen receptors localized in the sarcoplasm (700). Upon activation through ligand binding, androgen receptors undergo nuclear translocation and act as transcription factors to alter the mRNA expression of hundreds to thousands of genes (701). The administration of supraphysiological levels of anabolic steroids promotes appreciable skeletal muscle hypertrophy in males (702–707) and females (532, 708). Human and rodent studies imply that these effects are due to satellite cell-mediated myonuclear accretion (703, 709), enhanced mTORC1 signaling (presumably through noncanonical androgen signaling) (710), increased ribosome biogenesis (711), and heightened muscle protein synthesis (705, 712–714). However, a more recent study has shown that Pax7-DTA mice depleted of satellite cells experience a similar magnitude of skeletal muscle hypertrophy relative to control mice with testosterone administration (715), thus implying that satellite cells may not be needed for androgens to elicit anabolic effects. It has also been demonstrated that male androgen receptor knockout (ARKO) mice, but not female ARKO mice, exhibit impairments in muscle mass during adulthood (716), and similar data have been reported in male muscle-specific ARKO mice (717). Male muscle-specific ARKO mice have also been shown to exhibit impairments in plantaris hypertrophy after 28 days of synergist ablation versus wild-type mice (+54% vs. +115%) (718). The pharmacological blockade of the androgen receptor has been shown to slightly (albeit significantly) impair skeletal muscle growth in the gastrocnemius muscle of male rats that underwent 2 wk of hindlimb stimulation (719). Likewise, Yin et al. (720) reported that androgen receptor blockade

via flutamide prevents gastrocnemius hypertrophy in male rats that underwent 3 wk of ladder climbing. In men, longer-term testosterone suppression with a gonadotropin-releasing hormone analog has been shown to impair resistance training-induced skeletal muscle hypertrophy (713). Hence, there is a robust body of evidence to suggest that androgen signaling is involved with skeletal muscle hypertrophy, albeit it is notable that most of these data are prominently derived from testosterone administration studies or the blockade of androgen secretion and/or signaling.

Although the data above link androgen signaling to skeletal muscle hypertrophy, it is still unclear how androgen signaling under physiological circumstances affects this process. The necessity of circulating hormones in mechanical overload-induced hypertrophy was originally challenged by Goldberg who, as stated above, indicated that their role is minor in driving synergist ablation-induced hypertrophy in hypophysectomized rats (i.e., removal of the pituitary gland) (127). A series of studies published in the 1990s and 2000s suggested that transient elevations in circulating anabolic hormones [testosterone, growth hormone (GH), and IGF1] following bouts of resistance exercise are significant contributors to hypertrophic adaptations (721–726). However, several human studies have since indicated that the postexercise endocrine responses to bouts of resistance exercise do not correlate with transient anabolic signaling or long-term hypertrophic outcomes (727–731). Moreover, although there is limited evidence to suggest that androgen receptor protein content in skeletal muscle is associated with hypertrophic outcomes (732, 733), the most practical evidence against skeletal muscle androgen receptor signaling being a significant contributor to overload-induced hypertrophy comes from a comprehensive meta-analysis by Roberts et al. (734). These authors reported 12 hypertrophy outcomes from numerous resistance training studies involving male and female participants that spanned from 7 to 24 wk in duration (166, 735–742). The pooled effect size favoring relative (or body mass corrected) hypertrophy in males is small and not statistically significant (effect size: 0.07, $P = 0.31$). In explaining their findings, the authors rejected the notion that low circulating androgen levels, and presumably lower androgen signaling in skeletal muscle, are barriers to skeletal muscle hypertrophy in females.

Although some studies have examined the relationship between muscle androgen receptor protein content and hypertrophic outcomes, no study to date has extensively investigated canonical androgen receptor signaling events that occur with different loading paradigms. Assessing mTORC1 signaling in muscle tissue is straightforward given that there are relatively well-defined downstream targets that can be assayed for phosphorylation status. However, assessing whether canonical androgen receptor signaling is altered with mechanical overload is inherently more difficult given that nuclear lysates must be obtained from muscle tissue, the interaction of the androgen receptor to consensus DNA binding elements should be assessed, and transcriptional targets should be assayed. Given that the androgen receptor affects the mRNA expression of dozens to hundreds of genes, these collective endeavors can be cumbersome. Even if these assays are performed, extensive genetic screening would be needed to determine which androgen-sensitive mRNAs affected by mechanical overload promote skeletal muscle hypertrophy. Cardaci et al. (743) reported that androgen receptor DNA binding affinity is enhanced 3 h after a resistance exercise bout in men. Hence, replicating this approach with human time course resistance training studies and more downstream analyses may yield insightful information as to whether alterations in androgen receptor DNA binding affinity, as well as changes in nuclear androgen receptor concentrations and downstream mRNAs, correlate with hypertrophic outcomes. The transgenic androgen response element luciferase (ARE-Luc) mouse would also be an excellent model to utilize to further enhance our knowledge in this area (744). Specifically, this mouse model allows researchers to monitor androgen receptor DNA binding, and time course mechanical overload studies that examine muscle luciferase activity could yield insightful associations between androgen DNA binding activity and hypertrophy. Given these knowledge gaps and conflicting data in this area, more research is needed to determine the degree to which enhanced androgen signaling during periods of mechanical overload contributes to skeletal muscle hypertrophy.

As with testosterone, female sex hormones such as estrogen and progesterone operate through canonical nuclear receptor DNA binding and noncanonical protein kinase signaling (32). However, unlike exogenous testosterone administration, oral contraceptives (which consist of estrogens and/or progestins) have no meaningful impact on muscle hypertrophy in younger female participants who resistance train (512, 745–749). Furthermore, there is evidence to suggest that estrogen replacement in older women diminishes the myofibrillar protein synthesis response to a resistance exercise bout (750), and a recent meta-analysis by Javed et al. (751) indicated that estrogen-progesterone or estrogen-only replacement therapy does not prevent muscle mass loss with aging in women over the age of 50 yr. Hence, most evidence to date suggests that factors aside from sex hormone signaling are response for mechanical overload-induced skeletal muscle hypertrophy in females.

4.2.9. Involvement of inflammation via prostaglandin signaling.

Prostaglandins are lipid mediators formed through a multicatalytic reaction involving cyclooxygenase 1/2 (COX-1/2) enzymes and cell-specific prostaglandin synthases (752). Rodemann and Goldberg (753) were the first to report that prostaglandin F2α ($PGF_{2\alpha}$) increases protein synthesis in isolated rat hindlimb muscles. Two decades later, Trappe et al. (754) demonstrated in humans that 1,200 mg of ibuprofen, which inhibits the COX enzymes and blunts $PGF_{2\alpha}$ levels in skeletal muscle (755), prevents increases in muscle protein synthesis during a 24-h period following a high-volume eccentric resistance exercise bout. $PGF_{2\alpha}$ operates through the G protein-coupled prostanoid FP receptor to stimulate mTORC1 signaling in myotubes in vitro (756). There are also four known E-prostanoid (EP) receptors (EP1, EP2, EP3, and EP4), and EP4 may be especially relevant for skeletal muscle hypertrophy (237, 757, 758). Ho et al. (759) reported that prostaglandin E_2 (PGE_2) stimulates satellite cell proliferation through the EP4 receptor and that the genetic ablation of satellite cell EP4 receptor in mice impairs muscle repair after various forms of injury. These authors additionally reported that the acute administration of PGE_2 after muscle injury improves the morphology and function of muscle tissue. Hence, there are multiple lines of evidence indicating that prostaglandins likely contribute to anabolic signaling events in skeletal muscle.

Studies that have inhibited prostaglandin synthesis during periods of mechanical overload have yielded intriguing results. For instance, daily ibuprofen administration reduces plantaris hypertrophy in rats by ~50% after synergist ablation (760), and the daily administration of a COX-2-specific inhibitor in mice almost completely abrogates plantaris hypertrophy and muscle protein accretion 14 days after synergist ablation (761). In younger adults, Markworth et al. (762) reported that higher-dose ibuprofen administration (1,200 mg/day) blunts certain aspects of MAPK and mTORC1 signaling 3 and 24 h after a single resistance exercise bout, and others have reported that 1,200 mg/day of ibuprofen blunts increases in leg muscle volume by ~50% after 8 wk of resistance training compared to a group receiving 75 mg/day of acetylsalicylic acid (763). COX inhibition via ibuprofen infusion into muscle during and hours after an eccentric training bout prevents satellite cell proliferation 8 days after exercise (764). COX-2 inhibition in mice also prevents stromal cell proliferation up to 14 days after synergist ablation (761), and Mackey and colleagues (765) have reported similar findings in humans after a 36-km run. Peterson and Fyfe (28) authored a recent review citing multiple studies that examined the efficacy of cold-water immersion (CWI) as a means to potentially enhance recovery aspects during resistance training. Notably, this practice is popular in the athletic sphere given that certain lines of research indicate that CWI reduces postexercise soreness and promotes a more rapid restoration of muscle strength after rigorous exercise (766). However, limited research in this area indicates that CWI blunts certain aspects of mTORC1 signaling and satellite cell proliferation (767, 768). Although not explicitly stated, the potential contributions of reduced prostaglandin signaling cannot be discounted, given that cryotherapy has been shown to reduce tissue prostaglandins in other models of inflammation (769, 770). Hence, a reduction in exercise-induced inflammation via anti-inflammatory drugs or CWI may interfere with muscle repair or remodeling, and mechanisms that promote skeletal muscle hypertrophy may be subsequently impaired.

Like several mechanisms discussed in this review, there are also incongruent data in this area. Mikkelsen et al. (659) reported that indomethacin (a COX inhibitor) infusion into muscle during and after a resistance exercise bout did not affect myofibrillar or collagen protein synthesis rates during the 24–28 h postexercise period. Krentz et al. (771) reported that lower-dose ibuprofen administration (400 mg/day) in younger adults did not affect increases in biceps muscle thickness following 6 wk of resistance training, and Candow et al. (772) reported similar findings in postmenopausal women who consumed 400 mg/day of ibuprofen over a 9-week period. Lilja et al. (480) reported that higher-dose ibuprofen administration (1,200 mg/day) did not impair acute or chronic hypertrophy mechanisms (i.e., mTOR signaling, ribosome biogenesis, satellite cell content, myonuclear accretion, and muscle capillarization) in younger adults who partook in 8 wk of resistance training. Trappe et al. (773) reported that higher-dose ibuprofen administration (1,200 mg/day) enhanced hypertrophic outcomes in older participants, which contrasts with the data discussed above in younger participants indicating that higher-dose ibuprofen administration either does not affect or inhibits the hypertrophic response to resistance training. Damas and colleagues (310, 774) conducted a series of studies with an experimental design to test the relationship between changes in muscle damage and inflammation, myofibrillar protein synthesis, and muscle hypertrophy in previously untrained participants. Correlational analysis revealed that a greater magnitude of muscle damage and inflammation after the first four resistance exercise bouts does not confer a significantly greater hypertrophic response to 10 wk of resistance training. In addition, myofibrillar protein synthesis does not significantly correlate with muscle hypertrophy when damage and inflammation are highest (i.e., in response to the first resistance exercise session). After a progressive attenuation of muscle

damage and inflammation throughout resistance training, however, myofibrillar protein synthesis is strongly correlated with muscle hypertrophy induced by 10 wk of resistance training ($r = \sim 0.90$). As an interesting aside, Damas et al. (243) utilized a microarray approach to report that mRNAs related to inflammation and proteolysis are upregulated after the first bout of resistance exercise. However, a subsequent bout that occurred after 10 wk of resistance training in these same participants resulted in an upregulation in mRNAs related to muscle structure and contractile function, suggesting that increased training status results in a refined transcriptomic response.

Several lines of evidence published to date suggest that prostaglandin signaling in skeletal muscle is elevated in response to mechanical overload. Additionally, the human studies discussed in this section indicate that a greater reduction in prostaglandin synthesis occurs with higher doses of nonsteroidal anti-inflammatory drugs (1,200 mg/day), and this may partially abrogate resistance training-induced skeletal muscle hypertrophy compared with lower doses (i.e., 400 mg/day). However, findings are mixed and seemingly age dependent. Moreover, the necessity of inflammation, in general, for skeletal muscle hypertrophy to occur is confounded by the lack of associations discussed above by Damas and colleagues. Thus, more time course studies are needed to further elucidate the relevance of mechanical overload-induced inflammation in the hypertrophic process.

4.2.10. Involvement of β-adrenergic signaling in myofibers.

β2-Adrenergic receptor signaling operates through a canonical signaling pathway that involves *1*) ligand (catecholamine) binding to the G-coupled protein receptor, *2*) the intracellular activation of adenylyl cyclase, *3*) the production of cyclic adenosine monophosphate (cAMP), and *4*) the activation of protein kinases (e.g., protein kinase A, or PKA) (775). Active PKA phosphorylates and activates the cAMP response element binding protein (CREB) transcription factor (50). Several studies have shown that the administration of β-adrenergic receptor agonists to animals enhances skeletal muscle hypertrophy (776–786), which occurs independently of mechanical overload. Hinkle et al. (787) also reported that administration of the β-adrenergic receptor agonist clenbuterol to mice lacking β1-adrenergic receptors enhances skeletal muscle hypertrophy, whereas mice lacking β1/2-adrenergic receptors did not show this response. Woodall et al. (788) used genetic mouse models to determine that clenbuterol enhances muscle Akt activity, and transgenic mice conditionally overexpressing CREB-regulated transcriptional coregulators (Crtc) show a hypertrophic phenotype (789). Jessen et al. (790) reported that clenbuterol transiently upregulates skeletal muscle mTORC1 signaling markers in humans, and these researchers also reported that the selective β2-agonist salbutamol augments type II myofiber hypertrophy in college-aged men after 11 wk of resistance training (791). These studies, and others reporting similar findings (792), indicate that β2-adrenergic signaling can enhance mTORC1 activity in skeletal muscle. However, the administration of salbutamol (a short-acting β2-adrenergic receptor agonist) has been shown to concomitantly enhance the phosphorylation of CREB, AKT2, and the myofibrillar protein synthetic response to a single bout of resistance exercise in humans without affecting mTORC1 signaling markers (464).Thus, along with activating mTORC1 signaling, β2-adrenergic signaling seemingly stimulates skeletal muscle hypertrophy in an mTORC1-independent manner.

Select reviews such as those by Glass (793), Schiaffino et al. (10), and Sartori et al. (9) have highlighted the role that β2-adrenergic receptor signaling may have in promoting skeletal muscle hypertrophy. However, although research on this topic blossomed in the 1980s, it is perplexing that this area of the hypertrophy literature has been largely overlooked in recent years. Several recent hypertrophy reviews have prioritized the involvement of mTORC1 signaling, satellite cells, and ribosome biogenesis over β2-adrenergic signaling, which also reflects the low number of original articles on this mechanism in recent years. Although reasons as to why diminished interest in this area has occurred are difficult to posit, we speculate that certain discoveries in the field provide plausible explanations. First, clenbuterol administration was largely researched as a pharmacological means to increase meat yields in livestock, and this approach was abandoned shortly after food poisoning outbreaks in Europe linked to clenbuterol accumulation in bovine liver and meat products (794–796). Additionally, findings from the Bodine laboratory (797) indicating that mTOR inhibition in mice that were administered clenbuterol blunts, but does not completely abrogate, the hypertrophic effects of overload likely dampened enthusiasm in this area. The authors rightfully concluded that the anabolic effects of clenbuterol are mediated, in large part, through the activation of the mTOR signaling pathway. However, the authors also reported that canonical β2-adrenergic signaling might reduce atrophic signaling in an mTOR-independent manner. Indeed, more recent human findings by Jessen and colleagues (790, 791) indicating that β2-adrenergic agonists operate in an mTORC1-independent manner to enhance the protein synthesis response to resistance exercise are provocative, and these studies may instigate future research in this area.

Although the evidence regarding the anabolic effects of pharmacological β2-adrenergic agonists has been

informative, virtually no data exist on the potential anabolic effects of endogenous catecholamines during periods of mechanical overload. Several studies have shown that circulating epinephrine concentrations transiently increase during and after resistance exercise bouts (798–801), and epinephrine is a well-known β2-adrenergic receptor ligand (49, 802). Moreover, one report indicates that β2-adrenergic receptor blockade via propranolol does not affect strength outcomes following strength training interventions (789). However, mechanistic studies seeking to determine whether inhibiting β2-adrenergic receptor signaling during periods of mechanical overload affects anabolic signaling and/or skeletal muscle hypertrophy are lacking, and performing such studies will provide much needed insight in this area.

4.2.11. Involvement of angiotensin II signaling.

Research interest in angiotensin II signaling and hypertrophy was initially rooted in cardiac hypertrophy research (803–805). These findings motivated the Booth laboratory to investigate the role of angiotensin II signaling in skeletal muscle hypertrophy. Gordon et al. (806) reported that the pharmacological blockade of angiotensin II signaling through the angiotensin II type 1 (AT1) receptor reduced overload-induced hindlimb hypertrophy in rats 28 days after synergist ablation. Subsequent studies have demonstrated similar phenomena such as that the inhibition of angiotensin II production with an angiotensin-converting enzyme (ACE) inhibitor blunts the satellite cell proliferation response to synergist ablation in rat soleus muscles (807) and AT1 receptor blockade through losartan prevented skeletal muscle hypertrophy in rats following 4 wk of eccentric training (808). Notwithstanding, follow-up research in this area has been relatively sparse and conflicting. For instance, Zempo et al. (809) paradoxically reported that AT1 receptor global-knockout mice exhibited a similar magnitude of skeletal muscle hypertrophy relative to wild-type mice in response to 14 days of synergist ablation. Heisterberg et al. (810) reported that losartan administration did not affect various hypertrophy indexes after 4 mo of resistance training in older men. Another study by this group indicated that losartan generally did not affect the acute satellite cell or mRNA expression responses to one bout of exercise (811). Additionally, mouse studies suggest that heightened circulating angiotensin induces muscle atrophy through the hepatic production of proinflammatory mediators, which in turn leads to chronic elevations in muscle proteolysis (812). Hence, this collective evidence suggests that angiotensin II signaling through the AT1 receptor in skeletal muscle may coincide with overload-induced hypertrophy, albeit the conflicting evidence makes it difficult to conclude whether this hormonal mechanism exerts an appreciable role in the process.

4.3. *Emerging Mechanisms That May Be Involved with Skeletal Muscle Hypertrophy*

4.3.1. Mitochondrial biogenesis.

In vitro microbial research suggests that energy harnessed from the catabolism of four ATP molecules is required per peptide bond synthesized (813). Muscle proteolysis also requires ATP (583). Thus, although no formal estimates have been made, the bioenergetic requirement to support enhanced myofiber protein turnover and subsequent protein accretion during periods of resistance training, where mean fCSA values can increase ~15–30% in size on average (814), is appreciable. Indeed, the dysregulation of mitochondrial biogenesis and function leads to muscle loss. For instance, mice expressing a proofreading-deficient version of mtDNA polymerase gamma (PolG) show heightened mitochondrial fission and autophagy levels that coincide with muscle atrophy (815, 816). Moreover, mice overexpressing the transcriptional coactivator peroxisome proliferator-activated receptor γ coactivator-1α (PGC-1α), which is a key regulator of mitochondrial biogenesis (817), show a reduction in muscle atrophy induced by denervation, fasting, and hindlimb unloading (818, 819). Conversely, muscle-specific PGC-1α-knockout mice have been reported to show modest impairments in mechanical overload-induced plantaris hypertrophy after 14 days of synergist ablation (820). Uemichi et al. (466) demonstrated that 14 days of synergist ablation increased plantaris markers of mitochondrial remodeling to presumably enhance mitochondrial expansion and function. The investigators also used TEM to demonstrate that the area in myofibers occupied by mitochondria increased approximately fivefold after synergist ablation despite plantaris muscle mass only increasing approximately twofold. Thus, these several independent observations in rodents suggest that a critical mass of normally functioning mitochondria is needed within myofibers to maintain muscle mass and an expansion of the mitochondria may be needed to optimize load-induced skeletal muscle hypertrophy.

Although resistance training affects mitochondrial markers in humans, the data are mixed and this may be due to differences in training paradigms as well as different methodologies used to detect these markers. Costill et al. (152) were the first to report that resistance training increases mitochondrial enzyme activity markers, albeit this was reported to occur with higher-volume training.

Groennebaek and Vissing (821) authored a review including 16 human studies examining how chronic resistance training affected mitochondrial volume density and function. It should be noted that several studies cited in the review assayed muscle tissue CS activity as a surrogate of mitochondrial volume density given the findings of Larsen et al. (822) indicating that this metric exhibits a strong correlation with myofiber mitochondrial content as assessed through TEM imaging (r value = 0.84, $P < 0.05$). Additionally, older studies mentioned in the Groennebaek and Vissing review used TEM to assess the percentage of intramyofiber space occupied by mitochondria. Only two of the cited studies in the Groennebaek and Vissing review (195, 823) reported increases in mitochondrial volume density after 12 wk of resistance training, whereas the other 14 studies reported no changes or decreases. Three of the five studies that assessed markers of mitochondrial function (e.g., a tighter coupling of oxidative phosphorylation) reported improvements (824–826). These data, along with other studies (263, 827), motivated Parry, Roberts, and Kavazis (45) to author a review in 2020 positing that myofiber hypertrophy during resistance training may occur more rapidly than the expansion of the mitochondrial reticulum. However, subsequent findings by Ruple et al. (55) in college-aged males who partook in 10 wk of resistance training challenged this notion. In short, the investigators reported that mitochondrial volume density, assessed by immunostaining the outer mitochondrial TOMM20 protein, increased in type I and II myofibers and these increases outpaced fCSA increases in both fiber types. The authors also reported that CS activity values were not significantly altered with resistance training and that CS activity change score values exhibit a poor association with changes in type I and II myofiber mitochondrial area assessed by TOMM20 immunostaining.

The aforementioned reports have multiple implications. First, more research is needed to determine the veracity of using CS activity as a marker to track changes in mitochondrial volume density with resistance training. Second, increases in mitochondrial volume density in humans may precede load-induced increases in myofiber hypertrophy as observed by Uemichi et al. (466) in rodents. Pillon et al. (272), who analyzed numerous studies that examined the transcriptomic responses to resistance exercise, reported that PGC1-α mRNA is transiently upregulated after single bouts of resistance exercise. Although these data provide further indirect support for mitochondrial biogenesis being a response to resistance training, these microarray investigations may have captured an upregulation in the PGC-1α4 mRNA variant rather than the common PGC-1α1 variant. Notably, various studies in humans support that an upregulation in the PGC-1α4 isoform occurs with resistance training and that this could lead to hypertrophic signaling (e.g., downregulation in MSTN and upregulation in IGF1) while also upregulating glycolytic (rather than mitochondrion related) genes (828, 829). As a final note in this section, an expansion or remodeling of the mitochondrial reticulum during periods of mechanical overload may serve nonenergetic roles such as calcium buffering (and thus refining calcium signaling) (812), and the mitochondrial propagation of redox signaling may assist with myotube hypertrophy in vitro (813) and in rodents (814). Thus, although it appears that mitochondria play various roles in mechanical overload-induced skeletal muscle hypertrophy, much remains to be determined in deciphering all these roles.

4.3.2. Other bioenergetic adaptations in myofibers.

An emerging research theme in muscle biology involves the necessity of metabolic reprogramming and an enhanced uptake and utilization of glucose to provide substrates for myofiber growth during periods of mechanical overload. A seminal 1972 paper by Gollnick et al. (146) supported that weightlifters possessed a lower percentage of oxidative myofibers versus endurance-trained and untrained participants. Tesch and colleagues (167) indicated that 6 mo of conventional resistance training did not alter muscle enzyme activities related to the ATP-PCr or glycolytic systems, albeit earlier work by Costill et al. (152) reported that several glycolytic enzyme markers were affected with 7 wk of higher- versus lower-volume resistance training. In agreement with Costill et al., two proteomic investigations by the Roberts laboratory indicate that higher-volume resistance training induces a significant elevation in glycolytic proteins relative to lower-volume resistance training (262, 263). Verbrugge et al. (830) reported that muscle pyruvate kinase 2 (PKM2) is preferentially upregulated over the PKM1 isoform after 6 wk of resistance training in humans, and the same group found that the knockdown of both *Pkm1* and *Pkm2* blunts myotube hypertrophy in vitro. Valentino et al. (831) used microarrays to show that mechanical overload in mice activates the pentose phosphate pathway (PPP) leading to enhanced NADPH synthesis, and this mechanism was proposed to be necessary for heightened redox regulation during the early stage of hypertrophy. Other in vitro evidence suggests that glycolysis inhibition through 2-deoxy-D-glucose reduced murine and primary myotube size by ~40% (832), and the Ogasawara laboratory (833) reported that the inhibition of glycolysis reduces skeletal muscle mTORC1 signaling in rats following isometric contractions induced by hindlimb stimulations. Several

of these findings prompted Wackerhage et al. (834) to posit that metabolic adaptations accompanying mechanical overload-induced myofiber growth parallel metabolic reprogramming events that occur in cancer cells; specifically, both cell types increase the uptake and utilization of glucose for cell growth as posited by Otto Warburg in the 1920s (835). Others have similarly hypothesized that glucose and downstream metabolites are shunted to metabolic pathways in myofibers during hypertrophic growth to provide macromolecules (e.g., nucleotides, amino acids, and lipids) necessary for the anabolic processes (830, 831, 836). Thus, determining the roles these metabolic adaptations have in skeletal muscle hypertrophy represents an exciting new area of research for the field and will provide a more comprehensive description of the metabolism underlying muscle growth.

4.3.3. The muscle circadian clock.

Virtually all cells possess a circadian clock mechanism that is characterized by a ~24-h transcriptional-translational feedback loop (837). The circadian clock transcriptional program is commonly referred to as clock output, and this has been characterized in both mouse and human muscle (838–840). Clock gene expression patterns are similar between nocturnal rodents and diurnal humans when viewed in the context of rest-active cycle rather than the light- dark cycle. Beyond the commonality in core clock genes, comparisons of clock output have identified >400 common mRNAs that cycle in human and mouse skeletal muscle. Functional analysis of the common clock output mRNAs highlights the links between the circadian clock and substrate metabolism (e.g., PDK4), transcription factors (e.g., MYOD1), and proteostasis (e.g., TFEB) (841). Studies using genetic mouse models to disrupt the muscle circadian clock indicate that metabolism, mitochondrial function, and muscle function are impaired (842, 843).

Despite evidence demonstrating that clock function in muscle is critical for homeostasis, it is still unclear what the role of the muscle clock is in response to resistance exercise. In humans, Zambon and colleagues (844) provided the first and most extensive investigation as to how resistance exercise modulates the expression of circadian-regulated genes in skeletal muscle. The researchers had participants perform a session of unilateral leg resistance exercise at 1:30 PM after 8 days of controlling diet and physical activity. Muscle biopsies were obtained 6 h and 18 h after resistance exercise in both the exercised and nonexercised legs. Microarray results revealed that 704 genes and 1,479 genes are differentially expressed at 6 h and 18 h after the resistance exercise session, respectively. In addition, 40% of circadian rhythm-related genes are significantly altered 6 h after resistance exercise. Three of the core circadian clock genes (CRY1, PER2, and BMAL1) as well as the muscle-specific transcription factor MYOG were reported to also be upregulated 6 h after resistance exercise in the exercised leg. Other human data indicate that one bout of resistance exercise affects the muscle mRNA expression of core clock genes (844). However, the single-bout nature of these studies has not allowed investigators to determine whether exercise-induced alterations in the muscle circadian transcriptome are related to longer-term hypertrophic outcomes. Furthermore, the researchers did not vary the time of exercise, which would have provided stronger evidence that resistance exercise modulates circadian-related mRNA expression patterns.

It is also worth noting that a meta-analysis by Grgic and colleagues (845) indicates that the magnitude of muscle hypertrophy in humans is similar regardless of the time of day at which the resistance training is conducted. This can be viewed in one of two ways, including *1*) resistance training induces muscle circadian clock gene adjustments to better align metabolism and other cell functions around the training stimulus to better optimize skeletal muscle hypertrophy or *2*) although certain muscle circadian clock genes are responsive to resistance exercise as indicated above, resistance training has no appreciable influence on the muscle circadian transcriptome and this is not an involved mechanism in skeletal muscle hypertrophy. To test these hypotheses in humans requires the application of randomly timed exercise bouts throughout training, which is difficult to execute. Thus, the use of mouse genetic models will allow for direct testing of the requirement of the clock in adaptations to mechanical overload. Additionally, there are very few studies of resistance exercise or models of muscle hypertrophy that include true circadian design strategies, and this too warrants further investigation.

4.3.4. Microtubules and myonuclear and RNA trafficking.

The microtubule network in myofibers serves as an intracellular cytoskeletal scaffold that structurally harnesses myofibrils and other organelles. Elegant TEM work in the 1980s indicated that microtubules exist in the intermyofibrillar space and appear to wrap around myofibrils in a helical fashion (846). Boudriau et al. (847) subsequently used immunohistochemistry to show that slow- and fast-twitch myofibers possess extensive microtubule networks surrounding myofibrils and myonuclei.

Until recently, interest in the role myofiber microtubules have in exercise adaptations has been relatively

subdued. However, three recent studies have detailed critical roles that microtubules possess in myofibers, and there are stark implications regarding how the microtubule network may participate in overload-induced skeletal muscle hypertrophy. In 2021, Denes et al. (848) used an ex vivo RNA fluorescence in situ hybridization (FISH) visualization strategy to show that various mRNAs colocalize with microtubules in adult mouse myofibers, and this agrees with a 2021 report by Pinheiro et al. (849) indicating that mRNA distribution away from myonuclei in myofibers is dependent upon microtubule-mediated transport. Denes and colleagues also reported that the pharmacological inhibition of microtubule assembly leads to an aggregation of RNAs around nuclear envelopes and a robust downregulation in mRNA translation at the Z disks. These and other findings from this study led the authors to posit that microtubule-dependent RNA transport from myonuclei to ribosomes is essential for properly localizing muscle protein synthesis at the sarcomeres. The same month, Roman et al. (850) published a report demonstrating that the microtubule network transports myonuclei to contraction-induced muscle injury sites hours after exercise in mice. Additionally, these investigators performed in vitro experiments to illustrate that myotubes treated with compounds that slowed myonuclear migration exhibit a delay in sarcomere repair following laser-induced damage. On the basis of results from these experiments, the authors concluded that (contrary to the satellite cell-centric view of muscle repair) myonuclear migration is likely critical for the local delivery of mRNAs required for protein production and repair of damaged sarcomeres transiently following exercise bouts. An excellent review by Bagley, Denes, Wang, and others (51) discusses the implications of these papers for interested readers.

As an interesting aside, several investigations in cardiomyocytes have indicated that microtubule reorganization coincides with overload-induced cardiac hypertrophy (851–854), and Scarborough et al. (855) recently reported that that microtubules are indispensable for cardiac growth via spatiotemporal control of the translational machinery. However, no studies have directly sought to determine how mechanical overload in rodents or humans affects the expression or spatial orientation of microtubule proteins (e.g., α-tubulin and β-tubulin) or other proteins present in the microtubule network in skeletal muscle (e.g., nuclear lamins and desmin). Interestingly, α-tubulin is commonly used as a housekeeping protein, which is widely assumed not to be altered by various exercise stressors. In a 2016 article titled "Housekeeping proteins: how useful are they in skeletal muscle diabetes studies and muscle hypertrophy models?", Fortes et al. (856) reported that α-tubulin and γ-tubulin protein levels more than double in the extensor digitorum longus muscle of rats after 7 days of synergist ablation. Human data from the Bamman laboratory indicate that moderate and higher hypertrophic responders to 16 wk of resistance training exhibit an upregulation in muscle lysate α-tubulin protein levels, whereas lower responders do not (238). Both studies imply that myofiber hypertrophy may rely on the expansion or reorganization of the microtubule network given its putative role in RNA trafficking, protein synthesis regulation, and myofiber repair. However, this is highly speculative, and innovative investigations are needed to provide additional insight.

4.3.5. The gut microbiome-skeletal muscle signaling axis.

Several trillion bacteria inhabit the gastrointestinal tract, and these microbes affect physiological processes ranging from immune function to nutrient absorption (857). The continued development of ever more powerful sequencing technology underlies the heightened interest in the gut microbiome by allowing for the identification of individual bacterial species via metagenomic analysis. Various reviews have summarized the few studies that have investigated how exercise is able to alter the bacterial composition of the gut microbiome (858–860). A review by Mailing et al. (858) detailing how exercise affects the microbiome in humans indicates that *1*) 6 wk of endurance training increases the abundance of short-chain fatty acid (SCFA)-producing taxa and these effects are reversed after detraining (861), *2*) a trend for increased bacterial diversity occurs with 8 wk of endurance training (862), and *3*) a higher *Firmicutes*-to-*Bacteroidetes* ratio is associated with a higher aerobic capacity (863). Although informative, less research exists detailing the gut microbiome responses to resistance training. Cronin et al. (862) performed an 8-wk combined aerobic and resistance training intervention in which 90 participants were randomized to one of three groups including exercise training alone, exercise training with whey protein supplementation, and whey protein supplementation only. In short, the authors reported no significant changes in fecal taxonomic composition following the exercise interventions. Bycura et al. (864) reported how 8 wk of endurance training versus resistance training affected the gut microbiome in healthy, younger adults. Endurance training elicited more robust microbiome alterations compared to resistance training, indicating that resistance training either does not appreciably affect the microbiome or does so in a more subtle manner. Moore et al. (865) also examined fecal samples in older participants after 6 wk of resistance training. The authors reported that biome diversity metrics were not significantly altered despite this shorter period of resistance training causing a significant increase in

strength and skeletal muscle hypertrophy. Recent data in mice agree, in principle, with the two aforementioned human trials and also indicate that 4 wk of ladder climbing does not appreciably alter gut microbiome diversity metrics (866).

Although this preliminary evidence suggests that shorter-term resistance training does not appreciably impact the composition of the gut microbiome in humans, a preclinical study found that a healthy gut microbiome is necessary for skeletal muscle adaptation to exercise (867). Specifically, these authors reported that antibiotic-induced gut dysbiosis impairs soleus and plantaris muscle hypertrophy in mice subjected to 8 wk of loaded voluntary wheel running and this occurs despite drug-treated and untreated mice running similar distances. On the basis of these findings, the authors speculated that there are yet-to-be identified microbially derived metabolites that are required for optimal muscle adaptation to exercise training, which may include amino acids, bile acids, or SCFAs. Also notable are the earlier findings of Bäckhed et al. (868), who reported that germ-free mice, which completely lack commensal bacteria, display an atrophy phenotype. Finally, Castro et al. (869) recently determined that 12 wk of weighted ladder climbing in rats decreases the relative phyla abundance of *Pseudomonas*, *Serratia*, and *Comamonas*, while increasing *Coprococcus*. Although it remains to be determined, the authors speculated that the change in the composition of the gut microbiome with mechanical overload reduces inflammation, which improves metabolic and hypertrophic outcomes. This area of study is still in its infancy, and the goal of future studies will be to identify specific bacterial species and their respective metabolites that have direct or indirect roles in regulating muscle hypertrophy in response to mechanical loading.

5. A BRIEF DISCUSSION ON HOW SEX, RACE, AND AGE AFFECT OVERLOAD-INDUCED SKELETAL MUSCLE HYPERTROPHY

Skeletal muscle hypertrophy during periods of resistance training appears to be conserved between sexes. Evidence supporting this contention comes from recent meta-analyses showing that the degree of muscle hypertrophy in response to resistance training (when considering relative or body mass-adjusted values) is similar between males and females (734, 870), and similar data have been published since these meta-analyses (871). Moreover, mechanisms such as increased muscle protein synthesis, mTORC1 signaling, and the satellite cell response to resistance exercise are similar between sexes (222, 366, 731), which has been confirmed in preclinical models. However, these findings do not consider the fact that females are an understudied population in sports sciences (872, 873). As mentioned above, more data are needed in females to characterize how estrogen receptor signaling, among other inherent aspects of female physiology, may affect hypertrophic outcomes.

Although much more limited, there is evidence suggesting that race does not significantly affect the hypertrophic response to resistance training (874). Notwithstanding, more research is needed on diverse races, given that younger adult Caucasians have been the commonly examined population in many studies cited here.

Finally, although aging may impair the hypertrophic responses to mechanical overload, this is a more nuanced topic in the literature. Multiple studies have indicated that resistance training can lead to skeletal muscle hypertrophy in older adults (68, 722, 737, 740, 875–886), albeit some studies have indicated that aging impairs acute anabolic signaling and longer-term hypertrophic responses (880, 887–889). A recent meta-analysis by Straight et al. (890) suggests that increase in myofiber size with resistance training is impaired in older participants, which supports the notion that aging blunts hypertrophic outcomes. Studies that have obtained muscle biopsies have also indicated that participants >80 yr old show limited muscle plasticity in response to resistance training (520, 891) (e.g., limited increases in fCSA or satellite cell abundance). There are a variety of mechanisms that may be responsible for these age-related responses including, but not limited to, heightened low-grade inflammation with aging that blunts anabolic signaling in skeletal muscle (892, 893), older individuals showing a dampened anabolic response to protein and amino acid ingestion (894), a loss in higher-threshold motor units and myofibers (895), and impairments in skeletal muscle ribosome biogenesis and proteostasis in response to one or multiple bouts of resistance training (362, 519, 896).

As an interesting aside related to age-related responses to mechanical overload, recent rodent work has utilized senolytic cocktails (i.e., dasatinib and quercetin, or D + Q) to enhance skeletal muscle hypertrophy in older mice in response to overload. Specifically, Dungan et al. (897) reported that two gavage feedings of D + Q increases the plantaris hypertrophic response to 14 days of synergist ablation in older mice, which coincides with a blunted increase in senescence-associated beta-galactosidase-positive cells during the overload period. These researchers also reported that older mice present more senescent cells in the extracellular matrix in response to overload (but not in the basal state) relative to younger adult mice, and this is

independent of D + Q administration. Hence, the hypertrophic response may be impaired in older participants because of a heightened senescent cell accumulation in the extracellular matrix during resistance training. Likewise, more research is needed to determine whether senolytic cocktails enhance skeletal muscle hypertrophy in older participants who perform longer-term resistance training.

While the effect of aging on mechanical overload-induced skeletal muscle hypertrophy remains a salient issue in the field, it is practically undebatable that resistance training increases strength and functional outcomes in older individuals (898–902). Furthermore, a recent meta-analysis indicated that muscle-strengthening activities are inversely associated with the risk of all-cause mortality and diseases including cardiovascular disease, diabetes, cancer (overall), and lung cancer (903). Position stands by the National Strength and Conditioning Association (899) and the American College of Sports Medicine (904) provide resistance training recommendations in older persons for interested readers.

6. MOVING TOWARD A UNIFIED PERSPECTIVE ON A DEFINITION OF AND MECHANISMS INVOLVED WITH MECHANICAL OVERLOAD-INDUCED SKELETAL MUSCLE HYPERTROPHY

Several attempts in the literature have been made to define mechanical overload-induced hypertrophy in adult skeletal muscle. Russell and colleagues (905) suggest that hypertrophy is an increase in muscle mass and cross-sectional area at the whole tissue and cellular levels, and this largely agrees with Oxford's definition presented above in this review. However, more complex definitions exist in attempts to describe molecular nuance. Glass (906) defined skeletal muscle hypertrophy in adults as an increase in muscle mass, which manifests as an increase in the size, as opposed to the number, of preexisting skeletal myofibers. Roberts et al. (155) speculated that various forms of myofiber hypertrophy may occur, including conventional hypertrophy, or the proportional increase in contractile protein as myofibers increase in diameter, the disproportional increase in (or packing of) contractile protein as myofibers increase in diameter, or the disproportional increase in myofiber diameter relative to contractile protein accretion. Jorgenson et al. (1) suggest that conventional myofiber hypertrophy persists during various loading paradigms, and in some cases increases in fiber length can coincide to interactively promote tissue cross-sectional area changes. Damas, Libardi, and Ugrinowitsch (26) suggest that "true" hypertrophy occurs when there is an increase in the cross-sectional area of the myofibers or whole muscle, without the presence of exercise-induced muscle swelling. Finally, there is evidence to support that longitudinal myofiber hypertrophy may mechanistically differ from radial myofiber hypertrophy, and this is a continued area of investigation (1).

Each definition implies that muscle tissue and myofiber growth occur in tandem with contractile protein accretion. However, several points of contention exist regarding involved mechanisms. For instance, Jorgenson and colleagues (1) and Roberts and colleagues (155) argue that the current evidence is weak regarding mechanical overload-induced myofibril hypertrophy. There are also opposing viewpoints regarding whether myonuclear accretion via satellite cell fusion is obligatory for myofiber hypertrophy (545), whether myonuclei that are gained during resistance training demonstrate permanence or are lost during detraining (907–911), or whether hyperplasia contributes to skeletal muscle hypertrophy during extreme loading (912, 913). Ribosome biogenesis, rather than enhanced translational efficiency following bouts of overload, has been posited to be just as, if not more, critical in promoting muscle hypertrophy (5, 17). Researchers have exchanged viewpoints regarding whether edema is a significant contributor to the early stages of muscle hypertrophy (774, 914, 915), and the evidence is mixed concerning whether myofiber length increases appreciably contribute to hypertrophy with conventional resistance training (913, 916). Recent preliminary data in humans indicate that individuals may exhibit different morphological adaptations to the same resistance training program (917): specifically, some individuals may show tissue-level hypertrophy predominantly through fascicle length changes, whereas others may show tissue-level hypertrophy predominantly through fCSA increases. One of the more provocative questions related to myofiber hypertrophy was posed in 1982 by J. D. MacDougall and colleagues who pondered (56) whether

> ". . .skeletal muscle fibers possess an unlimited capacity for protein synthesis and enlargement, or is there a maximal or optimal size which can be attained?"

Despite the remarkable discoveries that have been made during the past four decades, this fundamental question remains to be answered.

The present authors agree that skeletal muscle hypertrophy in response to mechanical overload generally involves cross-sectional (or radial) growth at the tissue and myofiber levels and that this coincides with a proportional expansion of the extracellular matrix. Moreover, although less resolved, limited literature

Table 1. ***Summary of mechanisms discussed in this review***

Mechanism	Responses to Mechanical Overload	Knowledge of Current Role(s)	Knowledge Gaps
mTORC1 signaling	↑↑↑	mTORC1 signaling is critically involved with skeletal muscle hypertrophy through increased translation initiation and/or elongation.	Further interrogation of the upstream activating signals during mechanical overload
mTORC1-independent signaling	↑↑↑	MAPK signaling and other mTORC1-independent signals are transiently activated after a bout of resistance exercise to presumably affect aspects of transcription and translation.	Further elucidating the role MAPK signaling and other mTORC1-independent signals (e.g., YAP and TRIM28 phosphorylation) have in promoting skeletal muscle hypertrophy
Ribosome biogenesis	↑↑↑	Increased translational capacity	Determining whether ribosome specialization occurs with overload and, if so, determining whether this is a critical aspect of hypertrophy
Satellite cells	↑↑↑	Myonuclear accretion via fusion, muscle repair, and nonfusion roles	In humans, validating preliminary animal findings suggesting that satellite cells coordinate extracellular matrix adaptations during overload; also examining whether hypertrophy can proceed in the absence of satellite cell-mediated myonuclear accretion in humans with certain diseases where satellite cell counts are reduced (e.g., MYMK mutations); finally, determining how satellite cell fusion alters molecular processes in myofibers (single-cell studies) or myofiber morphology
Genetic variants	Inherently present; no changes to overload	Various single-candidate polymorphism studies show small hypertrophic advantages with certain genotypes.	Performing deep sequencing efforts to identify novel variants and adopting statistical approaches to examine the combinatorial effects of multiple variants
Epigenetic alterations	↑↑↑ and ↓↓↓	Methylation changes occur across hundreds of genes in the nuclear genome, and preliminary evidence suggests demethylation of mitochondrial genome with resistance training in humans.	Determining whether gene-specific methylation responses to overload are needed for hypertrophy to occur; further determining whether prolonged DNA demethylation during periods of mechanical overload confers more robust skeletal muscle hypertrophy
Muscle proteolysis	↑↑↑ early into training, but response subsides with increased training status.	Potentially needed for removing damaged proteins and organelles after initial stages of resistance training	Determining which proteolytic system(s) is primarily responsible for adaptive responses early (i.e., weeks) and later (i.e., months to years) into training; additionally, determining whether these proteolytic systems are required for muscle hypertrophy to occur in response to loading paradigms and/or whether synchronization between synthesis and proteolysis directs the degree of hypertrophy
Myostatin markers	↓↓↓	Numerous lines of evidence suggest that resistance training acutely and transiently decreases muscle MSTN mRNA levels.	Elucidating how MSTN pathway signaling (e.g., SMAD2/3 phosphorylation and the mRNA expression of downstream targets) is transiently affected during the initial and later stages of overload and whether these events are critically involved in the hypertrophic response

Continued

Table 1.—*Continued*

Mechanism	Responses to Mechanical Overload	Knowledge of Current Role(s)	Knowledge Gaps
Extracellular matrix remolding	↑↑↑	Markers of extracellular matrix remodeling are altered during periods of resistance training, but much of this work has been confined to mRNAs.	Broadening the scope of extracellular matrix remodeling markers during resistance training studies to determine whether remodeling is required or merely coincides with skeletal muscle hypertrophy
Angiogenesis	↑↑↑	Preliminary evidence suggests that capillary number per fiber prior to resistance training is associated with hypertrophic response to training.	Determining whether the magnitude of angiogenesis induced by resistance training (and/or enhanced microvessel function) enhances muscle hypertrophy
Muscle microRNA expression	↑↑↑ and ↓↓↓	Genes involved with IGF1/PI3K/AKT/mTOR signaling are directly and/or indirectly regulated by various miRNAs that are altered in response to overload.	Moving beyond microRNA-omics-based studies in humans to show a core set of microRNAs involved with or needed for skeletal muscle hypertrophy
Testosterone signaling	???	Transient postexercise responses in circulating testosterone concentrations do not correlate with intracellular anabolic signaling events (e.g., mTORC1 signaling or muscle protein synthesis) and hypertrophy. However, muscle hormone receptor protein content modestly correlates with anabolic outcomes in some studies.	Determining whether androgen DNA binding is altered during periods of overload and which of the identified hormone receptor-affected genes are involved with skeletal muscle hypertrophy
Inflammation through prostaglandin signaling	↑↑↑	Coincides with robust elevations in protein synthesis and satellite cell proliferation during in the initial phases of resistance training.	Determining whether certain aspects of inflammation (e.g., EP and FP receptor signaling) are needed for, or merely coincide with, skeletal muscle hypertrophy
β-Adrenergic signaling through endogenous catecholamines	???	The administration of β-adrenergic receptor agonists promotes skeletal muscle hypertrophy.	Determining whether intrinsic β-adrenergic receptor signaling via endogenous catecholamines, in part, promotes skeletal muscle hypertrophy during periods of mechanical overload
Angiotensin II signaling	???	Preliminary animal evidence suggested angiotensin II signaling is involved with overload-induced skeletal muscle hypertrophy. However, follow-up animal studies suggest angiotensin II signaling may blunt hypertrophic responses, and human data in this area are mixed.	Determining whether intrinsic angiotensin II signaling blunts, enhances, or does not affect hypertrophic outcomes in humans
Mitochondrial biogenesis	↑, ↓, or ↔	The increase in mitochondrial volume density may precede or concomitantly occur with muscle hypertrophy.	Demonstrating whether an expansion of the mitochondria is required for myofiber hypertrophy, or whether mitochondrial biogenesis, mitochondrial expansion, and myofiber hypertrophy merely coincide with one another
Other bioenergetic adaptations	↑↑↑	Resistance training can promote differential metabolic adaptations in skeletal muscle.	Determining whether metabolic adaptations (e.g., enhanced glycolytic flux) provide skeletal muscle with substrates needed for cell growth

Continued

Table 1.—*Continued*

Mechanism	Responses to Mechanical Overload	Knowledge of Current Role(s)	Knowledge Gaps
Muscle circadian transcriptome	???	The oscillation of core clock genes in muscle transcriptionally regulates hundreds of genes related to metabolism, protein turnover, ribosome biogenesis, and other processes.	Determining whether the muscle circadian transcriptome is altered (or disrupted) during periods of overload; if so, is this an involved mechanism with molecular adaptations (e.g., ribosome biogenesis or the altered expression of metabolic genes)?
Microtubule networks, and myonuclear and RNA trafficking	???	Studies suggest that *1*) microtubule-dependent RNA transport from myonuclei to ribosomes is essential for the translation process to occur and *2*) myonuclear trafficking to focal injury sites occurs through microtubules.	Determining whether microtubule network expansion scales with hypertrophy and if this process is needed to support RNA and myonuclear trafficking
Microbiome alterations	Minimal changes in bacterial species diversity	Preliminary evidence suggests that resistance training may not drastically alter diversity metrics in the gut microbiome.	Determining whether metabolites produced by the gut microbiome change in response to training; if so, do they act as signals to affect anabolic signaling pathways?

↑↑↑ or ↓↓↓, several independent laboratories have shown increases or decreases in markers associated with mechanism; ↑, ↓, or ↔, less evidence supports the mechanism being involved in mechanical overload-induced skeletal muscle hypertrophy; ???, involvement in mechanical overload-induced skeletal muscle hypertrophy has not been well elucidated relative to other discussed mechanisms. IGF1, insulin-like growth factor 1; PI3K, phosphatidylinositol 3-kinase; MSTN, myostatin; mTOR, mammalian/mechanistic target of rapamycin; mTORC1, mammalian/mechanistic target of rapamycin complex 1.

discussed here (55, 99) [and in other recent reviews (1, 155)] supports the idea that myofibril and mitochondrial content mostly scale with myofiber hypertrophy induced by resistance training in humans. However, there are limited data or knowledge gaps that require further investigation into how different modes of mechanical overload affect *1*) longitudinal tissue and myofiber growth, especially since most investigations examine radial growth; *2*) type I versus type II myofiber hypertrophy, which seems to be load dependent in humans albeit not well delineated (814); *3*) alterations in the size and number of myofibrils; *4*) the three-dimensional properties of myofibrils, the mitochondrial and sarcoplasmic reticula, and the cytoskeletal network in type I and II myofibers; and *5*) the time courses of *1*–*4* listed here. Additionally, a central tenet of this review is that several mechanisms are required for mechanical overload-induced skeletal muscle hypertrophy as shown in **TABLE 1**, and much remains to be learned in these areas as well.

7. CONCLUSIONS

Skeletal muscle hypertrophy research has rapidly evolved since the landmark report by Morpurgo in 1897. Pioneering discoveries in the field have motivated others to adopt innovative methodologies and drive the research boundaries in meaningful ways. Given the rapid advancements in molecular-based research techniques, investigations in upcoming years will continue to confirm or refute which of the discussed mechanisms are obligatory for (rather than coinciding with) load-induced skeletal muscle hypertrophy. More importantly, these efforts will likely unveil novel mechanisms that continue to reshape our thinking in this area of muscle biology.

CORRESPONDENCE

M. D. Roberts (mdr0024@auburn.edu); K. A. Esser (kaesser@ufl.edu).

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AUTHOR CONTRIBUTIONS

M.D.R. conceived and designed research; M.D.R., J.J.M., T.A.H., G.A.N., and A.N.K. prepared figures; M.D.R., J.J.M., T.A.H., S.M.P., A.L.M., G.A.N., M.D.B., A.N.K., P.T.R., R.O., C.A.L., C.U., and F.W.B. drafted manuscript; M.D.R., J.J.M., T.A.H., S.M.P., A.L.M., G.A.N., M.D.B., P.T.R., R.O., C.A.L., C.U., F.W.B, and K.A.E., edited and revised manuscript; M.D.R., J.J.M., T.A.H., S.M.P., A.L.M., G.A.N., M.D.B., A.N.K., P.T.R., R.O., C.A.L., C.U., F.W.B, and K.A.E. approved final version of manuscript.

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