Chapter 8
Skeletal Muscle Culture Under Spaceflight Conditions

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Historical Landmarks

1968—Yaffe describes establishment of a rat L6 myoblast cell line capable of generating spontaneously contractile myotubes in culture [1].

1975—Dietlein describes reduction in muscle volume and function associated with extended spaceflight during Apollo missions [2].

1977—Yaffe and Saxel describe the establishment of a mouse C2C12 myoblast cell line capable of generating myotubes in culture [3].

1982—Vandenburgh describes the ability of uni-axial stretching to promote the formation of contractile myotubes from primary avian myocytes within a collagen gel matrix [4].

1994—Kulesh et al. fly the first experiment aboard the Space Shuttle utilizing skeletal muscle cells (rat L8 myoblast cultures) resulting in the creation of a new, non-fusogenic myoblast variant known as the L8SF cell line which retained its altered phenotype even on return to Earth [5].

1997—Molnar et al. establish rat primary myoblast cultures in the rotating wall vessel (RWV) demonstrating myotube formation during microgravity analog conditions [6].

1999—Vanderburgh et al. fly the first experiment aboard the Space Shuttle to utilize cultured 3-D skeletal muscle organoids constructed from myoblasts to demonstrate that microgravity exposure can induce muscle atrophy even without the removal of extrinsic mechanical load [7].

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(continued)
2000—Dennis and Kosnik establish 3-D skeletal muscle organoids constructed from both myoblasts and fibroblasts that demonstrated superior contractile myotube properties than those constructed from myoblasts alone [8].
2005—Levenberg et al. establish 3-D skeletal muscle organoids created from myoblasts, fibroblasts, and endothelial cells capable of forming a functional microvascular network as well as contractile myotubes when transplanted into the whole animal [9].
2011—Van der Schaft et al. establish 3-D skeletal muscle organoids created from myotubes and endothelial cells in which microvascular formation and myotube alignment were driven by uni-axial load placed on the construct [10].
2013—Lee and Vandenburgh describe a contractile 3-D skeletal muscle organoid in which the removal of uni-axial load induced a dose-dependent myotube atrophy response and concomitant reduction in force production approximating that observed in intact muscle undergoing microgravity exposure [11].

8.1 Introduction

The physiological response of the human body to the microgravity environment encountered during spaceflight involves adaptations at numerous levels, ranging from those which affect the whole body, discrete physiological systems and/or tissues down to individual cell types. This chapter focuses primarily on the effect of microgravity exposure on skeletal muscle and how cell culture-based models have been used to investigate such effects. While the ultimate goal of such studies has been to understand the effects of spaceflight as it relates to maintaining skeletal muscle mass and function in crew members during and after spaceflight missions, such culture models also show potential for studying the underlying mechanisms at play in a variety of pathological conditions involving skeletal muscle in the terrestrial environment.

At the outset however, it must be clearly stated that while available tissue culture models provide an excellent means of studying the effects of microgravity exposure on the structure and function of skeletal muscle cells in isolation, information generated using these approaches must be viewed through a prism which recognizes the potential impact of various signals sensitive to spaceflight that emanate from other physiologic systems not represented within such skeletal muscle tissue culture analogs. For example, the complex, multi-cellular three-dimensional (3-D) architecture of skeletal muscle tissue (i.e., myofibers, satellite stem cells, neural cells, vascular cells) for the most part is not represented within skeletal muscle tissue culture analogs routinely available. In addition, in vivo biological signals which target skeletal muscle tissue that are derived from physiological systems known to be adversely affected by microgravity exposure, such as the endocrine, vascular, and neuronal systems, are absent in these culture models. Therefore, it is of the utmost importance that any conclusions drawn from skeletal muscle cell responses observed in

such tissue microgravity culture analogs be interpreted relative to additional effects already known to impact the whole body during spaceflight. As such, where appropriate the author has included information relevant to the similarities and the differences inherent between various tissue culture models of skeletal muscle structure and function in vitro relative to the response of whole skeletal muscle tissue in vivo.

8.2 General Effects of Microgravity Exposure on Skeletal Muscle

In general, microgravity exposure during spaceflight of both crew members and animals results in a rapid loss of muscle mass and function [12–17]**, especially in those muscles associated with the maintenance of body posture and ambulation in terrestrial gravity known as the "anti-gravity" muscles.** It is the negative impact of such skeletal muscle adaptation/de-adaptation upon crew member health, performance, and ultimately mission success which drives the need to understand the deleterious physiological and biochemical effects of spaceflight upon skeletal muscle cell function. While numerous muscle group and species-specific effects have been reported, in general terms microgravity exposure of the musculoskeletal system in vivo results in: (1) disruption of protein balance within the myofiber [18, 19]; (2) an overall reduction in both whole muscle and individual myofiber cross-sectional area (CSA) [20–24]; (3) an enhanced Type II myofiber selective atrophy response [22, 25, 26]; (4) evidence in some situations of a Type I to Type II myofiber phenotypic shift [24, 26–32]; (5) a reduction in myonuclear to sarcoplasmic volume ratio [22, 33]; (6) a reduction in neuromuscular junction (NMJ) size/complexity; and (7) a reduction in the number of capillary vessel/myofiber contacts (i.e., capillary bed tortuosity) within the muscle [14, 15, 23, 34, 35]. These biochemical, cellular, and histological alterations are all paralleled with concomitant decreases in overall skeletal muscle physiological function such as muscle tone/stiffness, neuromuscular activation patterns, strength, and endurance at the whole body level [12, 16, 36–40].

While some of the negative effects of microgravity exposure on skeletal muscle function can be directly attributed to the loss of myofibrillar contractile proteins such as myosin heavy chain (MHC) protein [41], or the disruption of myofiber cytoskeletal proteins responsible for maintaining sarcomeric integrity such as titin and nebulin [42, 43], it is still unclear whether or not these myofiber effects are primarily mediated via intrinsic or extrinsic factors. For example, microgravity-induced reductions in circulating levels of muscle growth factors, such as pituitary-derived growth hormone (GH) or liver-derived insulin-like-growth factor-1 (IGF-1) [44–46], most likely have subsequent down-stream effects on intra-myofiber signaling pathways within skeletal muscle tissue [47]. These include down-regulation of the hypertrophic Akt/mTOR pathway [48, 49] and up-regulation of the atrophic myostatin/activin IIB receptor pathway [50–52], which acting in concert appear to be primarily responsible for myofiber protein balance and the overall maintenance of

muscle mass in vivo [53–55]. In addition, the removal of mechanical load during spaceflight normally experienced by the musculoskeletal system under terrestrial conditions has already been shown to induce effects not only within the myofiber, such as disruption of normal gene transcription rates [56] and alterations in myonuclear-sarcoplasmic volume ratio [22], but also external to the myofiber but still within skeletal muscle tissue, such as reductions in the size and function of the NMJ [15] leading to possible myofiber type shifting [12, 23, 26, 57] and impaired neuromuscular function [35, 58]. Furthermore, the removal of mechanical load from skeletal muscle has also been shown to initiate intra-myofiber calcium-dependent proteolysis [59] and alterations in sarcoplasmic reticulum calcium pump (SERCA) expression [60, 61] due to an as yet undefined pathway potentially related to intrinsic membrane tension within the myofiber [62].

One additional level of complexity associated with the response of skeletal muscle specifically related to the mechanical unloading experienced during spaceflight is the apparent fiber type specific nature of these effects, linked not only to particular fiber types but also the species studied and the function of the specific muscle in which the fiber type resides. For example, during both short-term spaceflight [25] and bed rest [63], the human *m. vastus lateralis* muscle undergoes Type IIb myofiber selective atrophy whereas the human *soleus* and *gastrocnemius* muscle in the same leg undergo Type I myofiber selective atrophy following extended spaceflight [12] or bed rest [64]. Similar Type I myofiber selective atrophy has also been observed in the *soleus* muscle of the rat hind limbs following spaceflight [31] and terrestrial unloading resulting from hind limb suspension of the animals [65, 66]. It remains unclear why the same myofiber type in different muscles of the same individual crew member or animal exhibits different atrophy responses during spaceflight or terrestrial unloading. However, such myofiber selective responses appear related to the functional role of these particular muscles during standing/ambulation under terrestrial conditions (i.e., anti-gravity muscles), as well as the disruption of neural input to the myofibers due to reduced proprioceptive mechanoreceptor activation during spaceflight and terrestrial unloading in vivo [65, 67].

The complex response of whole skeletal muscle tissue to actual spaceflight or during terrestrial analogs of whole body microgravity exposure (i.e., bed rest, dry immersion, rodent hind limb suspension, etc.) clearly demonstrates that multiple cell types and a variety of signaling pathways are involved in the adaptive response of skeletal muscle tissue to microgravity. As such, in the context of this review it is important to recognize that most planar tissue culture experiments utilizing isolated skeletal muscle progenitor cells (i.e., satellite cells, primary myoblasts) or differentiated skeletal muscle cultures (i.e., myotubes), carried out under actual or terrestrial analog conditions of microgravity exposure, lack the complex multi-system, multicellular interplay required to truly mimic the overall response of intact skeletal muscle to spaceflight in vivo.

This is where tissue culture analogs in general and skeletal muscle tissue culture models in particular become extremely useful in investigating the cellular and biochemical responses of cells to a variety of environmental conditions such as microgravity exposure or mechanical unloading. By cataloging these responses while

manipulating specific experimental conditions in a defined fashion, skeletal muscle tissue culture analogs can be used to develop causal relationships between particular stimuli and specific cellular responses. This approach provides a wealth of information on the response of individual muscle cells and/or more complex, 3-D organoid skeletal muscle tissue culture analogs to a variety of altered stimuli, which in turn can then be integrated into a larger model of how intact muscle tissue responds in vivo to altered environmental conditions such as microgravity exposure. In addition, the knowledge gained utilizing such tissue culture analogs relative to understanding the underlying cellular mechanisms involved in the response of skeletal muscle to spaceflight (including those primarily caused by mechanical unloading) has direct application in understanding various terrestrial disease states, such as age-related sarcopenia [68], cancer cachexia [69], muscle cell autophagy [70], and obesity-related muscle atrophy [71].

8.3 Skeletal Muscle Tissue Culture Models

8.3.1 General Considerations

As discussed above, the response of intact skeletal muscle to spaceflight involves a complex interplay between muscle function, myofiber type, neuronal innervations, and various circulating factors that modulate myofiber behavior and function. While it is generally accepted that the majority of effects initiated within skeletal muscle during spaceflight are as a consequence of the removal of the mechanical loading normally experienced by the musculoskeletal system under terrestrial conditions, the effects of microgravity exposure per se (i.e., removal or disruption of a defined gravitational vector) should not be ignored. However, it should be remembered that such relatively subtle effects are difficult to isolate even under well controlled tissue culture conditions when compared to the more potentially profound effects of removing the almost continuous mechanical stimulation experienced by skeletal muscle cells created as a consequence of voluntary muscle contraction combined with terrestrial gravity loading in vivo.

Regardless of the predominant affect of spaceflight (i.e., mechanical unloading vs. removal of gravitational vector) on skeletal muscle cells, the complex multifactorial response of myofibers to spaceflight or ground-based analogs of spaceflight in vivo raises an as yet unresolved issue relating to initial source of the skeletal muscle cells. For example, there is clear experimental evidence that the response to spaceflight is dependent on the specific muscle studied, its physiological function in vivo, and the myofiber type(s) present within the muscle [12, 23]. For example, cultured skeletal muscle cells isolated from an adult *soleus* muscle (predominantly made up of Type I myofibers) may respond differently to spaceflight or decreased mechanical loading under terrestrial conditions to those cells isolated from an adult *gastrocnemius* or *m. vastus lateralis* muscle (containing both Type I and Type II myofibers). Any responses observed may be further complicated when species variations (e.g., avian vs. mammalian, rodent vs. human) and/or muscle age differences

(e.g., embryonic vs. adult tissue) are considered. As such, it is important to consider such "source" factors when interpreting observations made concerning the effects of microgravity exposure on cultured skeletal muscle cells in vitro. However, while skeletal muscle cells isolated from different muscles may initially retain some of their in vivo phenotypic characteristics, tissue culture studies which have specifically compared primary rodent myoblast cell populations derived from various muscles indicate that any such phenotypic programming appears to be lost after a relative short period of propagation under standard tissue culture conditions (i.e., three to four passages) resulting in myoblast cultures appear to have a uniform phenotype regardless of muscle origin [72].

8.3.2 Primary Skeletal Muscle Cell Cultures

In the case of skeletal muscle, the individual cell type most commonly used for tissue culture studies is the undifferentiated myoblast (derived from the muscle satellite cell population in vivo) or the differentiated myotube (consisting of multiple fused myoblasts forming a structure analogous to a myofiber in vivo) [73]. Primary myoblasts are isolated by mechanical and/or enzymatic disruption of fresh muscle tissue and can be isolated from a range of species and muscle types harvested from avian, amphibian, rodent, or human subjects. Skeletal muscle myoblasts are adherent cells requiring a culture substratum to attach and grow in culture. While primary myocytes can be directly cultured on a variety of tissue culture plastic surfaces, the use of extracellular matrix (ECM) coatings, such as collagen Type I or fibronectin, appear to be most suitable for ensuring cell attachment and proliferation in monolayer culture. Primary human myocytes become senescent within six to eight passages in culture, although addition of specific growth factors to the medium, such as fibroblast growth factor (FGF) or insulin-like growth factor-1 (IGF-1) [74, 75], have been reported to delay myoblast senescence while maintaining cell proliferation rates.

Since primary myoblast cultures are assumed to be derived from skeletal satellite cell populations, myoblast cultures have been used to study individual satellite cell responses to a variety of environmental and biochemical stimuli including microgravity exposure. In addition, under specific culture conditions myoblasts will fuse together to form a multi-nucleated structure known as a myotube, analogous to a myofiber in vivo. As such, primary myoblast cultures undergoing myotube formation have also been used to investigate the effects of various stimuli on the satellite cell–myofiber fusion process [76–78] shown to be central to the control of myofiber hypertrophy and the formation of new myofibers in response to injury in vivo [79]. In general, such primary myotube cultures will express markers of terminal differentiation, such as myosin heavy chain (MHC) [80] or creatine phosphokinase (CPK) [81], while organizing sarcomeric-like structures (i.e., striations) within the myotube [82]. While not usually spontaneously contractile, primary myotubes containing sarcomeres can be induced to contract after sarcolemma depolarization as a result of an externally applied electrical current or chemical depolarization [83].

8.3.3 *Skeletal Muscle Cell Cultures from Established Myocyte Cell Lines*

In order to overcome the limitations inherent in using primary myoblasts as a source for skeletal muscle cell tissue culture studies (such as a limited capacity for proliferation in culture and a reduction in myotube formation rate as culture age increases), investigators have turned to the use of a number of rodent myoblast cell lines that can be easily propagated in culture while still maintaining the ability to differentiate and fuse into myotubes (Table 8.1).

The three most commonly used, commercially available myoblast cell lines for studying skeletal muscle cell responses in vitro are the embryonic-derived rat omega (RMo) cell line [84], the adult mouse-derived C2C12 cell line [3], and the neonatal rat L6 cell line [1]. Each of these three cell lines has unique characteristics relevant to investigating the effects of various experimental conditions on specific skeletal muscle cell properties. For example, both rat-derived RMo and L6 myoblasts are capable of fusing to form multi-nucleated myotubes which are striated and exhibit spontaneous contraction even in the absence of neuronal cells [1, 84]. While contractility in RMo and L6 myotubes spontaneously arises, this contractile activity is capable of being "paced" by altering the myotube membrane potential either electrically [87] or chemically [88, 89]. Striated RMo myotubes also generate a complex intra-myofiber membrane system containing cholesterol (Fig. 8.1) similar to that observed in the sarcoplasmic reticulum system of intact human and rodent myofibers. Since the ability to regulate intra-myofiber calcium levels is a prerequisite for excitation–contraction (E–C) coupling within skeletal muscle myofibers in vivo [91], the formation of such intra-cellular membrane systems within cultured myotubes (Fig. 8.1) is almost certainly a requirement for spontaneous contractility to occur in vitro. Mouse-derived C2C12 myoblasts can also form striated, multinucleated myotubes but do not normally exhibit spontaneous contractile behavior. However, striated C2C12 myotubes can be induced to contract using an external

Table 8.1 Rodent myoblast cell lines commonly used to study muscle cell function in vitro

Myoblast cell line	Species	Tissue source	Myotube formation	Striations	Spontaneously contractile	References
RMo	Rat	Embryonic muscle	Yes	Yes	Yes	[84]
L6	Rat	Neonatal, quadriceps muscle	Yes	Yes	Yes	[1]
C2C12 (originally derived from C2 cell line)	Mouse	Adult, quadriceps muscle	Yes	Yes	No (inducible)	[3]
MM14	Mouse	Adult muscle	Yes	No	No	[85]
Sol8	Mouse	Adult, soleus muscle	Yes	No	No	[86]

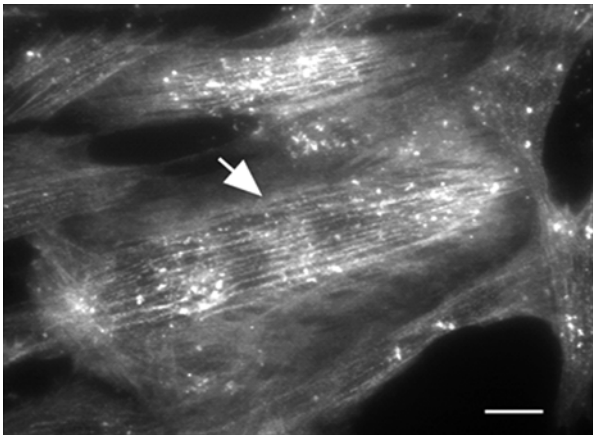

Fig. 8.1 Fluorescent micrograph of RMo myotubes immunostained for cholesterol. RMo myoblasts were differentiated into myotube structures for 7 days in culture medium containing 2 % horse serum on collagen Type I-coated Permanox plastic tissue culture chamber slides. Cells were fixed with freshly generated formaldehyde-PBS solution (pH 7.2), permeabilized using TX-100, and then stained with a monoclonal antibody directed against cholesterol as previously described [90]. Note the labeling of cholesterol-containing internal membrane components (*arrow*) aligned with myofibril direction within the RMo myotube (Bar—20 μm)

electrical current [92–94] to induce depolarization of the sarcolemma membrane, indicating that C2C12 myotubes possess the intra-cellular components required for E–C coupling and sarcomere contraction. One common issue encountered with the use of C2C12 cells to study muscle cell function in vitro is the loss of myoblast proliferative capacity and the ability to reproducibly form myotubes in culture. As such, it is very important to ensure that when utilizing C2C12 cells that the appropriate culture conditions are employed, such as high serum/low density seeding densities for myoblast propagation and low serum/high density seeding densities for myotube formation. By adhering to these conditions numerous multi-nucleated myotubes (Fig. 8.2) that express adult Type II MHC protein can be created in culture, thus providing for a well-defined and reproducible means of creating differentiated C2C12 myotube cultures for subsequent study.

8.3.4 Three-Dimensional Myoblast/Myotube Culture Approaches

There is wide recognition that conventional planar culture conditions which involve a single monolayer of adherent cells do not mimic the complex, three-dimensional (3-D) cellular architecture, intercellular interactions and biochemical signaling that occur within whole tissue in vivo. Recognition of this limitation has led many investigators to utilize 3-D tissue culture techniques to

Fig. 8.2 Light micrograph of C2C12 myotube cultures stained for myosin heavy chain (MHC) II protein using immune-peroxidase labeling. Proliferating C2C12 myoblasts were cultured in DMEM/F12 medium containing 10 % heat-inactivated fetal bovine serum (10%FBS.DMEM/F12) in 12 well culture plates coated with collagen Type I protein. Myoblasts were allowed to attach overnight in 10%FBS.DMEM/F12 after which time the medium was replaced with DMEM/F12 medium containing 2 % heat-inactivated horse serum (2%HS.DMEM/F12). Myoblasts were cultured for an additional 7 days with a single medium exchange at Day 3 of culture. Myotube formation begins at day 3–4 of culture under serum-deprived conditions. After 7 days, myotube cultures were fixed and stained using a rabbit anti-MHC II polyclonal antibody. Note the numerous large multi-nucleated myotubes staining for MHC II protein (*asterisks*) with little or no staining being observed in the remaining unfused myoblasts (*arrows*) (Bar—10 μm)

mimic such spatial interactions in a wide variety of cell types [95] including skeletal muscle [96, 97]. One of the simplest versions of this 3-D culture approach to study the effects of microgravity exposure on skeletal muscle is the formation of 3-D skeletal muscle organoids using the rotating wall vessel (RWV) tissue culture system [6, 98] originally developed by NASA as a means of modeling microgravity exposure (see Chap. 2). Both primary rat myoblast cells [6] and mouse C2C12 myoblasts [98] have been grown in the RWV system attached to collagen-coated Cytodex 1 micro-carrier beads to act as a culture substratum. 3-D skeletal muscle organoids generated in this fashion within the RWV consisted of myoblast cell/bead aggregates connected to each other by individual myoblast cells and/or myotubes. When 3-D organoids generated in the RWV were compared to identical 3-D organoids maintained under static culture conditions in Teflon culture bags, static 3-D organoids were much larger in size than those generated in rotational culture in the RWV [6]. These results were surprising considering the generally growth-promoting effects of RWV culture conditions on most other cell types related to mass transfer effects that result in better nutrient supply/waste product removal. Later studies utilizing mouse C2C12 myoblasts also grown on collagen-coated Cytodex-1 microcarrier in the RWV demonstrated the formation of complex networks of multinucleated myotubes [98] that was paralleled by a reduction in overall C2C12 myoblast proliferation rate and reduction in 3-D organoid size relative to static

control cultures. While similar to the effects previously observed in primary rat myoblasts grown in the RWV [6], these studies also indicated that C2C12-derived 3-D organoids grown under RWV conditions exhibited a less differentiated phenotype (i.e., reduced expression of myogenin, a-actinin, myosin, and tropomyosin) than 3-D organoids grown under static conditions [98]. The reduced differentiation observed in C2C12 3-D organoids grown in the RWV [98] was suggested to be a result of increased mechanical shear forces placed on the myoblasts during rotational culture as compared to static conditions. While RWV culture conditions are considered to produce relatively low levels of mechanical shear on individual cells during rotation, the shear effects created when larger 3-D organoids rotate around their own axis during RWV culture are less well understood. We have previously demonstrated that mechanical loading induces skeletal muscle cell membrane damage both in vivo [99, 100] and in vitro [81], resulting in the release of both CPK and fibroblast growth factor (FGF) from the sarcoplasm, the latter being a potent proliferative stimulus for myoblasts. As such, it is entirely possible that mechanical shear stress induced by RWV culture may result in a similar release of FGF that may account for the reduced level of differentiation observed when 3-D C2C12 organoids are grown under RWV conditions [98].

An alternative approach to creating modeled microgravity in myoblast/myotube cultures is the use of clinostat rotation of adherent monolayers grown in planar culture, rather than utilizing 3-D organoids maintained in suspension culture using the RWV system. Studies utilizing monolayers of rat L6 myoblasts grown under clinostat rotation demonstrate an inhibition of myoblast differentiation and myoblast fusion/myotube formation in modeled microgravity [101], although without a reduction in myoblast proliferation rate as observed in RWV culture [98]. These results suggest that the use of either 3-D myoblast organoids directly formed using the RWV system [6, 98] or monolayer myoblast cultures maintained under clinostat rotation [101] may not be the most appropriate model for investigating the effects of spaceflight on fully differentiated skeletal muscle cells (i.e., myotubes). However, these types of tissue culture analogs may be more appropriate for probing the potentially more subtle disruptive effects of microgravity exposure on myoblast/myotube fusion events [5, 102]. Since satellite cell (SC) activation and SC–myofiber fusion are important events in the maintenance and repair of muscle tissue in the terrestrial environment [79], understanding the underlying mechanisms which control these responses may also shed light on the etiology of a variety of terrestrial skeletal muscle diseases involving significant myofiber atrophy, such as age-related sarcopenia [68], muscular dystrophy [103], and cancer cachexia [70].

Skeletal muscle 3-D organoids can also be created by culturing myoblast cells embedded within a variety of polymerized extracellular matrix materials, such as collagen Type I gels or hydrogels containing hyaluronic acid (HA), fibrinogen, and synthetic peptides [11, 73, 96, 104]. Primary myoblast cells of avian, rodent, and human origin, as well as rodent myoblast cell lines have all been utilized to create such 3-D skeletal muscle organoids containing myotubes demonstrating that embedding myoblasts within a biologically compatible 3-D matrix does not, in of itself, inhibit myoblast proliferation, fusion, and/or myotube formation. Generation of

such 3-D skeletal muscle organoids allows application of defined amounts of external mechanical force (e.g., stretch) to be applied to the 3-D organoid [105, 106], or to create internal mechanical loads within the 3-D organoid by initiating myotube contraction using external electrical stimulation [11]. The ability to combine both defined external loading and/or intrinsic loading [11] allows the 3-D skeletal muscle organoid to be exposed to mechanical load profiles nearly identical to that experienced by skeletal muscle tissue in vivo. Indeed, intrinsic mechanical tension (created within the polymerized extracellular material as a consequence of the formation of myotubes in 3-D organoids tethered at both ends to prevent matrix contraction) [107, 108], or exogenous mechanical load by active stretching of the 3-D organoid has been shown to enhance myotube formation and promote the parallel alignment of myotubes within the 3-D organoid [11, 109, 110]. The importance of mechanical load in inducing a phenotype in culture resembling that of skeletal muscle tissue in vivo is highlighted by the up-regulation of a variety of protein markers associated with myoblast differentiation, myotube formation, and skeletal muscle tissue remodeling in vivo (i.e., sarcomeric tropomyosin, myogenin, creatine phosphokinase, IGF-1, and metalloproteinase 2) [11, 73, 105, 107, 109, 110] in these mechanically active tissue culture models.

Skeletal muscle 3-D organoids constructed from myoblasts (or a mixture of myoblasts and other relevant cell types such as fibroblasts and endothelial cells) exhibit many of the histological, biochemical, and mechanical load reactive properties of skeletal muscle in vivo [8]. As such, 3-D skeletal muscle organoids of this type exhibit the greatest physiologically relevant similarities to whole muscle in vivo of any skeletal muscle tissue culture analog developed to date. Hence, if available, such 3-D skeletal muscle organoids are the most appropriate in vitro analog model in which to investigate the in vivo effects of spaceflight or mechanical unloading on the structure and function of skeletal muscle.

8.4 Spaceflight Experiments Utilizing Skeletal Muscle Tissue Culture

To date, very few actual spaceflight experiments have been conducted on-orbit utilizing skeletal muscle cell cultures. In repeat experiments flown aboard two Space Shuttle missions, STS-45 and STS-63, exposure of a variant of the rat L6 myoblast cell line (known as Clone L8) to spaceflight resulted in both the inhibition of myoblast fusion and myotube formation [5]. During these flight experiments, L8 myoblasts were cultured under static, three-dimensional perfusion culture conditions [111]. In addition to the reduction in myoblast fusion and myotube formation rates observed in the original L8 myoblast cultures as a consequence of spaceflight, microgravity exposure also resulted in the conversion of the L8 cell line to a non-fusogenic variant cell line known as the L8SF clone. Interestingly, the non-fusogenic phenotype exhibited by the L8SF variant became permanent when these cells were returned to Earth and propagated in long-term culture [5]. If confirmed by

additional spaceflight experiments, the possibility that microgravity exposure may induce permanent phenotypic and/or genetic alterations associated with reduced myoblast fusogenic capacity may have significant negative implications for SC function as it relates to the repair or regeneration of damaged skeletal muscle in crew members. Interestingly, a reduced ability to induce SC activation and subsequent fusion of SCs with existing myofibers appears to be a major cellular "road block" in reversing the skeletal myofiber atrophy observed in age-related sarcopenia [68], adding credence to the concept that exposure to microgravity conditions during spaceflight may induce effects similar to premature aging.

The development of 3-D organoids as a tissue culture analog of skeletal muscle was in part driven by the need for an appropriate in vitro model to study the effects of spaceflight on skeletal muscle structure and function. The pioneering work of Vandenburgh and colleagues in developing such 3-D skeletal muscle organoids [4, 106, 108, 112, 113], also known as bioartificial muscles (BAMs) [96], led to a series of experiments being carried out on two Space Shuttle missions, STS-66 and STS-77, designed to assess the effects of spaceflight on these analogs [7]. The BAMs utilized in these spaceflight experiments were generated pre-flight using embryonic avian myoblasts embedded in a collagen/Matrigel ECM initially supported by a flat culture surface consisting of a wire mesh. After several days in culture, myotubes form within the collagen/Matrigel ECM causing the center of gel/cell sheet containing the myotubes to detach itself from the wire mesh. The central part of the cell sheet proceeds to roll itself into a long tubular structure while the ends of the "tube" remains anchored to the wire mesh (see reference [96] for images). Replicate BAMs were then transferred to a perfusion tissue culture system for subsequent flight aboard the Space Shuttle. Prior to spaceflight, BAMs contained numerous parallel aligned, multi-nucleated myotubes exhibiting striations and expressing sarcomeric tropomyosin. BAMs also expressed MHC protein and fibronectin consistent with a differentiated skeletal muscle tissue phenotype. BAMs were fixed during flight on Mission Day 9, or upon return to Earth. Ground synchronous controls were also processed in an identical fashion to the flight samples. Microgravity exposure of BAMs for 9 days during spaceflight induced a significant reduction in myotube cross-sectional area (CSA) of approximately 12 %, a reduction in the synthesis of non-collagenous, myofibrillar-associated protein (i.e., MHC and fibronectin), but did not appear to impact protein degradation rates [7]. These experiments were the first to demonstrate that microgravity exposure alone, absent the removal of mechanical load (produced either by active contraction or passive stretch), resulted in a muscle atrophy response analogous to that seen in intact muscle.

Interestingly, the rate of myotube atrophy observed in these space flown BAMs was very similar to that observed in myofibers from both intact human and rodent muscle after spaceflight [13–15, 23, 25], as well as in ground-based analogs such as human bed rest [100, 114] or rodent hind limb suspension [65, 115]. However, one significant difference observed between the effects of spaceflight on BAMs as compared to intact skeletal muscle is the distinct lack of increased myofibrillar protein degradation characteristically observed in whole skeletal muscle as a consequence of spaceflight or terrestrial unloading [18, 116, 117]. This difference may be related

to effects associated with the removal of the extrinsic mechanical loading (i.e., either passive or dynamic stretch) during spaceflight normally experienced under terrestrial conditions by intact muscle in both crew members and rodents as compared to the constant static loading experienced by the BAMs used in these experiments. Conversely, the maintenance of constant GH/IGF-1 levels in the tissue culture medium of the space flown BAMs as compared to a reduction in circulating levels of these same hormones observed in crew members [44] or space flown rodents [118] may explain the difference in protein degradation rates. Both the removal of extrinsic mechanical load or a reduction in pituitary-derived GH/IGF-1 signaling within intact skeletal muscle under terrestrial conditions has been shown to negatively impact myofiber protein balance [48], specifically leading to an increase in myofibrillar protein degradation via the ubiquitin-proteasome system [47, 59]. As such, it is very possible that the culture conditions experienced by these BAMs during spaceflight (no change in either extrinsic mechanical loading or culture levels of GH/IGF-1) may explain why no increases in myofibrillar protein degradation rates were observed in this space flown culture model as compared to that observed in intact skeletal muscle tissue after spaceflight or terrestrial unloading in vivo.

8.5 Modeling Spaceflight Exposure by Removal of Mechanical Load

One of the recurring themes in this chapter is the possibility that skeletal muscle adaptation to spaceflight is a consequence of two distinct yet related environmental factors experienced by the tissue, namely true microgravity exposure and/or the removal of the normal mechanical loading experienced by the intact musculoskeletal system while living under terrestrial gravity conditions. Actual spaceflight experiments utilizing both cultured monolayer [5] and 3-D skeletal muscle organoids [7] have shown that microgravity exposure alone, without alteration in the mechanical load profile experienced by skeletal muscle cells during spaceflight, causes individual cellular responses consistent with the spaceflight-induced atrophy response seen in intact skeletal muscle tissue in vivo. For example, myofiber atrophy is correlated with a reduction in mechanically induced sarcolemma damage during both spaceflight and 6°-head-down-tilt bed rest in human subjects [100], which in turn has been linked to a reduction in the release of FGF from myofibers via these sarcolemma wounds or "micro-tears." However, acute exposure of myotubes to brief periods of microgravity during parabolic flight inhibits the membrane–membrane fusion events required for membrane resealing and repair of such mechanically induced sarcolemma damage in vitro [102]. Such divergent responses suggest that the effects of microgravity exposure at the cellular level are not solely associated with removal of mechanical load from the muscle tissue as a whole.

In order to investigate the specific effects of mechanical unloading on skeletal muscle, a variety of mechanically active tissue culture models have been developed

which allow the application of defined extrinsic mechanical load to myoblasts and/or myotube cultures grown both in monolayer and 3-D organoid cultures. Extrinsic mechanical load stimulus can be applied directly by stretching the 3-D skeletal muscle organoid (as in the case of BAMs) [11], indirectly by stretching of the 3-D support matrix in which the myotubes are embedded (as is the case for collagen gels or fibrin hydrogels) [119], or stretching of a flexible (i.e., silicon) culture surface on which the myotubes are growing [81, 106, 120]. One of the most interesting and exciting applications of mechanically active 3-D skeletal muscle organoids relative to understanding the effects of spaceflight is the use of such models to first induce and then study the muscle atrophy response by mimicking the mechanical loading profiles experienced by intact, skeletal muscle when it undergoes a "terrestrial to spaceflight" transition in vivo. This can be achieved by first applying mechanical load to 3-D skeletal muscle organoids in the form of both external loading (i.e., stretch) combined with intrinsic loading (i.e., electrically elicited myotube contraction) [11], and then removing part or all of these load stimuli in order to mimic spaceflight conditions.

For example, Vanderburgh and colleagues [11] have used a 3-D skeletal muscle organoid culture (*a.k.a.* BAM) generated using primary mouse myoblasts which included regular periods of electrically induced myotube contraction over a period of 80 days, followed by a reduction in the amount of extrinsic mechanical load (i.e., stretch) placed on the BAM. The reduction in stretch of the BAM under terrestrial conditions resulted in a rapid myotube atrophy response characterized by reductions in myotube CSA content, tetanic force (Po), total protein synthesis rate, and non-collagenous protein content [11]. Similar atrophic responses were observed in BAMs flown aboard the Space Shuttle [7], even though the atrophy response aboard the space craft was induced solely by microgravity exposure and not the removal of either extrinsic or intrinsic mechanical loading as was observed in the case of terrestrial BAMs [11]. Interestingly, no significant increase in muscle protein degradation rates (a hallmark of spaceflight-induced muscle atrophy and terrestrial unloading in vivo) was observed in atrophied BAMs from either spaceflight or terrestrial conditions. Taken together, these data indicate that the underlying mechanism(s) involved in the initiation of the "hallmark" increase in myofiber protein degradation observed during myofiber atrophy in vivo as a consequence of either spaceflight or mechanical unloading in a terrestrial setting [16, 48, 59, 121] remains to be fully elucidated.

8.6 Knowledge Gained Applicable to Terrestrial Disease States

While the use of skeletal muscle tissue culture models for studying a variety of disease states in terrestrial populations has a relatively long history, most of these studies have utilized myotube cultures grown as conventional monolayers rather than 3-D skeletal muscle organoids. The more traditional myotube model

has been used to investigate myofiber-induced cancer cachexia [121–123], screening pharmaceutical agents [124], and nutritional supplements [125] potentially useful in combating muscle loss, understanding insulin signaling and glucose transport mechanisms as they relate to the etiology of Type II diabetes [126] and obesity [127], as well as investigating the underlying signaling pathways operating during muscle atrophy [121, 128] or hypertrophy [129]. However, the development of 3-D skeletal muscle organoids, driven in part by the spaceflight research community space, has significantly increased the ability of investigators to study a variety of other pathological conditions using tissue culture analogs much closer to the skeletal muscle tissue milieu existing in vivo.

For example, 3-D skeletal muscle organoids have been used to mimic myoblast behavior in aged skeletal muscle. Constructed from "high passage" myoblasts, these organoids exhibit many of the histological and biochemical characteristics of aged sarcopenic muscle [130], including myotube atrophy, decreased expression of metalloproteinase and IGF-1/IGF receptor mRNA, increased expression of myostatin mRNA, and an overall reduction in peak force production. All of these changes are consistent with the myofiber atrophy and impaired ability for muscle differentiation and regeneration observed in age-related sarcopenia [62, 68]. Interestingly many of the same histological and biochemical features reported in both actual and ground-based analogs of spaceflight exposure [13, 15, 23, 37] appear in this model of sarcopenic muscle, lending credence to the concept that spaceflight may induce "premature" aging of the skeletal muscle cells in vivo.

The use of 3-D skeletal muscle organoid cultures that exhibit histological, biochemical, and contractile properties nearly identical to that of intact skeletal muscle will provide many new opportunities for truly mechanistic study of skeletal muscle function as it relates to a wide variety of pathological conditions. The added benefit of being able to simultaneously modulate the levels of extrinsic (i.e., stretch) and intrinsic (i.e., contractile) mechanical load placed on 3-D skeletal muscle organoids, as well as allowing control of various circulating factors (i.e., GH, IGF-1, FGF, myostatin, follastatin, reactive oxygen species, etc.) in the tissue culture medium will allow the development of a range of models capable of explaining a wide number of skeletal muscle pathologies in a causal fashion.

8.7 Questions for Future Research

This review chapter has primarily focused on the use of conventional monolayer and 3-D organoid cultures of skeletal muscle cells as tissue culture analogs of skeletal muscle structure and function related to effects of spaceflight. It should also be obvious to the reader that such tissue culture analogs can also be utilized to understand the effects of a myriad of environmental, genetic, and epigenetic effects on skeletal muscle cells of interest in the terrestrial environment. By being able to manipulate mechanical loading levels and a wide range of culture conditions, 3-D skeletal muscle organoids generated from either normal or diseased

muscle can be used to investigate the underlying etiology and signaling mechanisms involved in almost any disease state which involves skeletal muscle tissue. However, while the highly specialized, tissue culture hardware required for achieving this level of experimental control are currently under development in a number of terrestrial laboratories, no such culture systems capable of operating in the space craft environment presently exist.

Specific research questions which remain unanswered relating to the effects of spaceflight on skeletal muscle structure and function center not only on the effects of microgravity exposure and unloading, but also the potentially negative combinatory effects of other environmental risk factors encountered by crew members during spaceflight or exploration class missions. For example, the potential effects of space radiation on satellite stem cell populations [131–133], elevated oxidative stress levels [134, 135], disrupted nutritional status [136, 137], and/or genetic risk factors [138] related to altered one-carbon metabolism (1-CM) [139] as it relates to skeletal muscle have yet to be investigated. Similarly, the effects of reloading on skeletal muscle after extended periods of inactivity/atrophy relative to the ability recover muscle mass [140, 141], as well as the capacity of atrophied muscle for tissue repair and regeneration after injury [77] remain unknown. The use of 3-D skeletal muscle organoid cultures where such combinatory effects can be mimicked will in part allow the investigation of many of these spaceflight-related issues, while furthering our understanding of how to combat these challenges in crew members.

While in the early stages of development, the creation of even more complex 3-D skeletal muscle organoids containing additional cell types such as endothelial, neuronal, and/or fibroblast cells [8–10] has demonstrated that it may be possible in the future to develop truly bioengineered skeletal muscle tissue nearly identical to intact skeletal muscle in vivo. Such bioengineered tissue will serve not only as an in vitro tissue culture analog of skeletal muscle structure and function, but potentially as means of repairing or replacing damaged or dysfunctional skeletal muscle tissue in vivo. The rapid and truly paradigm shifting advances being made in the area of 3-D tissue "printing" [142] using a variety of ECM compounds and primary cell types promises to accelerate all areas of tissue bioengineering, including that involving skeletal muscle tissue. For example, the ability to "print" a 3-D skeletal muscle organoid in three dimensions which contains myoblasts arranged for optimal formation of aligned myotubes while also having a network of capillary endothelial cells simulating a functional vascular system with strategically located motor-neuron cells has become a realistic and achievable goal [142]. Combining such "printed" 3-D skeletal muscle organoids with existing systems capable of providing defined contractile stimulation and manipulation of external mechanical loads only furthers our ability to mimic the tissue milieu/microenvironment within intact skeletal muscle. Analysis of these 3-D models will be advanced by the use of cutting edge technologies, like spatially resolved molecular pathology (SRMP) techniques [60], allowing the study of individual cellular components within skeletal muscle using a combination of laser capture microscopy and "state-of-the-art" genomic and proteomic analysis [60, 143]. Development of such bioengineered models of skeletal muscle will provide many opportunities to further our understanding of the effects

of spaceflight exposure on skeletal muscle, while providing a broad-based platform technology in which to probe the causes and potential cures for a wide variety of human muscle diseases.

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