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Standard Guide for Assessing the Skeletal Myoblast Phenotype[1]

This standard is issued under the fixed designation F3369; the number immediately following the designation indicates the year of original adoption or, in the case of revision, the year of last revision. A number in parentheses indicates the year of last reapproval. A superscript epsilon (ε) indicates an editorial change since the last revision or reapproval.

ε[1] NOTE—Table 1 was reformatted in May 2019.

1. Scope

1.1 Myogenic differentiation is a process regulated by specific transcription factors and signaling molecules that have been shown to induce a myogenic phenotype. Transcription factors mark the stages of myogenesis and act as benchmarks for use in myogenic assays.

1.2 This guide applies to mammalian cells but does not apply to non-mammalian cells as the myogenic markers for non-mammalian cells can be different than those described here.

1.3 This guide proposes appropriate markers to measure when conducting myogenic differentiation assays. This guide describes the stages for multipotent stem cell differentiation toward myoblasts and myotubes. This guide provides information about the appropriate methods to determine myogenic differentiation. This guide does not provide information about media, supplements, or substrates that drive differentiation toward a myogenic phenotype.

1.4 The purpose of this guide is to act as an aid for work performed in the area of skeletal myogenesis. Using this guide, researchers should be able to understand which skeletal muscle markers are best suited for experiments. This guide will improve consistency for studies of myogenic differentiation of multipotent stem cells by identifying appropriate markers for each stage leading to myocyte differentiation. It should be noted that myoblast differentiation *in vitro* may not be predictive of results that may be obtained *in vivo*.

1.5 *This standard does not purport to address all of the safety concerns, if any, associated with its use. It is the responsibility of the user of this standard to establish appropriate safety, health, and environmental practices and determine the applicability of regulatory limitations prior to use.*

1.6 *This international standard was developed in accordance with internationally recognized principles on standardization established in the Decision on Principles for the Development of International Standards, Guides and Recommendations issued by the World Trade Organization Technical Barriers to Trade (TBT) Committee.*

2. Referenced Documents

2.1 *ASTM Standards:*[2]

F2312 Terminology Relating to Tissue Engineered Medical Products

3. Terminology

3.1 Unless provided otherwise in 3.2, terminology shall be in conformance with Terminology F2312.

3.2 *Definitions:*

3.2.1 *myoblasts, n*—myoblasts repair or replace damaged muscle fibers by differentiating and fusing either with an existing muscle fiber or with each other to form multinucleated myotubes. Proliferating myoblasts are able to self-renew, but lose that ability once they exit the mitotic cycle.

3.2.2 *myogenic cells, n*—myogenic cells express a class of markers that suggest a muscle cell lineage fate or a cell's involvement in muscle fiber formation, or both.

3.2.3 *myogenic differentiation, n*—myogenic differentiation refers to the normal process by which early muscle precursor cells and myoblasts become more specialized cells capable of fusing with mature myoblasts or existing myotubes. Furthermore, the process of differentiation can be measured using markers to help describe cell behavior during culture. This guide will describe those different markers.

3.2.4 *myogenic terminal differentiation, n*—myogenic terminal differentiation refers to the normal end stage of myoblast maturation, where myoblasts can no longer proliferate and are able to fuse into myotubes. Experimental data to quantify this stage of differentiation is necessary in gaining additional information regarding cell behavior during culture.

3.2.5 *myotubes, n*—myotubes are long tubular structures with multiple nuclei, originating from mature myoblasts that

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[2] For referenced ASTM standards, visit the ASTM website, www.astm.org, or contact ASTM Customer Service at service@astm.org. For *Annual Book of ASTM Standards* volume information, refer to the standard's Document Summary page on the ASTM website.

fused together. Nuclei are centrally located following the initial fiber formation and as the myotube matures into a functional fiber the nuclei translocate to the periphery.

3.2.6 *primary myotube, n*—primary myotubes are multinucleated muscle cells present early during development.

3.2.7 *satellite cells, n*—satellite cells are normally quiescent cells that reside between the basement membrane and the sarcolemma of muscle fibers. Satellite cells are activated by extracellular cues associated with local damage. Once activated, satellite cells will re-enter the cell cycle to proliferate and supply a population of progeny (myoblasts). Satellite cells can adopt divergent cell fates that expand or maintain the satellite pool, or expand the number of myoblasts, or both. Proliferating myoblasts are responsible for muscle regeneration *in vivo* by fusing with other myoblasts or myotubes.

3.2.8 *secondary myotube, n*—seconary myotubes are multinucleated muscle cells present later in embryonic development that devleop in close apposition to primary myotubes.

3.2.9 *stem cells, n*—progenitor cells capable of self-replication, proliferation, and differentiation **(1)**.[3]

4. Summary of Guide

4.1 This guide consists of (*1*) recommended markers that are good predictors of myogenic differentiation, (*2*) markers that are *not* consistent predictors and are better used as complementary data, and (*3*) common methods that will enhance research results and data analysis such as real-time quantitative reverse transcription polymerase chain reaction, immunoblot, enzyme-linked immunosorbent assays, and microscopy.

4.2 This guide proposes a list of myogenic markers that have demonstrated a clear role in myogenic differentiation and muscle development both *in vitro* and *in vivo*.

4.3 Identifying markers in muscle development and function would enhance confidence following data acquisition. This guide will separate markers into two categories: primary and secondary.

4.4 Primary markers regulate myoblast differentiation and myotube formation.

4.5 Secondary markers are expressed and involved in muscle, but do not regulate myogenic differentiation, or are difficult to distinguish from other tissues that express them as well.

4.6 Methods used to measure and assess myogenic differentiation in stem cell and progenitor cell cultures are addressed in this guide, including (*1*) gene expression using polymerase chain reaction (PCR); (*2*) protein assessment using immunoblot; and (*3*) imaging.

4.7 Expression of primary markers does not guarantee myoblast differentiation or myotube formation, and should not be considered entirely sufficient until proven experimentally.

5. Significance and Use

5.1 This guide describes markers involved in myoblast differentiation that can be used to screen stem cells to help define myogenic capacity. Stem cells include pluripotent and multipotent stem cells capable of differentiating into several different mesenchymal cells, including skeletal muscle myoblasts.

5.2 To assess myogenesis in cells derived and not derived from muscle, markers are measured to accurately define the changes in transcription and structural proteins that regulate differentiation, fusion, and myotube formation. Discussion of these markers is important to understand why they are recommended.

5.3 *Myogenic Differentiation:*

5.3.1 Myogenic differentiation is a highly regulated process controlled by paired box (Pax) transcription factors and the myogenic regulatory factor (MRF) family. During early differentiation in adults, myogenic progenitors such as activated satellite cells or myoblasts express *Pax3* and *Pax7*. *Pax3* and *Pax7* transcription factors switch the cells toward a myogenic fate, and repress myocyte differentiation **(2)**, priming the cell for later MRFs. To form muscle, the family of MRFs is required to terminally differentiate myoblasts and form myofibers. These regulatory proteins belong to a superfamily of basic helix-loop-helix transcription factors that consists of myogenic differentiation factor 1 (*Myod1*), myogenic factor 5 (*Myf5*), myogenin (*Myog*), and myogenic factor 6 (*Myf6*). In the initial stages of myogenic differentiation, *Myod1* and *Myf5* are the first MRFs to be expressed, and trigger increased production of *Myog* and *Myf6* **(3)**. Increased intracellular *Myog* and *Myf6* induces terminal differentiation of myoblasts into myocytes, leading to fused myotubes.

5.4 *Forming Myotubes:*

5.4.1 While myogenic markers describe differentiation, fusion into multinucleated myotubes is an important factor in muscle biology. Myoblasts differentiate into a fusogenic phenotype characterized by multiple fusion markers. One marker of note is m-cadherin. M-cadherin is reported to be involved in myoblast fusion and to regulate myotube development **(4)**. Therefore, assessment of fusion markers in addition to myogenic differentiation markers would favor a cell phenotype capable of forming muscle. In support of this, studies have shown that despite expression of myogenic differentiation genes, cells not expressing m-cadherin were unable to fuse and form muscle. These results suggest that in addition to myogenic differentiation markers, fusion markers should be considered given their importance as indicators of whether a cell is able to fuse **(5)**. This guide will enumerate published methods to measure and quantify myoblast fusion markers.

6. Primary Myogenic Markers

NOTE 1—The markers discussed below are considered important regulators of a myogenic phenotype including satellite cell, myoblast, mature myoblast, and myotube.

6.1 *Paired Box 3 (Pax3) and Paired Box 7 (Pax7):*

6.1.1 In adult skeletal muscle, *Pax3*-positive and *Pax7*-positive cells positioned adjacent to the myofiber and underneath the basal lamina are called satellite cells. These are

[3] The boldface numbers in parentheses refer to the list of references at the end of this standard.

important in muscle regeneration and hypertrophy, and studies have shown that *Pax7*-positive cells strongly influence muscle regeneration (**6**). *Pax7* has been shown to play a critical role in adult skeletal muscle; progressive loss of *Pax7* and satellite cell populations impaired muscle regeneration in $Pax7^{fl/CreERT2}$ mice (**7**). This role is supported by demonstration that *in vitro* siRNA-mediated suppression of *Pax7* resulted in loss of *Myf5* expression, a critical step in myogenesis (**8**). More, *Pax7* transcription is an essential coactivator of other downstream muscle genes such as *Myod1* (**9**), and was shown to be sufficient for myogenic specification of muscle-derived stem cells (**10**). Like *Pax7*, *Pax3* is expressed in early muscle progenitor cells, and its absence causes severe muscle deficits. *Pax3* is one of the earliest markers expressed during muscle development and its expression continues in postnatal muscle, but in specific locations (**11**). For instance, *Pax3* β-galactoside positive cells were identified in mouse forelimbs but were sparse in the hindlimbs, indicating that *Pax3* is spatially dependent.

6.1.2 Taken together, *Pax3* and *Pax7* could indicate a myogenic phenotype, and their level of expression should be taken in reference along with additional experimental data to assess the myogenic phenotype. In support of this, reports using hydrogels of different stiffness values to culture mesenchymal stem cells demonstrated an increase in other myogenic regulatory factors, but were negative for *Pax7*. Lastly, when considering animal studies, spatial distribution of *Pax7*- and *Pax3*-positive cells should be carefully considered given their distribution in the body as noted above.

6.2 *Myogenic Factor 5 (Myf5) and Myogenic Differentiation Factor 1 (Myod1):*

6.2.1 Expression of both *Myod1* and *Myf5* is a key step in commitment of multipotential somite cells to the myogenic lineage. *Myf5* expression is induced in the dorsal-medial somites and is followed by expression of *Myod1*. As *Myod1* expression increases, *Myf5* expression *Myod1* is reduced. In animal knockouts, neither *Myod1* nor *Myf5* knockout mice alone disrupt normal muscle development, but when *Myod1* and *Myf5* are deleted, muscle development is inhibited (**12**). These findings underscore the specificity of *Myf5* and *Myod1* in the myogenic program, and highlights their expression in early stage myogenic cells. It is for this reason that *Myod1* and *Myf5*

TABLE 1 Primary Markers that Regulate Progression through the Myogenic Lineage

NOTE 1—Expression is indicated using shading and number of +'s. Light shading indicates low expression while darker shading indicates higher expression. In addition, a gradient is given using the number of "+'s" to indicate low expression with one '+' and high expression with multiple "+'s".

Myogenic Markers						
	Gene Name	Quiescent Satellite Cell	Activated Satellite Cell	Myoblast	Mature Myoblast	Myotube
Pax 3	*Pax3*	+++	++	++	+	+
Pax 7	*Pax7*	++++	+++	++	+	+
MyoD	*Myod1*	+	++	++++	++	+
Myf5	*Myf5*	+	+++	++++	++	+
MyoG	*Myog*	+	+	++	+++	++++
Myf6	*Myf6*	+	+	+++	+++	++++
MyHC	*Myh1*	+	+	++	+++	++++
Integrin α7	*Itga7*	++	++	+++	+++	++++
M-Cadherin	*Cdh15*	++	++	+++	+++	+
Desmin	*Des*	++	++	+++	+++	++++

are used so often in myogenic assays to determine relative expression of these genes.

6.2.2 Studies have also demonstrated that forced expression of *Myod1* induced a myoblast-like phenotype in non-muscle cells. *Myod1* is a master regulator (**13**) and functions by activating a feed-forward loop to control myogenic gene expression. *Myod1* has been shown to promote expression and production of *Myog* and *Myf6*. To demonstrate importance, *Myod1* has been transfected into many non-muscle specific cell types and generated expression of downstream myogenic genes like myogenin (**14, 15**). While forced expression of *Myod1* caused transdifferentiation of non-muscle cells into myoblast-like cells, non-muscle specific genes can still be expressed. When evaluating stem cells or progenitor cells derived from muscle and non-muscle sources, *Myod1* characterization is recommended to ensure that a gene which regulates several muscle pathways is considered. Likewise, *Myf5* expression is also considered an important gene regulator and is recommeded as a potential marker to evaluate myogenic phenotype.

6.3 *Myogenin (Myog):*

6.3.1 Myogenin is a key developmental regulator for skeletal muscle formation, is a marker of mature myoblasts, and while expression is reduced after muscle forms, it continues to be expressed in fully mature muscle fibers. The role of *Myog* is highlighted by the fact that knockout mice fail to form muscle during development (**16, 17**). This phenotype is consistent in cells that demonstrate reduced *Myog* expression and protein production, failing to fuse and form myotubes (**18**). In addition, loss of *Myog* during postnatal life still yields normal muscle development, but those developed fibers are significantly smaller compared to normal mice (19). Together, these data indicate that *Myog* might be critical in early muscle precursor cells and stem cells to form myotubes, but its role and downstream effect might change with maturation in either myoblasts or myotubes. Therefore, to assess a myogenic phenotype it is recommended that *Myog* be carefully considered as a marker of differentiation and maturation.

6.4 *Integrin α7 subunit (Itga7):*

6.4.1 In the basal lamina, satellite cells remain quiescent, expressing *Pax7* and low levels of integrin α7 (*Itga7*). A common marker of satellite cell isolations from muscle, *Itga7* is essential for myoblast immobilization and fusion (**20**), and increases as satellite cells differentiate into mature myoblasts. The role of *Itga7* in muscle development is emphasized by its ability to cause muscular dystrophy in *Itga7*-null mice. Deletion in mice has been shown to disrupt muscle healing, leading to fibrosis and exacerbating dystrophic muscle development (**21, 22**). Further, increases in eccentric exercise leads to increases in *Itga7*-positive stem cells ($Sca1^+/CD45^-$) in skeletal muscle (**23**), and α7-positive cells have been shown to increase muscle repair, hypertrophy, and protect against sarcolemma damage (**24, 25**). Given the specificity of α7 in myoblasts, its role in differentiation and muscle fiber formation, this guide encourages use of α7 as an assessment variable.

6.5 *Desmin (Des):*

6.5.1 Desmin is an intermediate filament protein and is muscle-specific. In skeletal muscle, desmin is expressed in differentiating myocytes, located at the periphery of the Z-disk in muscle fibers and aids in muscle contraction. It's expression is required for functional skeletal muscle to be formed. The first recognizable step in skeletal muscle myogenesis is the initiation of desmin synthesis in replicating myoblasts (**26**). Further, the amount and localization of desmin makes it an easy target to determine whether or not the differentiating cell possesses a skeletal myogenic phenotype (**27**). While there are studies that demonstrate desmin synthesis in cardiac and smooth muscle cells, smooth muscle desmin is located at the dense bodies of the cells, while in cardiac and skeletal muscle desmin is located at the Z-disk (**28, 29**). To determine differences between cardiac and skeletal muscle cells, the level of protein production can be examined. Skeletal muscle has elevated levels of desmin when compared to cardiac muscle. Lastly, it should be noted that mesenchymal stem cells also were reported to produce desmin filaments (**30**), suggesting that close attention should be given to experimental data following various differentiation and cell culture protocols. Desmin is a protein that if assessed improperly could be interpreted incorrectly. Thus, this guide recommends the use of desmin with Western blot or microscropy, or both, as an easy and effective way to distinguish between smooth, cardiac, and skeletal muscle cells.

6.6 *M-Cadherin (Cdh15):*

6.6.1 M-cadherin is a component of muscular adherens junctions and provides a physical interaction between myoblasts. When production of m-cadherin is suppressed, myoblasts possess a reduced ability to fuse (**31**). Interestingly, when m-cadherin expression was deleted in mice, muscle still developed due to alternative pathways available through other cadherins (**32**), indicating the overall importance of cadherins in muscle fiber formation. Based on these reports, which demonstrate that m-cadherin is a muscle-specific protein that regulates tube formation, this guide suggests that consideration be given to m-cadherin as a marker of myogenic phenotype in cells cultured for the purpose of myogenesis.

7. Secondary Complementary Markers

7.1 This list of markers in Table 2 is suggested to be used secondary to the markers listed in Table 1. While these markers are present in skeletal muscle cells they are also present in many other cell types. Thus, their specificity in muscle is lower making their use to assess myogenesis more difficult.

7.2 *Secondary Markers of Myogenesis:*

7.2.1 Vimentin (*Vim*) is an intermediate filament protein present in muscle. *Vim* is strongly expressed in developing fetal myotubes, and expression is reduced as the tube matures (**33**). This protein has also been reported in the literature to increase as desmin is reduced in response to TGF-β1 signaling (**34**), yet, *Vim* is produced in most mesenchymal cells. Therefore, it is recommended that *Vim* be considered a secondary marker.

7.2.2 Myocyte enhancement factor 2 (*Mef2*) is expressed in skeletal muscle and cardiac muscle. In both striated muscle tissues, *Mef2* is required to differentiate from muscle precursor to mature muscle cell, and its expression is regulated by *Myog* (**35, 36**). Due to expression in both cardiac and skeletal muscle,