Overview of Cell Types Capable of Contributing to Skeletal Muscle Repair and Regeneration

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Abstract

Skeletal muscle is the most abundant tissue in the human body, accounting for more than 30% of body weight, and is vital for the maintenance of posture, locomotion, and breathing. Skeletal muscle possesses a high intrinsic regenerative ability due to its resident stem cell, the satellite cell. However, muscle repair can fail in muscular dystrophies, during aging or after extensive trauma. A multitude of cell types are currently under investigation for their ability to support muscle regeneration in pathologies that induce tissue damage beyond the capacity of physiological regeneration. Even if the satellite cell is the most potent myogenic cell, factors including limited availability, difficulty of isolation, and in vitro expansion potential need to be addressed, and unorthodox cell types investigated, to develop the optimal cell type for a given therapeutic application. This chapter gives an overview of the advantages and disadvantages of cell types

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with myogenic capacity, focusing primarily on satellite cells, bone marrow-derived cells, muscle interstitial cells, and pluripotent stem cells.

1 Introduction

Skeletal muscle provides the body with the ability for controlled movement and can normally effectively repair and regenerate to counter various insults. However, failure to regenerate can occur in diseases including muscular dystrophies, trauma that severely damages a large area of a muscle (such as in road traffic accidents), or removal as a consequence of surgery, all of which are associated with disability. There is much investigation into how skeletal muscle can be repaired in response to this larger-scale damage, and the best cell source to use to repair skeletal muscle after such trauma. Here we review the properties of the resident skeletal muscle stem cell, the satellite cell (Katz 1961; Mauro 1961), and noncanonical cell types with myogenic potential. These unorthodox sources include CD133 cells (Negroni et al. 2009), bone marrow-derived stem cells (Gussoni et al. 2002), muscle interstitial cells (Cottle et al. 2017) such as pericytes (Birbrair et al. 2013a) and mesoangioblasts (Galvez et al. 2006), and myogenic differentiation of embryonic stem cells (Dinsmore et al. 1996) and induced pluripotent stem cells (Chal et al. 2016). Even if the satellite cell is the most potent myogenic cell (Relaix and Zammit 2012), factors such as low number, degree of difficulty of isolation, limited in vitro expansion, clinical safety, and sensitivity to genetic manipulation have to be considered against the properties of unorthodox myogenic sources when deciding the optimal cell type for a given therapeutic application (Meng et al. 2011). Here, we give an overview of the main cell types that can contribute to skeletal muscle regeneration in mouse and man, their isolation, and potential therapeutic application.

2 Skeletal Myogenesis

Skeletal muscle accounts for ~30% of the body mass of an adult woman and ~38% for men, with its primary functions being maintenance of posture, breathing, and locomotor activity (Janssen et al. 2000). It also has important roles in metabolism and thermoregulation. The basic contractile unit of skeletal muscle is the muscle fiber, containing myofibrils composed of sarcomeres arranged in series. Sarcomeres, in turn, contain the actin and myosin filaments that produce force by sliding past each other (Hanson and Huxley 1953). This highly ordered arrangement of sarcomeres gives skeletal muscle its characteristic striated appearance. Myosin heavy chain isoforms have varying ATPase activities that determine the speed of myofiber contraction, so forming “fast” or “slow” myofibers. Muscle fiber types also have different metabolic profiles to meet these varying requirements for ATP (Pette and Spamer 1986). Adult myofibers are adaptable, being capable of undergoing changes in fiber size (hypertrophy/atrophy) as well as fiber type (slow to fast and vice versa) (Greising et al. 2012).

Muscle fibers form by fusion of mononucleated muscle progenitor cells (myoblasts) during somitic and primary myogenesis in embryonic development. More myoblasts then fuse and donate their nuclei to grow myofibers during secondary myogeneisis in the foetal and perinatal periods, finally creating syncytia often containing several hundreds of myonuclei (Mintz and Baker 1967). However, some "progenitors" remain associated with myofibers to form a resident stem cell compartment and are termed satellite cells (Ciciliot and Schiaffino 2010). These stem cells become mitotically quiescent and reside in a niche on the surface of the myofiber, under the surrounding basal lamina.

Developmental and regenerative myogenesis is governed at the transcriptional level predominantly by members of the basic helix-loop-helix (bHLH) myogenic regulatory factor (MRF) family of MyoD, Myf5, myogenin, and Mrf4 (Weintraub 1993), as well as members of the myocyte enhancer factor (MEF2) family (Bharathy et al. 2013). The importance of these factors is shown by the ability of MRFs to activate the skeletal myogenic program after being introduced into various cell types derived from the three germ layers (Takano et al. 1998). MRFs dimerize with ubiquitously expressed bHLH E-proteins, and these heterodimers act as transcription factors by binding to the conserved DNA sequence CANNTG (E-box) that is present in most muscle-associated enhancer and promoter sequences (Moncaut et al. 2013; Zammit 2017).

Skeletal muscle displays a robust regenerative ability, especially if the connective tissue remains relatively intact. A finely tuned cascade of molecular events, recapitulating many deployed during developmental myogenesis, is initiated upon injury that ultimately yields a regenerated, vascularized, innervated, and contractile muscle (Chargé and Rudnicki 2004), with force/power re-established by 3 weeks in mouse (Rosenblatt 1992). However, trauma resulting in significant loss of muscle tissue cannot be successfully regenerated, resulting in compromised skeletal muscle function and strength.

Muscle bulk is also lost during normal aging in a process called sarcopenia (Delbono 2011), as a secondary process in cancer patients (cachexia) (Acharyya et al. 2005), or vascular injuries (Glass 2010), and in degenerative muscle diseases such as muscular dystrophies (Vilquin et al. 2011).

3 Skeletal Muscle Regeneration

Mechanical injuries to skeletal muscle lead to muscle fiber destruction by disruption of the basal lamina and plasma membrane. This allows calcium inflow, leading to degeneration induced by autodigestion followed by apoptosis (Ceafalan et al. 2014). Muscle regeneration following these events can be divided into three overlapping stages: destruction and inflammatory phase, repair phase, and remodeling phase (Ciciliot and Schiaffino 2010).

The destruction and inflammatory phase occurs between day 1 and day 3 after injury. Torn blood vessels allow inflammatory cells, such as polymorphonuclear leukocytes including neutrophils, to invade the necrotic injury site. Additionally,

connective scar tissue, mainly composed of fibrin and fibronectin derived from the blood of the initial hematoma, is formed immediately after the injury. Only a few hours after the onset of injury, the leukocytes are replaced by monocytes, which eventually differentiate into macrophages. Early invading M1 macrophages are present in high numbers at the injury site, peaking approximately 3–4 days after damage, with their numbers declining thereafter. M1 macrophages have two important roles, removal of necrotic tissue and secretion of pro-inflammatory cytokines, most notably tumor necrosis factor α (TNFα) and interleukin 6 (IL-6) and 1β (IL-1β) (Chazaud et al. 2009; Saini et al. 2016). A phenotype switch in invading macrophages then causes a population of M2 macrophages to appear at the site of injury at 2–4 days post damage. M2 anti-inflammatory macrophages secrete cytokines that are necessary to terminate inflammation, for example, interleukin 10 (IL-10). Additionally, M2 macrophages secrete factors that stimulate proliferation of satellite cells, as well as their myogenic differentiation (Chazaud et al. 2009; Saini et al. 2016), working together with growth factors released from the ruptured extracellular matrix and other cell types such as fibro-adipogenic precursors (Cantini et al. 2002; Järvinen et al. 2005).

The repair phase relies on satellite cells that activate and then undergo multiple rounds of proliferation to generate a pool of myoblasts, which subsequently fuse together, or into surviving myofibers, and hence repair myofiber damage (Zammit et al. 2002). In the remodeling phase, newly formed myofibers mature, and scar tissue is reorganized and contracts (Fukushima et al. 2001).

However, issues may arise during the regeneration process that causes aberrant remodeling of the muscle, such as a failure to resolve scar tissue. Myotubes within the same basal lamina may not fuse to each other, which could lead to clusters of small fibers. Myotubes may only fuse at one end, creating branched fibers (Ontell et al. 1982). This can lead to segmental necrosis, with a subsequent additional regenerative process at the damaged extremity of the myotube that might be impaired by scar tissue (Shen et al. 2005). Since the two myotubes that would have fused together are effectively separated, new myotendinous junctions might be formed (Baker and Poindextor 1991). Additionally, nascent myofibers can sometimes form outside the basal lamina, due to satellite cell migration or the contribution of non-muscle stem cells, and remain embedded in the interstitial tissue (Schmalbruch 1976).

4 The Satellite Cell: The Resident Stem Cell of Skeletal Muscle

Satellite cells were first reported in 1961, when Alexander Mauro observed an apparently quiescent cell lying on the surface of a myofiber but below the basal lamina, in frog and rat muscle (Mauro 1961). In the same year, similarly located cells were also described on intrafusal muscle fibers (Katz 1961). This specific location, on the edge of a myofibre, gave rise to the name satellite cell.

Satellite cells equate to approximately 2–5% of the number of myonuclei in mature muscle. There is a difference in amount of satellite cells per myofiber or

cross-sectional area between muscle fiber types though; in mouse, fast twitch muscle fibers (mainly IIb and IIx) of the extensor digitorum longus (EDL) have fewer associated satellite cells than slower twitch muscle fibers (IIa and I) of the soleus (Zammit et al. 2002). There is often a concentration of satellite cells at the neuromuscular junction in slow fibers in mouse and, at least in chicken, more satellite cells at the ends of the myofiber (Allouh et al. 2008). Number and potency of satellite cells can additionally be affected by exercise and age (Shefer et al. 2006, 2010).

Satellite cells are the primary source of myonuclei during postnatal development and early muscle growth, before becoming mitotically quiescent (Schultz et al. 1978). Their role then changes during adult life, when they are only sporadically required for muscle fiber homeostasis, fiber hypertrophy and repair (Zammit 2008). While the number of satellite cells drops significantly from postnatal to adult life, the adult population remains constant, even after multiple cycles of regeneration (Ceafalan et al. 2014). Indeed, the satellite cell pool can respond to 50 weekly cycles of repeated muscle damage with efficient regeneration (Luz et al. 2002). Why regeneration fails in muscle wasting diseases such as muscular dystrophy is unclear but is probably related to a deteriorating microenvironment (Aguennouz et al. 2011; Blau et al. 1983).

As stem cells, satellite cells maintain the regenerative pool via self-renewal (Collins et al. 2005). After injury, satellite cells are stimulated to activate and proliferate to generate large numbers of new myofibers within a few days (Whalen et al. 1990). Activation is governed by a cocktail of signals such as growth factors including HGF, FGF, and IGF and chemical signals including NO, derived from infiltrating cells and damaged myofibers. In mice, satellite cells ex vivo give rise to many $Pax7^{+}MyoD^{+}$ cells that are committed to myogenic differentiation, Pax7 expression then declines, and myogenin expression is induced as the cells irreversibly withdraw from the cell cycle and begin to differentiate (Zammit et al. 2004). Soon after exit from the cell cycle, myoblasts start to express structural proteins such as sarcomeric myosin and fuse into myotubes (Shefer et al. 2006). A small number of satellite cell progeny become $Pax7^{+}MyoD^{-}$ and return to quiescence to maintain the satellite cell pool (Zammit et al. 2004). Alternatively, it is proposed that the satellite cell pool is heterogeneous, with a small subset of self-renewing satellite "stem" cells responsible for generating satellite cells with myogenic commitment (Kuang et al. 2007; Tierney and Sacco 2016).

Identification of satellite cells based solely on endogenous molecular markers is complex (Table 1). The best and most commonly used marker for quiescent and activated satellite cells is the paired homeobox transcription factor Pax7 (Seale et al. 2000; Hernández-Hernández et al. 2017; Yin et al. 2013; Marg et al. 2014), since other markers used to characterize satellite cells, such as CD34 (Beauchamp et al. 2000) and syndecan-3/syndecan-4 (Cornelison et al. 2001; Pisconti et al. 2012; Sinha-Hikim et al. 2004), are additionally expressed by other cell types in skeletal muscle (Tedesco et al. 2010) (Table 1). Characterization of satellite cells on tissue sections can be performed using co-immunolabeling of Pax7 and laminin, to confirm their location under the basal lamina (Ortuste Quiroga et al. 2016). Not all markers recognize all satellite cells though, for example, $Myf5^{YFP}$ is expressed in a small

Table 1 Overview of markers used to identify and/or isolate murine and human satellite cells

Marker	Proportion of satellite cells (SC)	Cellular Localization	Human	Murine	Other notable/ relevant expression (mice)	Bulk isolation methods	Reference
Pax7	100% of quiescent and activated SCs	Nucleus	√	√	Central Nervous System	Cell sorting from GMO mice, e.g., mouse: $Pax7^{GFP}$	Seale et al. 2000; Zammit et al. 2006; Boldrin and Morgan 2012; Reimann et al. 2004; Sambasivan et al. 2009
Pax3	Quiescent SCs (only subset in certain muscles)	Nucleus	?	√	Melanocyte stem cells, brachial and femural arteries	Cell sorting from GMO mice, e.g., mouse: $Pax3^{GFP}$	Relaix et al. 2006; Goupille et al. 2011
Myf5	Most quiescent and proliferating SCs and myoblasts	Nucleus	√	√	Central Nervous System	Mouse: $Myf5^{nLacZ}$ cell sorting from GMO mice, e.g., $Myf5^{YFP}$	Tajbakhsh et al. 1996; Beauchamp et al. 2000; Bareja et al. 2014; Daubas et al. 2000
Syndecan-3 and -4	98% of quiescent and activated SCs	Plasma membrane	√	√	Brain, dermis, bone, smooth muscle, tumors	Cell sorting	Cornelison et al. 2001; Sinha-Hikim et al. 2004; Pisconti et al. 2012
VCAM-1	Quiescent and activated SCs	Plasma membrane	?	√	Activated endothelial cells	Cell sorting as part of panel of markers	Rosen et al. 1992
c-met	Quiescent and activated SCs	Plasma membrane	√	√	Many tissues and tumors	Not used	Cornelison and Wold 1997; Marg et al. 2014
Foxk1	Quiescent and activated SCs	Nucleus	?	√	Neurons	Not used	Garry et al. 2000
Cd34	Quiescent and activated SCs	Plasma membrane	√	√	Hematopoietic, endothelial, mast, and dendritic cells	Cell sorting as part of panel of markers	Beauchamp et al. 2000; Boldrin and Morgan 2012; Pisani et al. 2010

M-cadherin	Quiescent and activated SCs, myoblasts	Plasma membrane	√	√	N/A	Not used	Irintchev et al. 1994; Boldrin and Morgan 2012
Caveolin-1	Quiescent and activated SCs, myoblasts	Plasma membrane	√	√	Endothelial fibrous and adipose tissue	Not used	Gnocchi et al. 2009; Boldrin and Morgan 2012; Volonte et al. 2005
α7 integrin	Quiescent and activated SCs, myoblasts	Plasma membrane	√	√	Vessel-associated cells	Cell sorting as part of panel of markers	Blanco-Bose et al. 2001; Pawlikowski et al. 2009
β1 integrin	Quiescent and activated SCs	Plasma membrane	√	√	Many tissues	Cell sorting as part of panel of markers	Kuang et al. 2007; Xu et al. 2015
CD56 (NCAM)	Quiescent and activated SCs	Plasma membrane	√	√	Glia, neurons, natural killer cells	Cell sorting and beads	Betsholtz 2004; Agley et al. 2015
Cxcr4	Subset of quiescent SCs	Plasma membrane	√	√	HSCs, vascular endothelial cells, neuronal cells	Cell sorting as part of panel of markers	Sherwood et al. 2004; Bareja et al. 2014
Nestin transgene	Roughly 98% of quiescent SCs and myoblasts	Cytoplasmic GFP	N/A	√	Central and peripheral nervous system	Cell sorting from GMO mice	Day et al. 2007
SM/C2.6 antigen	Subset of quiescent SCs	Plasma membrane	X	√	Unknown	Cell sorting	Fukada et al. 2004, 2013
Calcitonin receptor	Adult satellite cells	Plasma membrane	?	√	Many tissues	Not used	Yamaguchi et al. 2012
Teneurin4	Quiescent SCs	Plasma membrane	?	√	Many tissues	Not used	Fukada et al. 2007

subset (Kuang et al. 2007), while *Pax3*^{eGFP} is expressed in a subpopulation in only certain muscles (Calhabeu et al. 2013; Relaix et al. 2006) (Table 1). It remains unclear though, whether such heterogeneity is related to the presence of dedicated satellite "stem" cells or reflects other factors such as altered stem cell characteristics e.g., through some cells having been activated fewer times or having undergone fewer divisions (Zammit 2017). Recent studies on gene expression in quiescent satellite cells have identified more potential markers that may help to resolve this issue (Machado et al. 2017; van Velthoven et al. 2017).

Isolation of satellite cells was first performed either from whole muscle extracts or single-cell suspensions (Kakulas et al. 1968; Pogogeff and Murray 1946; reviewed in Scharner and Zammit 2011). These explant cultures also contained non-myogenic cells including adipocytes, lymphocytes and fibroblasts. Pre-plating of dissociated cells on a non-collagen-coated dish allows enrichment of myogenic cells as fibroblasts preferentially adhere to this surface (Blau and Webster 1981). Another option is the fractionation of a cell suspension on Percoll density gradients (Richler and Yaffe 1970). A means to ensure purity of the population is to isolate satellite cells by explant culture from single myofibers, rather than muscle tissue. In this approach, a whole muscle, such as the EDL in mouse, is enzymatically digested to isolate individual myofibers with their associated satellite cells. Myofibers can then be plated, and large quantities of satellite cell-derived myoblasts soon surround the myofiber. This methodology can be successfully applied to both murine (Moyle and Zammit 2014) and human skeletal muscle (Marg et al. 2014).

Apart from explant cultures, techniques such as FACS and MACS can be used. Initially, FACS was performed to enrich for myogenic cells on the basis of cell shape (Baroffio et al. 1993) and also size/granularity (Montarras et al. 2005), with the surface marker neural cell adhesion molecule (NCAM) used in early studies (Walsh and Ritter 1981). Subsequently, FACS or MACS sorting of combinations of $CD45^{-}Sca\text{-}1^{-}CXCR4^{+}\beta1\text{-}integrin^{+}CD34^{+}c\text{-}met^{+}$ (Sherwood et al. 2004), $CD34^{+}CD45^{-}Sca\text{-}1^{-}$ (Montarras et al. 2005), syndecan-3^{+} and syndecan-4^{+} (Tanaka et al. 2009) from murine biopsies, or CD56+/Desmin+ for human biopsies (Agley et al. 2015) isolate a cell population highly enriched for satellite cells. Samples for sorting-based isolation techniques are commonly derived from adult muscle that is first cleared of non-muscle tissue (blood vessel, connective tissue, nerve bundles, and adipogenic tissue), mechanically minced and enzymatically digested. A collagenase-dispase enzyme mixture is normally used because dispase preserves surface antigens more effectively than trypsin or pronase (Yablonka-Reuveni 2011). A detailed protocol can be found in Motohashi et al. 2014.

Where available, an alternative to endogenous surface antigens for FACS is using mice genetically modified to express fluorescent proteins under control of various genes/promoters including *Pax3* (Montarras et al. 2005), *Pax7* (Bosnakovski et al. 2008), *Myf5* (Biressi et al. 2007), or *Nestin* (Day et al. 2007) (Table 1). Satellite cells from Pax3-GFP or Pax7-GFP mice are isolated according to their $GFP^{+}/CD34^{+}/CD45^{-}/Sca1^{-}$ profile; the presence of GFP is essential as it allows differentiation between endothelial ($CD34^{+}/GFP^{-}$) and myogenic cells (Montarras et al. 2005).

Current advances in cell profiling have allowed new markers for muscle satellite/stem cells to be identified, such as CD9, CD104 (integrin subunit beta 4) (Porpiglia et al. 2017), Asb5, and CD82 (Giordani et al. 2019) that can aid in the isolation of pure populations, but they are not yet in routine use.

The main advantage of direct isolation methods without extended culture steps was that the isolated satellite cells better maintained their regenerative ability, that was otherwise lost during explant culture: freshly isolated satellite cells show a significantly higher potential for regeneration than after in vitro expansion (Collins et al. 2005; Gilbert et al. 2010; Montarras et al. 2005). The high potential of "fresh" satellite cells in muscle regeneration was demonstrated in an elegant experiment showing that transplantation of a single myofiber with approximately seven associated satellite cells successfully regenerated and repopulated areas of skeletal muscle in dystrophic *mdx* mice (Collins et al. 2005). Transplantation of human fibers with their associated satellite cells into irradiated mouse muscle also results in robust engraftment, muscle regeneration, and proper homing of human $PAX7^+$ satellite cells to the stem cell niche (Marg et al. 2014). Short culture periods without subculture allows satellite cells to maintain their myogenic potency after transplantation (Ikemoto et al. 2007) but expanded cells perform poorly after transplantation compared to freshly isolated cells (Montarras et al. 2005). The loss of regenerative potential could be attributed to a reduction of "stem cell" characteristics and/or fraction during in vitro expansion, and gene expression changes drastically even during the isolation process (e.g., Machado et al. 2017). Such compromised potency is particularly problematic in therapeutic approaches, as it is necessary to expand the cells to obtain large amounts and myoblasts have not been effective in clinical trials for muscular dystrophies (reviewed in Negroni et al. (2011)). Similar to mice, only 5% of total myofiber nuclei in humans belong to satellite cells, and biopsies tend to be small, therefore minimizing the amount of useful satellite cells that can be obtained for therapeutic use (Lindström and Thornell 2009). The demonstration that viable and functional satellite cells can be obtained postmortem in man, however, opens up a potential new source (Latil et al. 2012).

Methods to increase the number/effectiveness of satellite cells for grafting without compromising their myogenic potential are thus important and include culture on soft hydrogels with a stiffness mimicking skeletal muscle (approximately 12 kPa) (Gilbert et al. 2010), artificial recreation of the native niche (Quarta et al. 2016), or manipulation of various signaling pathways such as prostaglandins (Ho et al. 2017) and p38 (Cosgrove et al. 2014).

Overcoming reduced potency due to in vitro expansion should facilitate widespread testing of satellite cells in clinical trials for degenerative diseases such as muscular dystrophies – an area that is currently lacking progress (Briggs and Morgan 2013). Even though transplantation of satellite cells has led to encouraging results in dystrophic mice, translation of these results into humans remains challenging (Negroni et al. 2016). Systemic delivery of autologous satellite cells or satellite cell-derived myoblasts is not currently possible, but it is an issue being addressed (e.g. Gerli et al. 2019). However, local intramuscular injection of in vitro expanded satellite cells might be effective in certain conditions. In patients suffering from

oculopharyngeal muscular dystrophy (OPMD), a major issue is difficulties swallowing due to wasting of the cricopharyngeal muscles. Intramuscular grafting of homologous expanded satellite cells into cricopharyngeal muscles during cricopharyngeal myotomy appears to increase quality of life and muscle function, although the relative contribution of surgery and grafting is currently unclear (Périé et al. 2014; Negroni et al. 2016).

5 CD133+ Cells

These are a subset of human cells characterized by expression of CD133, a transmembrane protein that is expressed at high levels in a variety of stem cells (Kobari et al. 2001; Zuba-Surma et al. 2009). Initially, contribution of $CD133^+$ cells to myogenesis was reported in $CD133^+$ circulating hematopoietic stem cells that were isolated from human blood samples (Torrente et al. 2004). Cultivated on a feeder layer of myogenic cells, $CD133^+$ cells express early myogenic markers such as Myf5, Pax7, and M-cadherin and fuse with the feeder cells to form mosaic myotubes. Later, $CD133^+$ cells were found in the interstitium of skeletal muscle, in addition to a location associated with satellite cells in neonatal healthy, as well as in postnatal, dystrophic muscle (Meng et al. 2014). $CD133^+$ cells are a heterogeneous population of multipotent stem cells, giving rise to several mesenchymal lineages in culture, and can contribute to muscle regeneration after grafting, but not after intravenous delivery (Meng et al. 2014). Grafted human skeletal muscle-derived $CD133^+$ cells disperse better in host muscle, with enhanced regenerative ability, compared to human myoblasts alone (Negroni et al. 2009). For isolation of tissue-derived $CD133^+$ cells, muscle biopsies can be enzymatically digested and red blood cells/dead cells removed from the single-cell suspension, before incubation with CD133 microbeads and MACS sorting (Meng et al. 2014).

$CD133^+$ cells do, however, have several drawbacks, including being rare, ~1% of mononucleated skeletal muscle cells (Benchaouir et al. 2007), being too fragile for FACS sorting (Meng et al. 2014), requiring a complex culture medium that is no longer commercially available (Negroni et al. 2009), as well as losing their myogenic potential after prolonged in vitro culture (Meng et al. 2014). Furthermore, MACS-based isolation of $CD133^+$ cells from cord blood is affected by significant variability in purity of the isolated population (10–85%) (Pelagiadis et al. 2012). Muscle-derived $CD133^+$ cells were used in a clinical trial, where autologous $CD133^+$ cells were grafted intramuscularly into the abductor digiti minimi of eight Duchenne muscular dystrophy (DMD) patients, resulting in an increase in capillaries per muscle fiber and a change in the ratio of slow to fast muscle fiber types (Torrente et al. 2007). This is despite the observation that DMD patient-derived $CD133^+$ cells exhibit a markedly decreased myogenic potential compared to those from a healthy individual (Meng et al. 2018).

6 Bone Marrow-Derived Stem Cells with Myogenic Potential

In 1998, Ferrari et al. reported that stem cells not resident in skeletal muscle could be involved in muscle regeneration. They demonstrated that bone marrow-derived stem cells (BMDCs) were capable of contributing nuclei to myofibers if injected into regenerating muscle of immunodeficient *scid/bg* mice (Ferrari et al. 1998). However, BMDCs could only contribute to muscle regeneration with satellite cells present. A few years later, LaBarge and Blau used a model causing irradiation-induced muscle damage, so ablating the endogenous satellite cell pool. Mice were then injected with an unfractionated bone marrow cell suspension expressing a fluorescent marker, into the tail vein. This study showed that BMDCs not only contributed to muscle regeneration; they additionally reoccupied the empty satellite cell niche and were capable of self-renewal, thereby becoming heritably myogenic (LaBarge and Blau 2002). However, depending on the intensity, irradiation-mediated ablation does not remove all satellite cells from their niche (Heslop et al. 2000), making it possible that regeneration attributed to BMDCs would not be as efficient if ablation had been absolute.

Contribution of BMDCs to total myonuclei is also vastly different between different muscles following bone marrow transplantation. For example, muscles including the intercostal muscles and tongue contain about 0.002% of BMDCs, while the frequency was 0.26% in the EDL, 0.06% in the tibialis anterior, and 5.2% in the panniculus carnosus. Data was collected 16 months post bone marrow transplant, showing that incorporation of circulating stem cells seems to occur naturally at different levels. However, injury might still be a factor in the different rates of incorporation, as the panniculus carnosus may be damaged by "scruffing" the mouse during handling so incorporates higher amounts of BMDCs than muscles subjected to less damage, such as tibialis anterior (Brazelton et al. 2003), even in the absence of acute injury (Naldaiz-Gastesi et al. 2016).

There is also evidence that BMDCs may contribute to skeletal muscle in man. Female patients that had received a bone marrow transplant from male donors 6–12 years previously, had rare skeletal muscle fibers (0.6%) with a nucleus that contained a Y chromosome. Although no Y chromosomes were present in the satellite cell niche on sections or in desmin-expressing myoblast cultures grown from isolated satellite cells, a rare Y chromosome-containing centrally located nucleus was detected, indicating that BMDCs had continued to add myonuclei to muscle (Strömberg et al. 2013). Thus, while BMDCs can contribute to muscle, it is extremely inefficient when the satellite cell pool is intact. In another study, a DMD patient was treated with a bone marrow transplant for X-linked severe combined immunodeficiency, and donor nuclei could still be found in 0.5–0.9% of myofibers 13 years after BMDCs transplantation (Gussoni et al. 2002). With the obvious caveat of a single patient, the efficiency of BMDCs incorporation into skeletal muscle appears unaffected by the stimulus of chronic muscle regeneration likely in the DMD patient.

The specific mechanism of how BMDCs contribute to muscle regeneration is not well understood, and a large proportion of BMDCs that incorporate into myofibers do not actually activate the myogenic program (Lapidos et al. 2004; Wernig et al. 2005). The translational consequence of BMDC transplant is seen when patients suffering from partial denervation in the elbow were treated with BMDC injection and tendon transfer. Subsequently, muscle fibrosis decreased by 52%, while myofiber diameter, the presence of satellite cells, muscle density, and motor unit amplitude all increased (Hogendoorn et al. 2014). The effect of BMDCs may be indirect, as intramuscular injection 7 days after injury lead to alteration of the immune response, by downregulation of IL-1b, IL-6, TNF-α, and TGF-β1 and upregulation of IL-10, resulting in overall better regeneration and revascularization (Helal et al. 2016). BMDCs can also generate an environment favoring muscle regeneration in minipigs – by stimulating local VEGF production and shifting the macrophage phenotype to one promoting muscle regeneration (Linard et al. 2018). Finally, BMDCs can give rise to $CD45^-/Sca\text{-}1^+/desmin^+$ cells capable of myogenic differentiation (Luth et al. 2008). These cells localize to both within and outside of the basal lamina of muscle fibers and can contribute to skeletal muscle regeneration (Kowalski et al. 2018). Due to their localization and shared marker profile, they are thought to be the origin of some interstitial muscle-derived stem cells (Dreyfus et al. 2004).

7 Muscle Interstitial Cells in Muscle Regeneration

The term muscle interstitial cells (MICs) encompasses a diverse set of cell types found in the interstitium of skeletal muscle, with the relationship of these various cell types to each other, still relatively unclear. Their location presumably allows MICs to sense mechanical or chemical changes to the skeletal myofibers. Most MICs are of mesoderm origin, often from the vasculature, and are generally assumed to interact with satellite cells, muscle fibers, immune cells, and fibroblasts in the event of muscle damage. MICs can be broadly divided into two main groups: cells that support muscle regeneration and those that may directly contribute myonuclei (Table 2).

Fibro-adipogenic precursors (FAPs) are a population of MICs that are defined by expression of specific marker combinations, which also dictate their isolation via FACS and so characterization. Two different groups defined cells belonging to this population as either $CD45^-/CD31^-/lin^-/Sca1^+/CD34^+$ or $CD45^-/CD31^-/lin^-/Sca1^+/\alpha7int^-$ (Joe et al. 2010), or $CD31^-/CD45^-/SM/C\text{-}2.6^-/PDGFR\alpha^+/PDGFR\beta^+$ mesenchymal progenitor cells (Uezumi et al. 2010). In general, FAPs are capable of differentiating into adipocytes, osteoblasts (with BMP7), and smooth muscle cells (using TGF-β) (Uezumi et al. 2011); they do not, however, differentiate into skeletal muscle cells if cultured in low serum conditions, or if transplanted into a skeletal muscle regenerative environment (Uezumi et al. 2010). Additionally, $CD45^-/CD31^-/lin^-/Sca1^+/CD34^+$ FAPs normally also express Osr1 at low levels, but Osr1 expression increases upon injury. This population contributes specifically to

Table 2 Muscle interstitial cells: characterization and contribution to myogenesis

Muscle interstitial cell types	Characterization	Direct contribution to myogenesis	Reference
FAP	$CD45^-/CD31^-/lin^-/Sca1^+/CD34^+$ (low Osr1)	No	Joe et al. 2010
	$CD45^-/CD31^-/lin^-/Sca1^+/\alpha7int^-$	No	Joe et al. 2010
	$CD31^-/CD45^-/SM/C\text{-}2.6^-/PDGFR\ \alpha^+/PDGFR\beta^+$	No	Uezumi et al. 2010
Side population	$Sca1^+\ CD4^-$, $CD5^-$, $CD8^-$, $CD11^-$, $CD45^-$, Gr-1^-, c-KIT^-	Yes	Gussoni et al. 1999
SP subpopulation/ main population	$CD34^+/Sca1^+$	Yes	Tamaki et al. 2002, 2003
Myoendothelial cells	$CD56^+/CD34^+/CD144^+$	Yes	Zheng et al. 2007
PICs (FAP subpopulation?)	$CD34^+/Sca1^+/Pax7^-/PDGFR\ \alpha^-$	Yes	Besson et al. 2011; Pannerec et al. 2013
PICs (FAP subpopulation?)	$CD34^+/Sca1^+/Pax7^-/PDGFR\ \alpha^+$	No	
$Twist2^+Pax7^-$	$Twist2^+Pax7^-$	Maintenance/ regeneration of fast IIb muscle fibers only	Liu et al. 2017

adipogenic infiltration and post injury resident FAPs (Stumm et al. 2018). During muscle regeneration, it is thought that FAPs indirectly interact with satellite cells, as proliferation of FAPs rapidly increases prior to satellite cell proliferation (Joe et al. 2010). Indeed, pharmacological inhibition of in vivo FAP expansion by the tyrosine kinase inhibitor Nilotinib results in a failure of satellite cells to expand in response to injury (Fiore et al. 2016). FAPs aid muscle regeneration by releasing diffusible factors or by remodeling the microenvironment using disintegrin and metalloprotease ADAM12 upon injury. However, production of extracellular matrix components such as collagen for fibrosis will limit muscle regeneration, so FAPs may also contribute to the debilitating phenotype of muscle-degenerating diseases such as muscular dystrophies (Uezumi et al. 2011). Consistent with this notion, ADAM12 suppresses muscle regeneration and increases fibrosis if overexpressed (Jørgensen et al. 2007).

MICs with inducible myogenic potential include several cell types. Side population (SP) cells were first identified as BMDS multipotent hematopoietic stem cells that were characterized by their ability to efflux the vital fluorescent DNA dye Hoechst 33342 (Goodell et al. 1996). While isolating cells from the muscle of wild-type mice, Gussoni et al. also found a subpopulation of cells able to efflux Hoechst 33342 (Gussoni et al. 1999). SP cells are characterized by being $Sca1^+$, but negative for bone marrow and lineage markers (CD4, CD5, CD8, CD11, CD45, Gr-1, c-KIT), and display a high hematopoietic potential (Gussoni et al. 1999;

Jackson et al. 1999). The conditions used for FACS isolation of SP cells, such as Hoechst dye concentration however, can influence the composition of the SP fraction, making them a heterogeneous population (Montanaro et al. 2004; Rivier 2004). The regenerative potential of SP cells was highlighted after transplantation into lethally irradiated *mdx* mice, where they gave rise to dystrophin-positive myofibers (Gussoni et al. 1999). SP cells are juxtaposed to the platelet endothelial cell adhesion molecule (PECAM) expressing endothelium and blood vessels. Additionally, SP do not express Pax7 or desmin and only undergo myogenic differentiation if either co-cultured with primary myoblasts or injected into regenerating muscle (Asakura et al. 2002).

A potential subpopulation of the SP cells was proposed by Tamaki et al. who described $CD34^+/Sca1^+$ cells with multi-lineage potential from the skeletal muscle interstitium that were negative for hematopoietic markers. These cells express bHLH factors myogenin and MyoD and contribute to postnatal de novo myofiber formation, as well as adipocytes and endothelial cells (Tamaki et al. 2002). However, it was later determined that SP cells made only an extremely minor contribution to myogenic and endothelial generation in vitro, with 99.9% coming from the main population (Tamaki et al. 2003).

Myoendothelial cells have also been isolated from man and express both satellite and endothelial cell markers (CD56, CD34, and CD144), being located between myofibers, but outside of the basal lamina. Myoendothelial cells efficiently regenerate skeletal muscle after transplantation and differentiate into myogenic, osteogenic, and chondrogenic cells in vitro (Zheng et al. 2007).

The stress mediator PW1 has been used to define a population of MICs with myogenic potential called PW1(+)/Pax7(−) interstitial cells (PICs) (Mitchell et al. 2010). PICs are isolated from minced muscle tissue by FACS, by gating first on $CD45^-$ cells, followed by selection of $CD34^+$ and $SCA1^+$ cells. Of that population, PW1 and either PDGFα-positive or PDGFα-negative PICs can be selected, with both populations lacking Pax7 expression (Besson et al. 2011; Pannerec et al. 2013). PICs self-renew and differentiate into myofibroblasts, muscle fibers, and can occupy the satellite cell niche (Mitchell et al. 2010) but can also differentiate into adipocytes and compromise muscle regeneration (Yao et al. 2016). PICs contributing to adipogenesis share expression of PDGFRα with FAPs, while PICs capable of contributing to muscle regeneration are negative for PDGFRα (Pannerec et al. 2013). It is now thought that some PICs are a subpopulation of FAPs, sharing lineage markers CD34 and Sca1. The myogenic potential of PICs relies on the eventual expression of Pax7, acquired shortly before differentiating, as PICs from $Pax7^{-/-}$ mice exclusively adopt a smooth muscle fate (Mitchell et al. 2010).

Finally, the MIC $Twist2^+Pax7^-$ cells contribute exclusively to the regeneration of adult-type IIb/x myofibers, but are not involved in primary or secondary myogenesis. Ablation of $Twist2^+Pax7^-$ cells in mouse leads to type IIb-specific fiber atrophy, indicating their importance in maintenance and regeneration of these subtypes of fast muscle fibers (Liu et al. 2017) (summarised in Table 2).

8 Pericytes

Considering the affiliation that the MICs have with blood vessels and endothelium, it is unsurprising that intramuscular vasculature is a source of cells that support muscle regeneration. Pericytes are located beneath the basal lamina of small blood vessels and express ALP and NG2. Pericytes are essential for the formation of new blood vessels, being the first cells to invade freshly vascularized tissue (Nehls et al. 1992), where they adopt an angiogenic phenotype (Diaz-Flores et al. 1992) and guide establishment of new blood vessels (Ozerdem and Stallcup 2003). Additionally, pericytes promote endothelial cell survival (Kale et al. 2005), prevent vessel regression (Enge et al. 2002), and are crucial for vessel maturation (Hellström et al. 2001). Angiogenesis is important for muscle regeneration, since only 5 h in an oxygen-deprived environment causes necrosis in up to 90% of surrounding skeletal muscle (Labbe et al. 1987), which can lead to limb amputation where revascularization fails (Conrad et al. 2011).

Two types of pericytes are present in skeletal muscle, and while both type 1 (Nestin-GFP^{-}/NG2^{+}) and type 2 (Nestin-GFP^{+}/NG2^{+}) pericytes are in close proximity to endothelial cells, only type 2 pericytes can express Pax7 (Birbrair et al. 2013b) and contribute to myogenesis (Birbrair et al. 2013a). Pericytes are also capable of differentiating into smooth muscle, adipocytes, or osteoblasts under specific culture conditions but will also spontaneously form multinucleated myotubes if exposed to skeletal muscle differentiation medium. Interestingly, proliferating pericytes do not express MyoD, Pax7, or Myf5, which only appear in terminally differentiated and fused pericytes. Moreover, early postnatal skeletal myogenesis in vivo is supported by pericytes, whose contribution is additionally increased in dystrophic environments or by cardiotoxin injury (Dellavalle et al. 2011). It is of note that involvement of pericytes in myogenesis differs between muscles, for example, being higher in the diaphragm than in the tibialis anterior. Pericytes may also influence maintenance of satellite cell quiescence, as ablation of NG2^{+} pericytes in mouse enhances proliferation of Pax7^{+} cells (Kostallari et al. 2015). An aging microenvironment reduces participation of type 2 pericytes in skeletal muscle regeneration (Birbrair et al. 2013b), although culture in three-dimensional hydrogel rescues some myogenic potential (Fuoco et al. 2014). However, pericytes are also likely to regulate muscle fibrosis. Type 1 pericytes expressing ADAM12, give rise to collagen-producing cells in cases of muscle injury (Dulauroy et al. 2012; Birbrair et al. 2013b) and respond to TGFβ, an essential cytokine for formation of fibrous tissue (Massagué 2012), with increased collagen production in vitro (Birbrair et al. 2013b). However, recently it has been proposed that the multipotency of pericytes in vitro or after transplantation may just arise from the culture conditions (Guimarães-Camboa et al. 2017), which may actually be a useful property for a potential therapeutic cell.

Human pericytes or perivascular cells differ slightly from murine pericytes. These cells are associated with arterioles and capillaries in skeletal muscle and do not express classical myogenic markers such as Myf5, MyoD, myogenin, M-cadherin, nor Pax7

when isolated. Human pericytes do, however, rapidly induce expression of these markers in vitro, or in vivo upon intramuscular transplantation (Crisan et al. 2008).

9 Mesoangioblasts

Mesoangioblast designates a common precursor cell for both vascular and extravascular mesodermal derivatives. Originally isolated as a population of cells from the mouse embryonic dorsal aorta – mesangioblasts express early endothelial and myogenic markers (De Angelis et al. 1999). Freshly isolated murine mesangioblasts express MyoD and Myf5 but retain expression of early endothelial markers including SCA1, CD34, Flk-1, or VE-cadherin in vitro, but not later ones such as vWF. A small proportion (5–30%) additionally express α-SMA, a protein normally expressed in both smooth muscle cells and pericytes (Minasi et al. 2002) and transiently in skeletal myoblasts (Springer et al. 2002). In long-term culture, mesangioblasts lose expression of MyoD and Myf5 and their ability to spontaneously undergo myogenesis but regain myogenic potential if co-cultured with myoblasts (Cossu and Bianco 2003). Aorta-derived myogenic clones lose expression of CD34 during clonal expansion, while endothelial progenitors retain it (Sancricca 2010). Mesoangioblasts isolated from adult murine skeletal muscle have smooth muscle/pericyte markers and have been shown to contribute to skeletal myogenesis during development, regeneration, and upon transplantation (Dellavalle et al. 2011; Tedesco et al. 2010). Human mesangioblasts isolated from skeletal muscle biopsies are similar to pericytes, in that they do not express MyoD, Myf5, myogenin, M-cadherin or NCAM, nor typical endothelial markers CD31, CD34 (unlike murine mesangioblasts), and VEGF2 (Dellavalle et al. 2007; Morosetti et al. 2006). Markers used to isolate and characterize human and murine mesoangioblasts by FACS are given in Table 3.

The ability of mesangioblasts to undergo skeletal myogenesis was also shown by making artificial muscle using human or mouse mesangioblasts to generate multinucleated myotubes in a PEG-Fibrin hydrogel in vitro. Transplantation of such murine mesangioblast/PEG-fibrin hydrogel artificial muscles onto the tibialis anterior resulted in de novo muscle-like tissue, complete with host-derived blood vessels. Indeed, replacement of the murine tibialis anterior with a mesangioblast/hydrogel construct allowed functional recovery – demonstrating the potential for generation of patient-specific replacement muscle if suitable scale-up can be achieved (Fuoco et al. 2015).

Table 3 Marker expression profiles of murine and human mesoangioblasts

Murine mesoangioblasts	Positive	AP, NG2, Sca1, CD34, CD44, CD117, CD140s, CD140b
	Negative	CD31, CD45, CD56, CD133
Human mesoangioblasts	Positive	AP, NG2, CD13, CD44, CD49f, CD90, CD140a, CD140b, CD146
	Negative	CD31, CD34, CD45, CD133, CD56

In pathological settings, intra-arterial delivery of murine mesangioblasts in a limb-girdle muscular dystrophy mouse model (Sampaolesi et al. 2003) or intramuscular injection in *mdx* mice (Berry et al. 2007) increases muscle regeneration. The potential of mesangioblasts to effectively contribute to muscle regeneration in DMD was shown using a microchip engineered model of muscle regeneration. Co-culture of either healthy myoblasts or mesangioblasts with DMD donor-derived myotubes revealed that restoration of dystrophin expression was significantly increased with mesangioblasts (Serena et al. 2016). At least in facioscapulohumeral muscular dystrophy though, human mesangioblasts can vary in their ability to support skeletal muscle regeneration dependent on the characteristics of the muscle from which they are derived (Morosetti et al. 2007, 2011).

Mesoangioblasts have been used in a first-human phase 1/2 clinical trial via intra-arterial delivery for patients suffering from DMD, showing that the procedure is safe, although negligible beneficial effects were seen in muscle function, in part, likely due to the advanced stage of disease at grafting (Cossu et al. 2015). Generally speaking though, mesoangioblasts seem a useful tool for therapy, as they are readily isolated from postnatal tissue, can be extensively expanded in vitro while maintaining their myogenic potential, are easily modifiable to carry therapeutic genes, and are safe for delivery in man (Sancricca 2010).

10 Pluripotent Stem Cells

Differentiation of pluripotent stem cells to skeletal muscle has been achieved with and without genetic manipulation of the pluripotent derivatives. Induction of murine embryonic stem (ES) cells can be achieved by stimulation with DMSO; this however causes differentiation to cardiac, smooth, and skeletal muscle in similar amounts. ES cells would only differentiate specifically to skeletal muscle when simultaneously modified to express MyoD (Dinsmore et al. 1996). Even though transdifferentiation of ES cells into skeletal muscle has been possible for more than 20 years, one of the major difficulties was a low efficiency (Klinger et al. 2003). A way to bypass this efficiency problem is by generation and isolation of mesenchymal precursors from ES cells and subsequent differentiation into skeletal muscle cells (Barberi et al. 2005). Recently, protocols have emerged that allow for direct differentiation of human or murine ES cells first into Pax7-expressing skeletal muscle progenitors and then into mature myocytes (Borchin et al. 2013; Caron et al. 2016; Shelton et al. 2014, 2016; Swartz et al. 2016). Apart from allowing for a higher efficiency in generation of skeletal muscle cells, no genetic manipulation is used in these protocols, making these cells a feasible tool for therapeutic applications, with the caveat of ethical considerations.

Another approach to generate pluripotent stem cells for the subsequent differentiation into skeletal muscle is using inducible pluripotent stem cells (iPSCs). First described by Takahashi and Yamanaka in 2006, iPSCs are mature somatic cells that are reprogrammed to achieve a pluripotent state by the overexpression of four transcription factors: Oct3/4, Sox2, c-Myc, and Klf4 (Takahashi and Yamanaka

2006). IPSCs allow researchers to investigate early developmental processes without the limitations of scarcity of embryonic cells or the ethical concerns associated with the use of embryonic tissue. Importantly, patient cells can be reprogrammed, which has been performed for many conditions, including patients suffering from muscular dystrophies (Tedesco et al. 2012; Smith et al. 2016) and laminopathies (Steele-Stallard et al. 2018), also permitting ex vivo correction of pathogenic mutations (Tedesco et al. 2012). Subsequent differentiation into defined lineages provides powerful insight into pathomechanisms and provides a valuable tool for screening/testing potential therapies (Young et al. 2016). Again, commitment of iPSCs to the myogenic line can be achieved by MyoD overexpression (Maffioletti et al. 2015), but single-cell RNA-seq indicates lack of extracellular cues (insulin) or an inhibitory signaling loop (initialized by overexpressed BMP4) can impede the process (Cacchiarelli et al. 2018). Conditional expression of Pax7 in human ES/iPSCs can direct them to not only make muscle but also allow them to contribute to the myogenic progenitor pool (Darabi et al. 2012), occupying the satellite cell niche and responding to repeated injuries, and so conferring an enduring regenerative potential (Incitti et al. 2019). Indeed, gene expression in such grafted iPSC-derived satellite cells matures in vivo, from their initial embryonic/fetal signature to that more closely resembling postnatal myoblasts (Incitti et al. 2019). Transgene-free protocols are now emerging though, to generate human satellite-like cells and myofibers from iPSC (Chal et al. 2016).

Recently, muscle tissue has been constructed ex vivo from iPSCs (Rao et al. 2018) and now multi-lineage isogenic artificial muscle with myofibers, pericytes, endothelium, and motoneurones all being derived from the same iPSC source (Maffioletti et al. 2018). This artificial muscle was successfully grafted into mouse (Maffioletti et al. 2018), meaning volumetric muscle could be addressed by isogenic artificial muscles derived from the patient's own iPSCs. It will be important to determine how such isogenic artificial muscles perform in an in vivo regenerative setting.

11 Summary

In this brief overview, we have highlighted the range of cells that are able to contribute to the skeletal myogenic lineage. The preeminent resident stem cell of skeletal muscle is the satellite cells, which is essential for successful and complete muscle regeneration, since genetic ablation of $Pax7^{+}$ cells leads to a block in regeneration, with no/few myoblasts detectable in the injured area (Lepper et al. 2011; McCarthy et al. 2011; Relaix and Zammit 2012). In the absence of satellite cells, noncanonical muscle precursors are unable to act as a replacement for repairing muscle. Such non-regenerated muscle tissue is prone to infiltration by inflammatory cells and formation of fibrotic and adipose tissue, showing the importance of prompt and full regeneration.

Satellite cells are not a uniform population though (Biressi and Rando 2010). Recent clustering analysis on single-cell RNA-seq of satellite cells from uninjured

muscle segregates them into multiple populations (Cho and Doles 2017), suggesting that they move along an activation continuum with fluid boundaries, with single-cell mass cytometry (CyTOF) analysis (Porpiglia et al. 2017) also indicating potential subsets. These studies used fluorescent markers to FACs isolate satellite cells, which predefine the population analyzed, and activation will have already been initiated in many of these cells. Gene expression is also dynamic, so such studies provide a snapshot of the mean expression profile of the population at the point of fixation, but new markers will further aid in identification/isolation of satellite cells (Giordani et al. 2019; Hicks et al. 2018; Porpiglia et al. 2017). More recent transcriptomic analysis has used satellite cells fixed much closer to quiescence, allowing more detailed examination of the gene expression profile of quiescent cells (Machado et al. 2017). Employing such methodology at the single-cell level will help further define the nature of satellite cell heterogeneity. Satellite cells remain the most potent cell contributing to myogenesis, and factors including availability, difficulty of isolation, in vitro expansion limitations, loss of potency ex vivo, delivery to patients or difficulty of genetic manipulation are being addressed to enhance their usefulness as therapeutic tools (e.g., Gilbert et al. 2010; Ho et al. 2017). However, alternative myogenic cell types are also being considered and tested for therapy.

At present, the main unorthodox skeletal myogenic progenitors are broadly classed as MICs, those associated with blood vessels and BMDCs. A difficulty in dissecting the contribution of these different cell types to myogenic differentiation lies in their inherent heterogeneity and complex interactions. A panel of markers are normally required for isolation, since many antigens are promiscuous, e.g., Sca1 is found in FAPs and endothelial cells, and CD34 is expressed by both FAPS and satellite cells. Indeed, these populations can overlap, with some PICs, for example, now classed as a subpopulation of FAPs. More subpopulations are being identified as analysis methods are increasingly refined and achieve characterization at an enhanced depth. Single-cell RNA sequencing of mononucleated cells isolated from uninjured muscle shows that cells can be clustered into three major groups: $CD45^+$ blood cells, $CD31^+$ endothelial cells, and Lin^- cells (showing the greatest group diversity) (Giordani et al. 2019) with two newly characterized populations emerging as $Itga7^+/Vcam^-$ or Scx^+ cells.

There may also be a closer relationship between myoblasts and such non-canonical myogenic precursors from in vitro evidence. Some unfused mononucleated cells derived from cultures of multinucleated myotubes derived from immortalized human myoblasts, show a distinct mesenchymal state, with many not expressing myogenin or Myf5, but being positive for ID2 and ID3 (inhibitors of myogenic differentiation) and SPHK1 (Zeng et al. 2016). SPHK1 plays a role in epithelial to mesenchymal transition and expression correlates with invasiveness in cancer cells (Xu et al. 2017). Could transition of some myoblasts to a more mesenchymal state also be an origin for some MICs in skeletal muscle?

Techniques allowing us to characterize cell population at high resolution are constantly evolving, and we can expect that the future will shed more light on the heterogeneity within satellite cells and other cell types with myogenic potential, and the complex cell interactions that govern skeletal muscle regeneration.

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