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REVIEW

It takes all kinds: heterogeneity among satellite cells and fibro-adipogenic progenitors during skeletal muscle regeneration

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ABSTRACT

Vertebrate skeletal muscle is composed of multinucleate myofibers that are surrounded by muscle connective tissue. Following injury, muscle is able to robustly regenerate because of tissue-resident muscle stem cells, called satellite cells. In addition, efficient and complete regeneration depends on other cells resident in muscle – including fibro-adipogenic progenitors (FAPs). Increasing evidence from single-cell analyses and genetic and transplantation experiments suggests that satellite cells and FAPs are heterogeneous cell populations. Here, we review our current understanding of the heterogeneity of satellite cells, their myogenic derivatives and FAPs in terms of gene expression, anatomical location, age and timing during the regenerative process – each of which have potentially important functional consequences.

KEY WORDS: FAP, Satellite cell, Fibro-adipogenic progenitor, Muscle, Regeneration

Introduction

Up to 40% of total body mass is made up of skeletal muscle, which is essential for movement, structural support and whole-body metabolism (Baskin et al., 2015). It is composed primarily of contractile multinucleate myofibers. These myofibers are surrounded by muscle connective tissue, which maintains the structural integrity of muscle and functionally transmits muscle contractile force to tendons and bones.

Adult vertebrate skeletal muscle has a remarkable capacity for regeneration, with restoration of complete function by one month, even after severe damage, in both mice and humans (Warren et al., 2007; Laumonier and Menetrey, 2016). Following acute injury, myofibers undergo degeneration and necrosis. As myofibers are post-mitotic, regeneration of skeletal muscle is mediated by tissue-resident stem cells, the satellite cells.

Satellite cells are essential for muscle regeneration (Fig. 1). In response to injury, quiescent satellite cells activate, transition to myoblasts and differentiate into myocytes, which fuse and regenerate multinucleate myofibers (Relaix and Zammit, 2012; Dumont et al., 2015). In addition to regenerating myofibers, some satellite cells self-renew, returning to quiescence and the myofiber-associated niche. Whether satellite cells differentiate or self-renew is tightly regulated to ensure both efficient regeneration of muscle and maintenance of the satellite cell pool (Dumont et al., 2015).

In addition to satellite cells, muscle regeneration requires the coordination of multiple cell types for complete restoration of muscle. These cell types include immune cells (macrophages, eosinophils, neutrophils and regulatory T cells), vascular endothelial cells and pericytes (Wosczyna and Rando, 2018). Of particular note are another population of cells, the fibro-adipogenic progenitors (FAPs; Joe et al., 2010; Uezumi et al., 2010) (Fig. 1). FAPs are a population of fibroblasts embedded in the muscle connective tissue, and during regeneration they play two crucial roles. First, FAPs synthesize the transient connective tissue fibrosis necessary to maintain the structural integrity of regenerating muscle (Goetsch et al., 2003; Serrano and Muñoz-Cánoves, 2010). Second, they provide trophic support for myogenic cells (Murphy et al., 2011; Wosczyna et al., 2019).

Regeneration is a complex cellular process. It requires the temporally and spatially regulated interplay between satellite cells, FAPs and other populations of cells resident in muscle. Furthermore, it has become apparent that satellite cells, their myogenic derivatives and FAPs are heterogeneous populations. The nature and functional consequences of this heterogeneity are long-standing questions. Recently, the introduction of single-cell sequencing and mass cytometry technology has led to the publication of multiple single-cell analyses of skeletal muscle that provide new insights into the heterogeneity of satellite cells, their myogenic derivatives and FAPs. Additional functional insights have been provided by new genetic and transplantation experiments. In this Review, we discuss current advances in our understanding of heterogeneity in satellite cells, their myogenic derivatives and FAPs with regards to gene expression, anatomical location, age, and timing during the regenerative process and functional capacity.

Overview of satellite cell-mediated skeletal muscle regeneration

Satellite cells were first identified and named based on their unique ‘satellite’ position between the sarcolemma and basement membrane of myofibers (Fig. 1; Mauro, 1961). They were postulated to be muscle stem cells, but it was not until they were found to specifically express the transcription factor PAX7 (Seale et al., 2000) that their function could be formally demonstrated. The generation of *Pax7*^{CreER} mice (Table S1) enabled inducible genetic labeling and ablation of satellite cells in the adult and established that satellite cells are both necessary and sufficient for muscle regeneration (Lepper et al., 2011; Murphy et al., 2011; Sambasivan et al., 2011).

Satellite cells are generally thought to uniformly lie in a quiescent G0 state in their niche underneath the basement membrane and adjacent to myofibers (Montarras et al., 2013). In homeostatic muscle, satellite cells constitute less than 10% of myofiber-associated nuclei (Bischoff and Franzini-Armstrong, 2004). Yet, the number of quiescent satellite cells varies stereotypically in different muscles: more satellite cells are associated with slow myofibers (e.g. in the predominantly slow soleus muscle), whereas fewer are associated with fast myofibers [e.g. in the fast extensor digitorum longus (EDL) muscle] (Collins et al., 2005). In addition, culture of C2C12 or MM14

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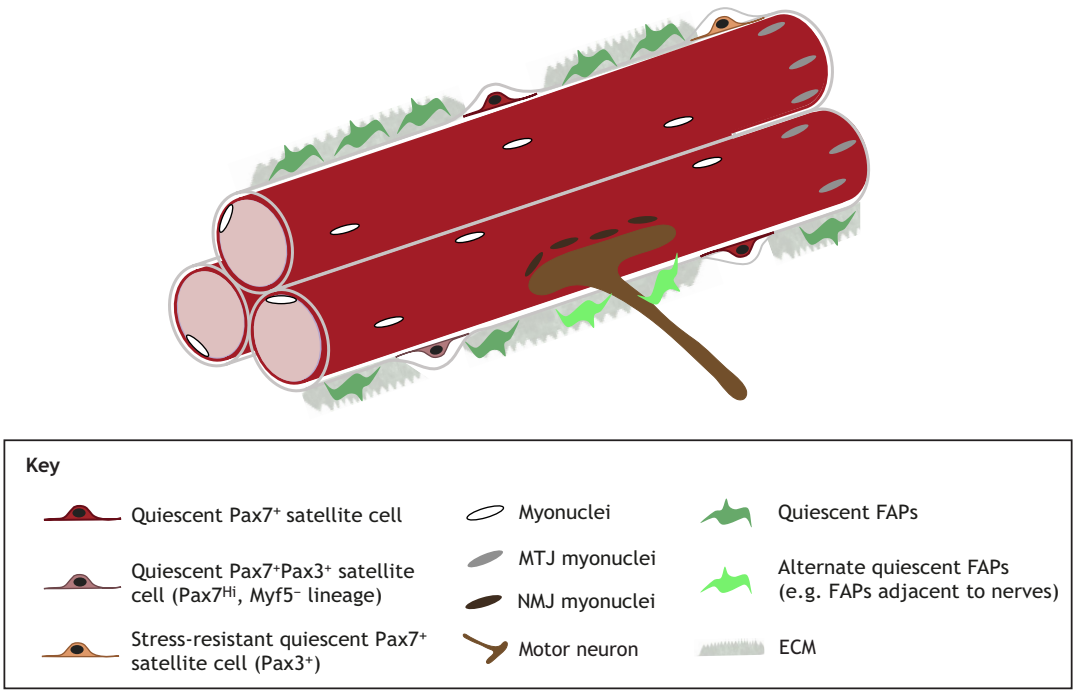

Fig. 1. Populations of satellite cells, FAPs and myonuclei in uninjured skeletal muscle. Genetic studies support the existence of at least two subpopulations of quiescent satellite cells. Stem quiescent satellite cells have been identified by expression of high levels of Pax7 (Rocheteau et al., 2012) and by absence of expression of Myf5 (Kuang et al., 2007). Stem cells resistant to stress express Pax3 (Der Vartanian et al., 2019; Scaramozza et al., 2019). Myonuclei adjacent to neuromuscular junctions (NMJ) and myotendinous junctions (MTJ) are distinct from myonuclei along the main body of the myofiber (Jacobson et al., 2001; Koch et al., 2004; Petrany et al., 2020). There is currently no consensus on whether there are multiple subpopulations of quiescent FAPs. Muhl et al. (2020) identified FAP subpopulations localized to different muscle regions, including adjacent to the motor neurons. ECM, extracellular matrix.

satellite cell lines or primary satellite cells associated with myofibers revealed that subpopulations, termed reserve cells, are more resistant to differentiation (Clegg et al., 1987; Miller, 1990; Yoshida et al., 1998; Olguín and Olwin, 2004; Zammit et al., 2004). These observations suggest that quiescent satellite cells are a heterogeneous population, with differences in functional capacity.

In response to injury or damage, satellite cells activate, leave their niche and re-enter the cell cycle (Fig. 2; reviewed by Relaix and Zammit, 2012). Activated satellite cells increase in size and have a greater RNA content (Chargé and Rudnicki, 2004; Fukada et al., 2007; Rodgers et al., 2014). Upon activation, satellite cells either become committed myoblasts that express sustained levels of MYOD and differentiate to regenerate myofibers (Lahmann et al., 2019) or self-renew to replenish the satellite cell pool. Asymmetric cell division of activated satellite cells is thought to be crucial for establishing these two subpopulations of activated satellite cells (Shinin et al., 2006; Kuang et al., 2007; Feige et al., 2018). An outstanding question is whether these two subpopulations are distinct and exhibit unique molecular signatures.

Satellite cells destined to regenerate myofibers follow a stereotypical series of steps (Fig. 2). Upon activation, myoblasts proliferate and migrate to the site of injury. MYOD+ myoblasts differentiate into myocytes that express myogenin (MYOG) and fuse to regenerate myofibers (Chargé and Rudnicki, 2004). Regenerating myofibers, with their hallmark centralized nuclei, transiently express embryonic myosin heavy chain (MyHCemb) and then mature to express slow or fast myosin (Chargé and Rudnicki, 2004). Thus, the process of myogenesis during regeneration follows a defined trajectory: quiescent PAX7+MYOD− satellite cells activate and become committed PAX7−MyoD+ myoblasts, then differentiate to MYOG+ myocytes and finally fuse into myosin heavy chain+ (MyHC+) myofibers. Although PAX7, MYOD and MYOG have been key transcription factors defining the different myogenic populations, it has been an area of interest whether other previously unrecognized myogenic populations are present and whether cell surface markers [allowing for fluorescence-activated cell sorting (FACS) isolation] identify these populations.

Both regenerated and uninjured myofibers are syncytial, meaning they are composed of hundreds of myonuclei sharing a common cytoplasm and sarcolemma. How homogeneous these hundreds of myonuclei are within one myofiber was largely unknown. The accumulation of *AchR* (also known as *Chrna2*) and *AchE* transcripts adjacent to the neuromuscular junction and *Col22a1* transcripts near the myotendinous junction argued that individual myonuclei are molecularly distinct (Jacobson et al., 2001; Koch et al., 2004). The identification of myofibers that express more than one MyHC isoform (MyHCI, MyHCIIA, MyHCIIX, MyHCIIB) also raises the question of whether individual myonuclei are specialized to express one type of MyHC and whether individual myonuclei in a mixed myofiber may express different MyHCs (Dos Santos et al., 2020). Recent single-nucleus RNA-seq experiments, which we discuss below, shed light on this debate.

Elucidating satellite cell heterogeneity based on gene expression

For many years, researchers have tried to determine the degree of heterogeneity in the quiescent satellite cell population in muscle (reviewed by Biressi and Rando, 2010). One of the first pieces of molecular evidence for heterogeneity came from the identification in cultured isolated myofibers of a rare population of satellite cells that do not express CD34, M-cadherin or MYF5 (Beauchamp et al., 2000). Subsequently, the Rudnicki lab (Kuang et al., 2007) used

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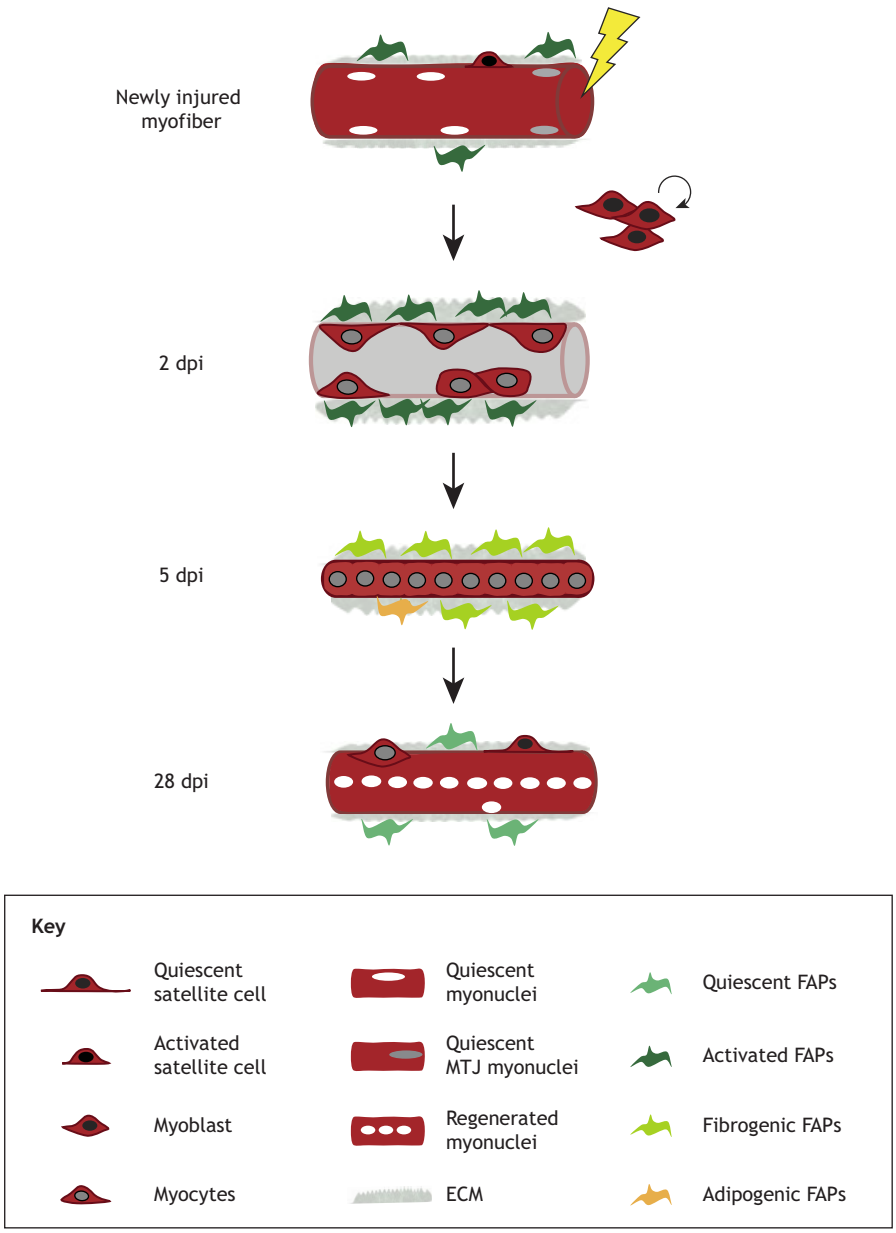

Fig. 2. Populations of satellite cells, their myogenic derivatives and FAPs during skeletal muscle regeneration. In response to injury, quiescent satellite cells and FAPs are activated and these activated cells are distinct from their quiescent counterparts (e.g. McKellar et al., 2020 preprint). Activated satellite cells differentiate into myoblasts and then myocytes, which fuse to form regenerated myofibers with characteristic centralized nuclei. Activated FAPs differentiate into fibrogenic or adipogenic FAPs and presumably transition to a quiescent FAP phenotype when regeneration is largely complete at 28 dpi (e.g. McKellar et al., 2020 preprint). ECM, extracellular matrix; MTJ, myotendinous junctions.

Myf5$^{cre/+}$;*Rosa*$^{YFP/+}$ mice to show that the majority of quiescent satellite cells express yellow fluorescent protein (YFP) (and so are *Myf5*+ or derived from *Myf5*+ cells), but 10% are negative for YFP (and so have never expressed *Myf5*) and are more stem-like.

Distinct subpopulations of quiescent satellite cells have also been identified based on the expression levels of *Pax7* and the related transcription factor *Pax3* (Rocheteau et al., 2012; Der Vartanian et al., 2019; Scaramozza et al., 2019). Rocheteau and colleagues identified two populations of quiescent satellite cells based on *Pax7* expression using transgenic *Pax7-nGFP* mice (Table S1) (Rocheteau et al., 2012). They showed that *Pax7*^{Hi} cells expressed higher levels of *Cxcr4* and *CD34* (markers of stemness; Beauchamp et al., 2000; Sherwood et al., 2004) and lower levels of *Myog* (a marker of differentiation). Upon activation, *Pax7*^{Hi} cells took longer to perform the first cell division compared with *Pax7*^{Lo} cells, and most *Pax7*^{Hi} cells performed asymmetric DNA segregation during cell division, with the more stem-like daughter retaining template DNA. These results suggest that *Pax7*^{Hi} cells are a subpopulation of quiescent satellite cells that are more stem-like than *Pax7*^{Lo} cells. It has also been shown that a subset of *Pax7*$^{+}$ satellite cells co-express *Pax3* (Montarras et al., 2005; Relaix et al., 2005a). Two independent studies (Der Vartanian et al., 2019; Scaramozza et al., 2019) demonstrated that *Pax7*$^{+}$ satellite cells that also express *Pax3* are resistant to stress (environmental and irradiation). In normal conditions, *Pax3*$^{+}$ satellite cells have limited contribution to muscle regeneration, but under stress they possess extensive regenerative potential (Scaramozza et al., 2019). During muscle homeostasis, *Pax3*+ satellite cells have also been found to contribute more readily to myofibers during muscle homeostasis (de Morree et al., 2019). Why only subsets of satellite cells express the *Pax3* transcript is unclear, but PAX3 protein levels are additionally regulated by alternative polyadenylation (Boutet et al., 2012; de Morree et al., 2019). Overall, these studies identify heterogeneity in quiescent satellite cells present in uninjured muscle, but the relationship between the identified subpopulations has yet to be resolved.

The advent of single-cell RNA-seq and multi-parameter mass cytometry (CyTOF) have enabled the detailed characterization of

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cell populations within a tissue (Bandura et al., 2009; Macosko et al., 2015; Zheng et al., 2019). Recently, multiple single-cell analyses have been conducted on skeletal muscle (Table S2) and have investigated the composition of quiescent satellite cells (Table S2). Several studies found only a single population of quiescent satellite cells (Cho and Doles, 2017; De Micheli et al., 2020a; Oprescu et al., 2020; Rubenstein et al., 2020), whereas others found two subpopulations – one more quiescent or stem-like and one more primed to activate (Dell'Orso et al., 2019; De Micheli et al., 2020b; Kimmel et al., 2020, 2021; Yartseva et al., 2020) – or even more (Barruet et al., 2020; Perez et al., 2021). However, recent re-analysis and integration of all available single-cell and single-nuclei data calls into question whether subpopulations of quiescent satellite cells can be identified by these technologies (McKellar et al., 2020 preprint). Several studies have revealed that the dissociation of quiescent satellite cells induces stress, and the expression of genes encoding for heat shock proteins, immediate early genes (e.g. *Fos* and *Jun*) and other stress-related genes leads to the induction of an artificial cell population that resembles activated satellite cells 4 h post-injury (Machado et al., 2017, 2021; van den Brink et al., 2017). McKellar and colleagues' re-analysis of all available datasets reveals that subpopulations of quiescent satellite cells are largely found in studies in which these cells were isolated via FACs and/or were cultured and therefore are likely experimentally-induced artifacts (McKellar et al., 2020 preprint). Thus, single-cell and single-nuclei studies do not currently support the existence of distinct subpopulations of quiescent satellite cells. However, it should be noted that these technologies are not well suited for the discovery of rare populations due to the small number and incomplete transcriptome of captured cells (McKellar et al., 2020 preprint).

Another question yet to be resolved is whether upon activation and asymmetric cell division some satellite cells have a unique set of markers that indicate that they will self-renew, whereas others have a different set of markers that indicate that they will differentiate. Several single-cell and single-nuclei studies examining regenerating muscle have only found a single population of activated satellite cells (Table S2; McKellar et al., 2020 preprint; Oprescu et al., 2020; Yartseva et al., 2020). However, two studies that examined multiple time points post injury and conducted pseudo-time analyses distinguished two populations of activated satellite cells (Porpiglia et al., 2017; De Micheli et al., 2020a). A third study of cultured primary satellite cells also identified two clusters of activated satellite cells (Kimmel et al., 2020). The lack of consensus among these studies likely derives from the difficulty in sampling enough activated satellite cells, as they are a relatively small and transitory population. An additional complication is a lack of a definitive marker of activated satellite cells. Although activated satellite cells have canonically been designated as *Pax7*+ cells which express MYOD protein (Grounds et al., 1992; Yablonka-Reuveni and Rivera, 1994), a recent study has shown that proliferating, activated satellite cells express oscillating levels of MYOD and so MYOD does not definitively mark these cells (Lahmann et al., 2019). A further difficulty is that subpopulations of activated satellite cells may be distinguished by lowly expressed genes or genes subject to post-transcriptional or post-translational modifications (e.g. Bernet et al., 2014). Thus, whether single-cell RNA-seq experiments can discriminate different subpopulations of activated satellite cells is unclear. To help resolve the question of heterogeneity in activated satellite cells, future single-cell studies will need to focus on the early time points post injury [1.5-3 days post injury (dpi)] and explicitly examine cell cycle status.

Elucidating myogenic cell and myonuclei heterogeneity based on gene expression

An area in which single-cell technology has been fruitful is the elucidation of populations of differentiating myogenic cells. Single-cell analyses of regenerating muscle have readily been able to identify proliferating myoblasts and differentiating myocytes as distinct populations (Porpiglia et al., 2017; Dell'Orso et al., 2019; De Micheli et al., 2020a; McKellar et al., 2020 preprint; Oprescu et al., 2020; Yartseva et al., 2020). Having distinguished these populations, these studies have been useful for providing additional markers (see Table S2) and insights into the cell-cycle regulators and the metabolic state of myoblasts and myocytes.

Myofibers are multinucleate, and whether myonuclei in different locations along mature myofibers are molecularly and functionally distinct has been a long-standing question. It has been difficult to assess transcriptional heterogeneity of myonuclei owing to their shared cytoplasm, but with the advent of single-nucleus RNA-seq technology, the transcriptional diversity of myonuclei has now been investigated. Four studies have identified multiple distinct populations of myonuclei (Chemello et al., 2020; Dos Santos et al., 2020; Kim et al., 2020; Petrany et al., 2020). Specifically, myonuclei associated with the myotendinous junctions, neuromuscular junctions, spindle-fibers and perimysium are molecularly distinct and the bulk of myonuclei express only one MyHC isoform. Interestingly, myonuclei populations and their transcriptomes differ during postnatal growth, age and muscular dystrophy (Chemello et al., 2020; Petrany et al., 2020; Perez et al., 2021). Together, these studies demonstrate that myonuclei have distinct transcriptional profiles that are associated with specialized regions and functions of the myofiber. How and when this specialization occurs is not known, but presumably it happens after myocyte fusion, as satellite cells, myoblasts and myocytes do not appear to contain specialized subpopulations correlating to the specialized myonuclear populations.

Spatial and temporal heterogeneity of satellite cells during muscle regeneration

The anatomical location of satellite cells imparts significant heterogeneity in their gene expression, numbers and behavior. Satellite cells in different anatomical regions are derived from different embryonic regions (Randolph and Pavlath, 2015). Satellite cells in the trunk, limb, diaphragm and tongue arise from the somites. In contrast, satellite cells in the extraocular muscles arise from the prechordal mesoderm and cranial paraxial mesoderm of the first pharyngeal arch, masseter satellite cells arise from the first and second arches, and pharyngeal satellite cells from the third and fourth arches. Reflecting their different embryonic origins, extraocular, head, masseter and limb muscle satellite cells have different gene expression profiles (Harel et al., 2009; Sambasivan et al., 2009; Ono et al., 2010). Recently, two groups have explicitly profiled gene expression of satellite cells derived from the extraocular, masseter and tibialis anterior (TA) muscles. Notably, they found that satellite cells in the TA uniquely express HoxA and HoxC genes, as a result of both cell-intrinsic and -extrinsic (i.e. host environment) determinants (Evano et al., 2020; Yoshioka et al., 2021).

Not only does the number of satellite cells vary between different muscles (Collins et al., 2005; Ono et al., 2010; Keefe et al., 2015), but their behavior differs. During homeostasis, satellite cells continuously contribute to uninjured myofibers, but the degree of contribution varies between slow and fast myofibers and in different anatomical muscles (Keefe et al., 2015), potentially reflecting cell-intrinsic differences among satellite cells in different muscles

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(de Morree et al., 2019). During regeneration, satellite cells from different muscles are also functionally heterogeneous (see discussion below). Even within individual muscles, heterogeneity in satellite cell behavior exists. In uninjured muscle, satellite cells that reside in closer proximity to endothelial cells have been found to be more quiescent (EdU label-retaining; Verma et al., 2018). This suggests that endothelial cells may be crucial for inducing or maintaining a more stem-like (reserve) satellite cell population in muscle.

Although regeneration of myofibers proceeds in a stereotyped manner, several studies have found that the satellite cell-mediated rate of regeneration can vary. The time to activation is accelerated when a previous or contralateral muscle injury has occurred (Rodgers et al., 2014). In these circumstances, satellite cells primed for activation (termed G-alert) reach their first division sooner than quiescent G0 cells. Similarly, in response to environmental stress, *Pax3*+ satellite cells exhibit accelerated cell-cycle kinetics and appear to be 'alerted' compared with *Pax3*− cells (Der Vartanian et al., 2019; Kimmel et al., 2020). Differences in activation have also been found in satellite cells derived from different muscles: in myofiber cultures, satellite cells of the soleus muscle (composed mainly of slow MyHCI and IIa myofibers) activate more quickly than cells derived from the EDL muscle (composed of fast MyHCIIb myofibers) (Motohashi et al., 2018). Surprisingly, *in vivo* satellite cells derived from either the EDL or soleus regenerate myofibers at a faster rate than satellite cells derived from the TA (composed of MyHCIIx and b fibers) when transplanted into the TA muscle (Collins et al., 2005). Therefore, both fiber type and muscle-specific effects regulate the kinetics of associated satellite cells.

Satellite cells and regeneration are also significantly affected by age. With age, muscle mass and function declines (Narici and Maffulli, 2010) and concomitant with this, satellite cell number is generally thought to decline (Collins et al., 2007; Shefer et al., 2010). Aged satellite cells differ from young satellite cells in gene expression (Liu et al., 2013; Bernet et al., 2014; Sousa-Victor et al., 2014) and their epigenetic profile (Liu et al., 2013). In addition, as discussed below, multiple studies have demonstrated that aged satellite cells have compromised regenerative capacity. Finally, two recent studies have demonstrated that age affects satellite cell kinetics: satellite cells derived from older mice are delayed in their regenerative response compared with those derived from younger mice (Kimmel et al., 2020, 2021).

In vivo functional heterogeneity of satellite cells

Transplantation studies, pioneered by Mikel Snow, Terence Partridge and Miranda Grounds (Snow, 1977, 1978; Partridge et al., 1978; Watt et al., 1982) have been essential in evaluating the regenerative capacity and potential functional heterogeneity of satellite cell populations *in vivo*. Such experiments have established that satellite cells from different muscles are functionally different (Collins et al., 2005; Ono et al., 2010). For example, soleus-derived donor satellite cells produce not only a higher number of myofibers, but more satellite cells than TA-derived satellite cells (Motohashi et al., 2018). A comparison of satellite cells derived from extraocular muscles with those derived from limb muscles also revealed that extraocular satellite cells have a significantly higher rate of engraftment when transplanted into the TA (Stuelsatz et al., 2015). Interestingly, the Motohashi study also showed that only donor satellite cells derived from the slow soleus, and not donor cells from the TA, yielded slow MyHCI+ regenerated myofibers when transplanted (Motohashi et al., 2018). Together, these studies indicate that satellite cells derived from different anatomical muscles and associated with different fiber types have different functional capacities.

Transplantation studies have revealed satellite cell subpopulations with distinct functional capabilities. As mentioned earlier, Kuang and colleagues distinguished YFP− and YFP+ satellite cells derived from $Myf5^{cre/+};Rosa^{YFP/+}$ mice (Kuang et al., 2007). Transplantation experiments showed that YPF− cells generated both YFP− and YFP+ satellite cell populations and produced more myofibers than YFP+ cells, indicating that YFP− cells are more stem-like. Similarly, when satellite cells were isolated based on *Pax7* expression ($Pax7^{lo}$ and $Pax7^{Hi}$), $Pax7^{Hi}$ cells generated both $Pax7^{Hi}$ and $Pax7^{lo}$, although they did not differ in their fiber engraftment potential following transplantation (Rocheteau et al., 2012).

Functionally distinct satellite cell subpopulations have also been identified based on their cell-cycle behavior. Using H2B-GFP mice, Chakkalakal and colleagues distinguished a subpopulation of rarely-dividing satellite cells, termed label-retaining cells (LRCs) (Chakkalakal et al., 2012). Transplantation of LRCs showed that they give rise to more satellite cells and myofibers than non-LRC satellite cells (Chakkalakal et al., 2012, 2014). Recently, further heterogeneity within the LRC population, based on *Mx1* lineage, was demonstrated (Scaramozza et al., 2019). Transplantation of *Mx1*+ and *Mx1*− LRC satellite cells showed that *Mx1*+ LRCs possessed a greater self-renewal potential and myofiber contribution than *Mx1*− LRCs, suggesting that *Mx1*+ LRCs are a more potent stem cell than *Mx1*− LRCs (Scaramozza et al., 2019). *Mx1*+ LRCs co-express *Pax7* and *Pax3*, and *Pax3* is required for their enhanced self-renewal and fiber contribution following transplantation (Scaramozza et al., 2019). Interestingly, under normal regeneration conditions, the majority of satellite cell self-renewal and myofiber contribution is through *Mx1*−*Pax3*−*Pax7*+ satellite cells; only under conditions of elevated stress or multiple injuries is this reserve population of *Mx1*+*Pax3*+*Pax7*+ LRCs recruited.

Transplantation studies have also been crucial for investigating satellite cell functional heterogeneity introduced by aging. Several studies have shown, by comparing heterochronic transplantation of old with isochronic transplantation of young satellite cells into young regenerating muscle, that aged satellite cells are defective in their ability to engraft (Chakkalakal et al., 2012; Bernet et al., 2014; Cosgrove et al., 2014; Sousa-Victor et al., 2014). These studies have identified several molecular changes – an increase in *Spry1* expression and p38α/β MAPK activity and de-repression of $p16^{INK4a}$ – that lead to these cell-intrinsic functional defects. Not only do aged satellite cells differ from young ones, but heterogeneity exists within aged satellite cells. Transplantation of small numbers of aged satellite cells revealed that only a small percentage of these cells are functional stem cells (Cosgrove et al., 2014). Chakkalakal and colleagues demonstrated that the slower-dividing, more quiescent LRC aged satellite cells are a subpopulation better able to engraft and differentiate into myonuclei (Chakkalakal et al., 2012).

In summary, transplantation experiments have been crucial for revealing that satellite cells are functionally heterogeneous – differing depending upon the anatomical muscles or myofiber type they reside in, their age, lineage, gene expression and cell cycle behavior.

Identification of FAPs and their role in muscle regeneration and homeostasis

In uninjured skeletal muscle, fibroblasts are a population of cells that reside in the interstitial space between myofibers and are the major

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source of extracellular matrix (ECM) surrounding myofibers (Sasse et al., 1981; Kühl et al., 1982). ECM is crucial for muscle structure and function; it provides stability to the myofibers, blood vessels and nerves and transmits the contractile force from myofibers to tendons and bones (Grounds et al., 2005; Passerieux et al., 2007). Studying muscle connective tissue fibroblasts was initially difficult because of the lack of unique molecular markers and efficient methods to isolate these cells. Fibroblasts were originally identified and isolated via their *in vitro* functional properties; fibroblasts more readily bind to plastic dishes than myogenic cells and so have been isolated by pre-plating (Richler and Yaffe, 1970; Rando and Blau, 1994). Markers such as vimentin and α-smooth muscle actin have been used to identify muscle connective tissue fibroblasts, but these markers are not specific to these cells (e.g. Sato et al., 2003). Subsequently, other fibroblast markers were discovered: the transcription factors TCF4 (TCF7L2; Murphy et al., 2011) and OSR1 (Vallecillo-García et al., 2017; Stumm et al., 2018), fibroblast activation protein α (Roberts et al., 2013) and the receptor PDGFRα (Uezumi et al., 2010). Further lineage studies using tamoxifen-inducible $Tcf4^{CreERT2}$ (Murphy et al., 2011), $Osr1^{GCE}$ (Stumm et al., 2018) and $Pdgfr\alpha^{CreER}$ (Wosczyna et al., 2019) mice demonstrated that these markers label the muscle connective tissue fibroblasts (Table S1). In addition to these studies, two independent groups identified cell surface markers that allow fibroblasts to be isolated via FACS: CD31− CD45− α7 integrin− Sca1+ (Joe et al., 2010) and CD31− CD45− PDGFRα+ (Uezumi et al., 2010). These fibroblasts were named FAPs based on their ability to differentiate into either fibroblasts or adipocytes (Joe et al., 2010). Subsequent experiments showed that these cells are also capable of differentiating into osteocytes in the presence of BMP2 (Wosczyna et al., 2012).

There has been debate in the literature as to the relationship between muscle connective tissue fibroblasts and FAPs (e.g. Wosczyna and Rando, 2018). It is likely that FAPs and fibroblasts identified by TCF4 and OSR1 are largely overlapping (Stumm et al., 2018). A common feature of all these fibroblasts is that they are distinct from myogenic cells and express PDGFRα (Joe et al., 2010; Uezumi et al., 2010, 2014; Murphy et al., 2011; Heredia et al., 2013; Malecova et al., 2018; Stumm et al., 2018; Biferali et al., 2019; Theret et al., 2021). For the sake of simplicity, we collectively refer to all adult muscle connective tissue fibroblasts as FAPs in this Review. Nevertheless, an unresolved question has been whether there are one or more distinct subpopulations of FAPs in uninjured and injured skeletal muscle.

During regeneration, FAPs are responsible for the transient fibrosis that is necessary to maintain the structural integrity of regenerating muscle (Fig. 2). Immediately following injury, FAPs rapidly proliferate and reach peak levels 3-5 dpi and then decline to basal levels by 14 dpi (Joe et al., 2010; Uezumi et al., 2010; Murphy et al., 2011; Lemos et al., 2015; Wosczyna et al., 2019). The rapidly expanding FAPs synthesize the ECM and transient fibrosis that occurs during regeneration. As regeneration concludes, this fibrosis is resolved by decreasing the number of FAPs and dampening their expression of pro-fibrotic genes (Lemos et al., 2015). Unresolved fibrosis impairs muscle function and is characteristic of diseased muscle (Serrano and Munoz-Canoves, 2017). Thus, the number of FAPs and their molecular characteristics are tightly regulated to allow for transient fibrosis, but avoid maladaptive persistent fibrosis.

During regeneration, FAPs can also adopt the alternative fate – becoming adipocytes rather than fibrogenic (Joe et al., 2010; Uezumi et al., 2010, 2011). Transient adipogenesis is a feature of regeneration after muscle injury (Lukjanenko et al., 2013) and this fat deposition is presumably mediated by FAP-derived adipocytes. As the long-term deposition of intramuscular fat by adipocytes is generally considered maladaptive, because it impairs muscle contractile strength and metabolism (Coen et al., 2010; Biltz et al., 2020), FAP differentiation into adipocytes is tightly controlled. Recently, both cell intrinsic (Mueller et al., 2016; Wosczyna et al., 2021) and cell extrinsic (Heredia et al., 2013; Kopinke et al., 2017) mediators of FAP differentiation into adipocytes have been described.

FAPs not only contribute to transient muscle fibrosis and adipogenesis during regeneration, but they also regulate satellite cells. Genetic ablation of FAPs using $Tcf4^{CreERT2}$;$Rosa^{DTA}$ (Murphy et al., 2011) or $PDGFRa^{CreERT2}$;$Rosa^{DTA}$ (Wosczyna et al., 2019) mice demonstrated that *in vivo* FAPs prevent satellite cells from prematurely differentiating, thereby promoting proliferation of satellite cells during the early phase of regeneration. Some *in vitro* experiments co-culturing FAPs with satellite cells have corroborated these results and found that FAPs promote satellite cell expansion (Quinn et al., 1990; Fiore et al., 2016; Lukjanenko et al., 2019), whereas others report that FAPs promote satellite cell differentiation (Joe et al., 2010; Mozzetta et al., 2013). Thus, it is possible that different subpopulations of FAPs play different roles in regeneration.

Finally, FAPs have further additional functions in muscle regeneration. FAPs play an important phagocytic role in removing necrotic debris (Heredia et al., 2013). FAPs are also a significant source of secreted signals that regulate satellite cells, macrophages and regulatory T cells (Biferali et al., 2019). The multiple roles of FAPs during regeneration suggest that there may be several subpopulations of FAPs that differ in their properties, functions or fate.

In addition to their functions in regeneration, strikingly FAPs have been found to be essential for maintenance of skeletal muscle. Genetic ablation of fibroblasts using $Pdgfra^{CreERT2}$;$Rosa^{DTA}$ mice (Wosczyna et al., 2019; Uezumi et al., 2021) or transgenic *FAPα-DTR* mice (Roberts et al., 2013) resulted in a dramatic decrease in the cross-sectional area of myofibers and consequent sarcopenic-like loss in muscle mass. Therefore, FAPs are necessary to maintain homeostatic muscle. How FAPs mechanistically regulate muscle homeostasis is just starting to be elucidated; FAPs secrete BMP3B (GDF10), which maintains myofiber size via AKT signaling and neuromuscular junctions via stabilization of Schwann cells (Uezumi et al., 2021). Whether these FAPs in uninjured homeostatic muscle differ molecularly and functionally from activated FAPs during muscle regeneration has yet to be discovered.

Identification of FAP subpopulations via gene expression analysis

Single-cell technologies have allowed us to delve into FAP molecular characteristics and elucidate whether there are different subpopulations of FAPs. Multiple studies have examined the composition of quiescent FAPs in uninjured muscle (Table S2). Several studies have concluded that there is only one population of quiescent FAPs (Giordani et al., 2019; Marinkovic et al., 2019; Pawlikowski et al., 2019 preprint; De Micheli et al., 2020a,b; Petrilli et al., 2020; Kimmel et al., 2021), whereas others have identified two FAP populations (Malecova et al., 2018; Oprescu et al., 2020; Rubenstein et al., 2020; Perez et al., 2021). Another study identified nine populations of quiescent FAPs that are located in different muscle regions (Muhl et al., 2020). Thus, there currently is not a consensus on the number of quiescent FAP populations. Interestingly, the transcriptional repressor HIC1 has emerged as a

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marker of quiescent FAPs – although it is not exclusively expressed in FAPs (Scott et al., 2019).

A question that has been elucidated by the use of single-cell technology is whether quiescent FAPs differ from FAPs during regeneration (i.e. activated FAPs). Multiple studies have found that quiescent FAPs are molecularly distinct and cluster separately from activated FAPs in injured muscle (Table S2; Malecova et al., 2018; Marinkovic et al., 2019; Pawlikowski et al., 2019 preprint; Scott et al., 2019; Oprescu et al., 2020; Kimmel et al., 2021). Although there is consensus that quiescent and activated FAPs are molecularly distinct, each of these studies stratified activated FAPs via different markers (Table S2). Future studies that integrate these datasets will be required to determine the relationship between identified populations of activated FAPs.

As FAPs can differentiate into fibrogenic or adipogenic cells (Joe et al., 2010; Uezumi et al., 2010, 2011), an outstanding question is whether fibrogenic versus adipogenic FAPs are molecularly distinct. The first group to investigate FAPs during regeneration found one population that was not present in uninjured muscle and unique to differentiation (Malecova et al., 2018). This population was described as fibrogenic based on high expression of known fibrogenic genes. Subsequently, two studies have observed more than one population of FAPs that segregate by fate. Kimmel and colleagues observed pre-adipocytes, adipocytes and myofibroblasts in cultured FAPs (Kimmel et al., 2021). McKellar's integrative analysis of multiple papers found three FAP populations: a stem FAP, a pro-remodeling FAP (which appears to be fibrogenic) and an adipogenic FAP (McKellar et al., 2020 preprint). Newly identified markers of fibrogenic versus adipogenic FAPs will be useful for future studies determining what drives FAPs to these two different fates.

Overall, these analyses from single-cell data indicate that there are multiple subpopulations of FAPs. Quiescent FAPs in uninjured muscle are distinct from FAPs following injury, and fibrogenic and adipogenic FAPs are distinguishable. The relationship between these different subpopulations is currently unclear, but it will be important to resolve and distinguish whether these subpopulations have different functions in muscle homeostasis and regeneration.

Spatial and temporal heterogeneity of FAPs

An outstanding question is whether FAPs vary in different anatomical regions. Intramuscular connective tissue is organized into three interconnected sheaths: epimysium, perimysium and endomysium (Sanes, 2004). The epimysium enwraps whole muscle, perimysium surrounds fiber bundles or fascicles and endomysium ensheathes individual myofibers. Each of these layers contains distinct proteoglycans and collagens (Jarvinen et al., 2002; Purslow, 2020). This difference in ECM expression in the different layers suggests that FAP populations within each layer may vary. Furthermore, there is some evidence that FAPs derived from different anatomical muscles are different in their growth rates and expression of matrix metalloproteases (Archile-Contreras et al., 2010). Whether FAPs in these different sheaths are molecularly distinct has recently been investigated by single-cell RNA-seq (Muhl et al., 2020). The authors found distinct populations of endomysial (six subclusters) and perimysial (two subclusters) FAPs, as well as FAPs associated with nerves and a newly described FAP population adjacent to the perimysium. Although these populations are distinguished by gene expression and location within the muscle, it has yet to be determined whether they are functionally distinct. Also, it is still unknown whether epimysial FAPs are molecularly and functionally distinct.

Another important question is whether FAPs differ temporally – over the course of regeneration or throughout the lifetime of an individual. During regeneration, the expansion and clearance of FAPs is tightly regulated (Lemos et al., 2015; Contreras et al., 2019; Marinkovic et al., 2019; Contreras et al., 2020; Eisner et al., 2020). A series of studies have shown that FAPs produce and secrete different factors at different times during regeneration to provide an optimal regenerative milieu (Lemos et al., 2012; Mozzetta et al., 2013; Kuswanto et al., 2016; Juban et al., 2018; Biferali et al., 2019; Lukjanenko et al., 2019). For example, FAPs secrete IL33 during the initial phases of regeneration (0-1 dpi; Kuswanto et al., 2016), whereas IL10 and IL6 are secreted at 3 dpi (Lemos et al., 2012). Congruent with these data, single-cell data have also segregated FAP subpopulations based on their progression during the regenerative process (Table S2). Oprescu et al. (2020) observed activated FAPs immediately following injury at 0.5-2 dpi, *Wisp1*+ FAPs at 3.5-5 dpi, *Dlk1*+ FAPs at 10 dpi and *Osr1*+ FAPs at 21 dpi. *Wisp1*+ FAPs are a fibrogenic population (based on co-expression of ECM genes) and have previously been identified as a distinct FAP population during regeneration (Lukjanenko et al., 2019). Likewise, a progression of FAP populations during regeneration has been shown, with activated FAPs at 2 dpi, remodeling or fibrogenic FAPs at 5 dpi and resolved FAPs at later post-injury time points (De Micheli et al., 2020a).

FAPs also differ over the lifetime of an individual. The quantity and quality of connective tissue changes with age, leading to increased muscle stiffness and reduced muscle function (Haus et al., 2007). Consistent with this, FAPs from aged versus young mice differ in their transcriptional profile (Lukjanenko et al., 2019; Kimmel et al., 2021). Aged FAPs also differ functionally such that, during regeneration, aged FAPs expand and are cleared more slowly and are more fibrogenic and less adipogenic (Lukjanenko et al., 2019).

Functional heterogeneity of FAPs

Transplantation experiments have been an important experimental paradigm to test the fate and function of FAPs. Initial studies of FAPs isolated from uninjured muscle found that they do not engraft when transplanted into uninjured muscle, consistent with a hypothesis that healthy uninjured muscle does not support fibroadipogenic infiltration (Joe et al., 2010). However, it has subsequently been shown that FAPs isolated from uninjured muscle are able to engraft into uninjured muscle in which FAPs have been genetically ablated (Uezumi et al., 2021), into regenerating muscles after a $BaCl_2$ injury (Uezumi et al., 2010; Wosczyna et al., 2019) or into muscles with adipocyte infiltration after a glycerol injury (Joe et al., 2010; Uezumi et al., 2010; Wosczyna et al., 2021). Therefore, it appears that FAPs from uninjured muscle are only able to engraft when transplanted into a disturbed muscle environment. Transplantation experiments have provided strong evidence that the muscle environment determines whether FAPs become either fibrogenic or adipogenic. Multiple studies have found that FAPs transplanted into muscle after a glycerol injury differentiate into adipocytes (Joe et al., 2010; Uezumi et al., 2010; Wosczyna et al., 2021). Uezumi and colleagues explicitly tested whether the local host environment dictates FAP fate by conducting a challenge experiment: FAPs derived from muscle damaged by a myotoxin or glycerol injury were transplanted into muscle damaged by the opposite injury type (Uezumi et al., 2010). FAPs from a glycerol-injured muscle transplanted into a toxin-injured muscle yielded few donor cells, whereas FAPs from a toxin injury transplanted into a glycerol-injured muscle yielded an accumulation of donor-derived adipocytes. These experiments demonstrate that the host

environment strongly affects the fate of transplanted FAPs. However, the fate of donor FAPs is not entirely dictated by the host environment. For example, the intrinsic expression of miR-206 in FAPs inhibits FAP differentiation into adipocytes via repression of pro-adipogenic *Runx1* – even when transplanted into glycerol-injured muscle (Wosczyna et al., 2021). Thus, although the host environment appears to have a dominant role in determining FAP fate, intrinsic FAP determinants also play a role.

The important function and therapeutic potential of FAPs in regulating muscle has also been elucidated by FAP transplantation experiments. Transplantation of FAPs into regenerating muscle reveals their function in regulating satellite cells and myofiber regeneration. For example, transplantation of young versus aged FAPs into injured muscle showed that young FAPs more strongly supported the expansion of activated satellite cells and demonstrated that the secretion of WISP1 by young FAPs (and not aged FAPs) was a key paracrine signal mediating this function (Lukjanenko et al., 2019). Transplantation of FAPs into uninjured muscle has been crucial for establishing that FAPs are essential for maintaining myofiber size and overall muscle mass (Wosczyna et al., 2019; Uezumi et al., 2021). In contrast, transplantation of pathogenic FAPs (derived from denervated muscle) into uninjured muscles leads to muscle atrophy and fibrosis and so demonstrates the important role of FAPs in regulating muscle pathology. Recently, transplantation experiments have also revealed the therapeutic potential of FAPs. Transplantation of FAPs that have been metabolically rewired by a short-term high-fat diet improved the size of dystrophic myofibers (Reggio et al., 2020) and suggested that FAPs are an important source of signals that can positively affect diseased myofibers.

Overall, transplantation experiments are a powerful tool to dissect the fate and function of FAPs and the role of intrinsic and extrinsic determinants of these processes. To date, these experiments have largely used FAPs isolated from uninjured hindlimb muscle. In the future, such transplantation experiments could be used to test whether different FAP subpopulations – isolated at different times during regeneration, from diverse muscles of different ages or from endomysial, perimysial or epimysial sheaths – are indeed functionally heterogeneous and/or have different fates.

Conclusions and future perspectives

In vertebrate skeletal muscle, satellite cells and FAPs are essential cell populations that contribute to muscle homeostasis and are required for muscle regeneration. However, as a multitude of studies have demonstrated, satellite cells and FAPs are heterogeneous populations. Satellite cells and FAPs vary between different anatomical muscles and even differ within individual muscles. Such anatomical heterogeneity perhaps is a consequence of the structural, functional and metabolic differences of the muscles they reside in. In addition, satellite cells and FAPs from aged compared with young muscles differ in gene expression and their functional properties.

Recent single-cell analyses have provided important insights into satellite cells and their myogenic derivatives (Table S2). Such analyses have been able to distinguish quiescent from activated satellite cells and delineate the series of myoblasts and myocytes that differentiate into myofibers during regeneration (Porpiglia et al., 2017; Dell'Orso et al., 2019; De Micheli et al., 2020a; McKellar et al., 2020 preprint; Oprescu et al., 2020; Yartseva et al., 2020) as well as show that there are several distinct populations of myonuclei (Chemello et al., 2020; Dos Santos et al., 2020; Kim et al., 2020; Petrany et al., 2020). However, current single-cell papers have surprisingly not definitively detected distinct subpopulations of quiescent satellite cells. Based on the identification of reserve cells in satellite cell cultures and *My5* lineage, *Pax3* and *Pax7* studies (Beauchamp et al., 2000; Montarras et al., 2005; Relaix et al., 2005b; Rocheteau et al., 2012; Der Vartanian et al., 2019; Scaramozza et al., 2019), different populations of quiescent satellite cells seem likely. However, single-cell analyses are challenging because of the difficulty of isolating and analyzing truly quiescent satellite cells and the potential rarity of these subpopulations (McKellar et al., 2020 preprint); clearly this is an important issue to resolve. Another unresolved question is whether satellite cells from different anatomical muscles or even within different regions within particular muscles (e.g. near capillaries or associated with slow myofibers; Motohashi et al., 2018; Verma et al., 2018) are transcriptionally distinct and would segregate into distinct subpopulations. Currently, satellite cells from only a small number of muscles (e.g. extraocular, masseter, TA, EDL and soleus) have been explicitly compared and functionally tested. A survey of a wider array of muscles may reveal new populations of satellite cells with advantageous and potentially therapeutically useful properties.

Single-cell analyses have also elucidated heterogeneity in FAPs by identifying several FAP subpopulations. Quiescent FAPs found in uninjured muscle are distinct from FAPs activated during regeneration (Malecova et al., 2018; Marinkovic et al., 2019; Pawlikowski et al., 2019 preprint; Scott et al., 2019; Oprescu et al., 2020; Kimmel et al., 2021) and, within regenerating muscle, FAP subpopulations have been identified that differ in fate (fibrogenic versus adipogenic; Malecova et al., 2018; McKellar et al., 2020 preprint; Kimmel et al., 2021) or appearance in the regenerative process (De Micheli et al., 2020a; Oprescu et al., 2020). Resolving the lineage relationships between these subpopulations and determining their functional significance is an important area for future research. Also yet to be discovered is whether FAPs from different anatomical muscles are transcriptionally and/or functionally distinct. The different developmental origins of FAPs and the important role of these cells in differentially regulating the morphogenesis of anatomical muscles (Sefton and Kardon, 2019) suggests that FAPs residing in different adult anatomical muscles are molecularly distinct. Determining whether FAPs from different muscles differ molecularly and have unique functional capabilities may yield unique insights into their developmental and regenerative functions, as well as their therapeutic potential.

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Competing interests

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