Muscle interstitial cells: a brief field guide to non-satellite cell populations in skeletal muscle

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Running Head: Muscle interstitial cells, a brief field guide.

Summary

Skeletal muscle regeneration is mainly enabled by a population of adult stem cells known as satellite cells. Satellite cells have been shown to be indispensable for adult skeletal muscle repair and regeneration. In the last two decades, other stem/progenitor cell populations resident in the skeletal muscle interstitium have been identified as "collaborators" of satellite cells during regeneration. They also appear to have a key role in replacing skeletal muscle with adipose, fibrous or bone tissue in pathological conditions. Here, we review the role and known functions of these different interstitial skeletal muscle cell types and discuss their role in skeletal muscle tissue homeostasis, regeneration and disease, including their therapeutic potential for cell transplantation protocols.

Keywords: skeletal muscle, interstitial cells, pericytes, mesoangioblasts, mesenchymal progenitors, fibro-adipogenic progenitors, *Pw1*, interstitial cells, stem cells, muscle regeneration

Introduction

The primary role of skeletal muscle is to generate movement, maintain posture and support soft tissues, contributing also to body metabolism and temperature control. Muscle contraction and force generation is mediated by the interaction of actin and myosin proteins within the complex sarcomere unit. Aligned sarcomeres units make myofibrils, bundles of which span the length of each muscle fibre (myofibre). In turn, numerous bundles of myofibres make up each muscle [1]. These multinucleated, syncytial cells are formed during the process of myogenesis [2]. However, as myofibre nuclei are post-mitotic they are unable to contribute to growth and repair [3].

It is generally accepted that satellite cells, a population of muscle stem cells that reside beneath the basal lamina of myofibres, are responsible for the regenerative capacity of adult skeletal muscle. Satellite cells are a heterogeneous group of stem cells of embryonic somitic origin that normally reside in a quiescent state until activated by damage or growth signals [4-6]. The vast majority of mammalian satellite cells can be identified by expression of the paired-box transcription factor Pax7, which is satellite cell-specific in skeletal muscle. Many other proteins mark the majority of satellite cells, including Integrin-α7, M-Cadherin, Caveolin-1, CD56/NCAM, CD29/Integrin-β1, Syndecan 3 and 4 (reviewed in [4,7,8]). However, these markers are also expressed by other populations of cells within the muscle tissue, so they should be used in combination to ensure specificity (Table 1). Once activated, satellite cells undergo defined proliferation/differentiation or self-renewal processes to contribute either to tissue repair or replenishment of their stem cell pool [9-11].

Other stem/progenitor cell populations present in the adult skeletal muscle (Figure 1) have been identified as capable to contribute to or to modulate muscle regeneration. The role of these populations in normal muscle homeostasis and function is still under

investigation, although some populations, including pericytes/mesoangioblasts, Pw1+ cells, and CD133+ cells hold special interest as therapeutically useful cell types to substitute satellite cells in clinical applications, such as stem cell transplantation. Other populations, referred here as mesenchymal progenitors have been investigated as pharmacological targets for tissue remodelling.

Here, we provide an overview and discuss the role and known functions of these non-satellite cells residing in adult skeletal muscle, focusing on studies published in the last decade. Additional information on other cell populations (e.g. muscle resident "side population") can be found in Table 1. We direct the reader to other review articles for a more comprehensive analysis of developmental origins of muscle stem cells, molecular networks, functions and use in cell-based therapies [4,8,12-18]. We define satellite cells as a Pax7+ cells located underneath the basal lamina, and interstitial cells as those resident between myofibres and outside their basal lamina. This will help to distinguish satellite cells from occasional Pax7+ cells in the muscle interstitium, which could either be separate interstitial stem cell populations or satellite cells trapped outside the basal lamina following myofibre remodelling (Paolo Bianco, personal communication).

Pericytes and mesoangioblasts

Pericytes are a heterogeneous group of contractile cells which encircle the endothelium of micro-vessels, first described by Rouget in 1873 [19]. Present in all vascularised tissues, pericytes regulate blood vessel growth, homeostasis and permeability, in addition to other tissue-specific roles (reviewed in [14]). In skeletal muscle, blood vessels run adjacent to myofibres resulting in the close association and likely cross-talk between pericytes and satellite cells [20-22]. Indeed, pericytes have been shown to regulate post-natal myogenesis and satellite cell quiescence [23]. At rest, pericytes are embedded within the

vascular basement membrane, which separates them from other periendothelial mesenchymal cells [14,22].

A major limitation in the study of muscle pericytes is the lack of a specific marker to distinguish them from other satellite and non-satellite cell populations (reviewed in [12,14]). Therefore, they are mainly defined by their anatomical location and by the combined expression of multiple genes. Additionally, expression of accepted pericyte markers differs between species, cellular subpopulations and developmental stage [14,21,24,25]. Furthermore, not all muscle pericytes have the potential to contribute to skeletal myogenesis. The subpopulation of pericytes with skeletal myogenic capacity can be distinguished in vivo by expression of tissue non-specific alkaline phosphatase (TNAP) in murine and human muscle [21,26]. Skeletal muscle pericytes/perivascular cells with myogenic potential can also be identified by the expression of CD146 ([27] and Paolo Bianco and Mara Riminucci, personal communication) or Nestin [24], although Nestin is also expressed in satellite cells [28]. The extent to which these three populations overlap is currently unknown. Interestingly, a recent report has confirmed that human pericytes isolated from skeletal and smooth muscle tissues are functionally different, and that only the pericytes isolated from skeletal muscle are able to contribute to skeletal muscle regeneration [29].

Mesoangioblast is a term for vessel-associated mesodermal stem/progenitor cells expanded *in vitro*, initially utilised for cells isolated from the murine embryonic dorsal aorta [30]. Mesoangioblast markers depend on the stage of development at which they are isolated; embryonic mesoangioblasts deriving from the dorsal aorta express mostly endothelial markers such as VE-cadherin and CD34 [31,32]. Cells similar to embryonic mesoangioblasts can be derived from adult skeletal muscle pericytes, expressing varying degrees of pericyte markers such as neuro-glial 2 proteoglycan (NG2), platelet-derived

growth factors receptor beta (PDGFR-β), alpha smooth-muscle actin (αSMA), desmin and, most importantly, TNAP, whilst being negative for endothelial and myogenic makers [14,21,24-26]. Mesoangioblasts also express Pw1 (see below), which was shown to be essential for proper stem cell function [33]. To simplify relationships with specific resident skeletal muscle cells, mesoangioblasts are considered as the activated progeny of pericytes in the same way that myoblasts are the activated progeny of satellite cells (Figure 1).

Although myogenic pericytes may have a lower myogenic capacity than myoblasts, their advantageous traits of expansion, migration and extravasation upon intra-arterial delivery in dystrophic models [21,25,34-37] make them suitable candidates for cell therapies of muscle disorders. Additionally, activated mesoangioblasts can self-renew or migrate under the basal lamina and contribute to the satellite cell pool during skeletal muscle growth, chronic and acute muscle regeneration [25,26,35,38]. Importantly, Pax7+ cells are also found when expanded mesoangioblasts are transplanted. Notably, a first-in-human phase I/II clinical trial based upon intra-arterial delivery of pericyte-derived mesoangioblasts in five boys with Duchenne muscular dystrophy was recently completed [39]. The study showed a good safety profile (one adverse event with no clinical sequelae) and functional parameters were transiently stabilised in two out of three ambulant patients. Although donor-derived dystrophin was detected in one patient, several aspects of the current protocol will need optimization in order to reach clinical efficacy. Examples of future improvements may include: 1) enrolment of younger children; 2) increase of cell dose (e.g. by means of iPS cell-derived progenitors) [40]; 3) modulation of inflammation [41,42]; 4) enhanced engraftment or differentiation by acting on properties of donor cells [43,44] or recipient patients [37].

Finally, several recent publications have implicated a role for pericytes in fibro-

adipose infiltration of tissues (reviewed in [45]. In muscle, some pericytes may be precursors of myofibroblasts, interstitial cells which regulate fibrosis. Indeed, Birbair and colleagues have shown that type 1 non-myogenic pericytes contribute to fatty-fibrotic accumulation in aged and regenerating muscle [24,46]. Therefore, pericyte-based therapies should focus on promoting myogenic differentiation whilst repressing adipo/fibrotic differentiation.

Fibro-adipogenic / mesenchymal progenitors

Other resident muscle interstitial progenitor populations may have increased propensity to differentiate towards non-myogenic cell types. One such population has been identified upon expression of PDGFR-α, CD34 and stem cells antigen-1 (Sca1). Initial reports, based on in vitro experiments, showed that these cells could be an important source of pro-differentiation signals for myoblasts during the process of muscle regeneration and that were able to differentiate into myofibroblasts and/or adipose cells [47,48]. Consequently, they were named fibro/adipogenic progenitors (FAPs) or mesenchymal progenitors (MPs). Recent reports have demonstrated that these cells are also capable of osteogenic and chondrogenic differentiation *in vivo* [49], therefore we will refer to these cells using the more general term “mesenchymal progenitors” (Figure 1). However, they should not be confused with “mesenchymal stem cells”, whose markers and properties are still a matter of active discussion [50]. We apologise to the reader for the oversimplification of the model (and the possible occasional inappropriate nomenclature) and redirect them to excellent reviews that clarify terminology and lineage relationships of mesenchymal stem/progenitor cells in other mesodermal tissues [50,51].

It has been shown that during acute muscle injury, these mesenchymal progenitors activate, rapidly expand and then disappear [47,48,52]. Once activated, they have been

shown to interact with satellite cells and with the regenerating muscle environment, promoting satellite cell differentiation and myofibre formation [47,48]. However, it was recently shown that upon ageing, mesenchymal progenitors have a deleterious effect on satellite cell function, repressing satellite cell myogenesis [53]. Moreover, epigenetic reprograming of mesenchymal progenitors by treatment with HDAC inhibitors has been shown to drive them toward a myogenic lineage and improve regeneration of dystrophic mice [54], opening a therapeutic avenue for these progenitors. Interestingly, mesenchymal progenitors have been isolated from human muscles [55] and demonstrated to contribute to adipocyte formation [56].

The expansion of resident mesenchymal progenitors has been shown to be mediated by the cytokine Interleukin 4 (IL-4), produced by eosinophils during the early phases of regeneration [57]. Also, a recent report from Rossi lab has demonstrated that pro-inflammatory cytokines (i.e. tumour necrosis factor, TNF) produced by the first wave of infiltrating macrophages induces apoptosis of mesenchymal progenitors. During chronic injury, where pro-inflammatory and anti-inflammatory macrophage populations coexist [58], changes in the cytokine milieu (i.e. higher levels of transforming growth factor β1, TGFβ1), prevent the apoptosis of mesenchymal progenitors and induces their differentiation into persistent matrix-producing cells [52]. The cytokine combination to induce adipogenic differentiation remains to be determined, although certain types of injury (with different inflammatory response and therefore different cytokine environment) can highly increase differentiation to the adipogenic lineage [48]. Lemos and colleagues findings are in line with a previous report demonstrating that a subpopulation of mesenchymal progenitors expressing the metalloproteinase ADAM12, is one of the major sources of fibrotic tissue accumulation after muscle damage [59]. This subpopulation of mesenchymal progenitors rapidly differentiates into myofibroblasts upon TGFβ1 stimulation and it may represent a more committed fibrogenic-progenitor. Interestingly,

ADAM12+ mesenchymal progenitors share features with pericytes, being associated with blood vessel walls and expressing NG2, a marker of pericytes [59].

Muscle-resident fibroblasts are the cell population classically thought to be responsible for extracellular matrix remodelling and accumulation of fibrosis in pathological conditions such as muscular dystrophies. However, they also support healthy myogenesis, as ablation of transcription factor 4 (Tcf4)-positive muscle fibroblasts has been shown to impair muscle regeneration through premature differentiation of satellite cells and reduction of the satellite cell pool [60]. Interestingly, Tcf4 is expressed by both fibroblasts and mesenchymal progenitors, making it difficult to decipher the individual roles of each cell type using current experimental strategies. Specifically, whether some of the functions currently accounted to fibroblasts may be in fact performed by different populations of mesenchymal progenitors (and vice versa). Additionally, in chronic injury models, resident myoblasts, endothelial and hematopoietic cells have been shown to trans-differentiate into fibroblastic cells, advancing dystrophic pathology [60,61], with a mechanism of transdifferentiation that occurs through an intermediate mesenchymal stem cell step [61]. Therefore, the definition of which cells are fibroblast or mesenchymal progenitors and their origin may be even more difficult than expected.

Besides their role during muscle regeneration and chronic pathologies, resident mesenchymal progenitors may have a role in skeletal muscle homeostasis. They secrete a number of Wnt ligands and myokines such as IL-6 [47,48,52]. Moreover, it has been demonstrated that interstitial mesenchymal cells are the main producers of collagen VI in resting muscle [62]. Collagen VI fibres are abundant in the endomysium of skeletal muscle and are a regular component of the satellite cell niche [63]. Mutations in collagen VI encoding genes cause several diseases associated with muscle weakness in humans [64,65], and collagen VI deficient mice show myofibre degeneration, reduced strength and

deficient satellite cell self-renewal [63,66-68]. Interestingly, mesenchymal progenitors of synovial origin secreted collagen VI when engrafted into muscle [69], indicating that this may be one of the functions of muscle-resident mesenchymal progenitors.

PW1+ interstitial cells

In 2010, Mitchell et al. isolated a Pax7- non-satellite cell muscle-resident population located in the skeletal muscle interstitial space and capable of myogenic differentiation [18,70]. Apart from their location, these cells are characterised by the expression of the PW1/paternally expressed gene 3 (Peg3), and were named as PICs (PW1+ interstitial cells). In addition, PICs were mostly Sca1+ and CD34+. Lineage tracing experiments demonstrated that PICs do not share the same embryonic origin of satellite cells, and have increased potency, since they are capable to generate smooth and skeletal muscle cells and adipocytes [70].

Interestingly, satellite cells and mesoangioblasts also express PW1 [32,33,70]. Another report using a PW1 reporter mouse demonstrated that the combination of PW1, Sca1 and PDGFRα markers may be used to separate all the different stem cell populations in skeletal muscle [71]. Using this isolation strategy, the PW1+/Sca1+/PDGFRα+ cells are the most abundant subpopulation and comprise the totality of the fibro-adipogenic mesenchymal progenitors with pro-adipogenic potential. Interestingly, this population is similar to the recently described resident brown adipocyte progenitors in the skeletal muscle which were isolated also with the Sca1 marker [72]. PW1-/Sca1+ cells were also functionally similar to the FAPs/MPs, though just with pro-fibrotic potential. In the referred study, this is the only cell subpopulation having a fibroblastic fate. It is therefore tempting to propose that fibro-adipogenic mesenchymal progenitors may be a heterogeneous population of muscle-resident Sca1+ cells that upon pro-fibrotic environmental cues (e.g.

TGFβ) will turn into fibroblastic cells, or upon still poorly characterised signals, will acquire PW1 expression and become adipogenic. The PW1+/Sca1+/PDGFRα- population comprises a small group of cells with myogenic potential but negative for Pax7, defined by the authors as “non- satellite cell progenitors with myogenic potential”, although they hold some pro-adipogenic potential in vitro. These cells may account for the Sca1+ primary myoblast subpopulations described in some reports in the early 2000s [73-77]. Finally, the PW1+/Sca1-/PDGFRα- subpopulation included Pax7+ satellite cells and Pax7- cells which were positive for adult myogenic pericyte markers (e.g. NG2+/PDGFRβ+/Myf5-). Interestingly, PW1 is expressed in pericyte-derived mouse and human mesoangioblasts, where it regulates their myogenic ability and migration capacity [33].

CD133+ cells

CD133 (Prominin 1) was identified as a surface marker of both neural and haematopoietic stem and progenitor cells [78], and its expression has been used to characterise a population of human blood and muscle-derived myogenic stem cells. A small fraction of adult peripheral blood cells expressing CD133 was initially shown to display myogenic potential [79]. Muscle-resident human CD133+ cells are found both in the muscle interstitium and underneath the basal lamina of myofibers, co-expressing Pax7 [80]. When expanded *in vitro*, CD133+ preparations contained a heterogeneous population of cells expressing myoblast, pericyte and mesenchymal genes [80,81]. Additionally, expression of CD133 is unstable in culture and influenced by culture media; a thorough expression analysis has not been performed on freshly isolated cells due to their rarity [80-82]. When injected intramuscularly, human CD133+ cells effectively engraft in the muscle and contribute to myogenesis with a proportion entering the satellite cell compartment [80-82]. Transplanted human CD133+/Pax7+ cells are functional, and capable of regenerating

mouse muscle following injury [80]. Taken together, the variability of genes and anatomical location implies that CD133-positivity may distinguish a heterogeneous set of stem cells with high myogenic capacity. This makes them an interesting candidate for cellular therapies and indeed they were tested in a pilot, phase I autologous clinical study for Duchenne muscular dystrophy based upon intramuscular transplantation without genetic correction [83]. However, whether the proportion of cells that extravasates and engrafts into muscles downstream of the injection site derives from the population expressing pericyte markers or whether the CD133+/Pax7+ population is able to be safely injected systemically is currently unknown. Although there have been no reports on the contribution of mouse CD133+ cells to skeletal muscle regeneration (probably due to technical reasons), the use of reporter mouse models for CD133 expression in other stem cell niches [84], may allow future lineage-tracing studies in murine skeletal muscle.

Concluding remarks and future prospects

Adult muscle growth and regeneration is fuelled by satellite cells. However, a growing milieu of interstitial stem or progenitor cells have been described both in resting and regenerating skeletal muscle, which are able to crosstalk with satellite cells, myoblasts, myofibres and cells of vascular and hematopoietic origin. These interstitial cells can differentiate into vascular, fibrogenic, adipogenic, osteogenic, and chondrogenic lineages in pathological conditions (e.g. [24,55,56,85]), although their function and lineage relationships in healthy tissue (where non-myogenic differentiation pathways have a supportive role, or are absent or are repressed) is still far from being understood.

Moreover, there is an urgent need to improve the characterisation and distinction of the different populations of muscle interstitial progenitors, in order to determine whether particular cell types identified in different studies might actually be analogous and to find

out which of them should be enhanced (or repressed) to foster efficient myogenesis. In the near future, advanced flow cytometry techniques such as spectral flow cytometry [86,87] or flow cytometry coupled with mass spectrometry (mass cytometry or CyTOF) [88,89] which able to discriminate between many factors at the same time, could allow researchers to answer these questions.

A question likely to arise from this in-depth analysis is when does the differential expression of markers correspond to a subpopulation, or to a separate progenitor population? Additionally, how definitive are these populations? During normal growth and regeneration, some interstitial muscle progenitors are known to have lineage plasticity. A well-characterised example of this is pericytes becoming Pax7+ stem cells residing under the basal lamina [26]. Whether these cells are identical to satellite cells and whether they can transdifferentiate back to the pericyte lineage is unknown. Moreover, lineage plasticity between many of the muscle resident cell populations has been demonstrated to increase greatly in pathogenic conditions [61,90], implying that cellular relationships and composition of the cellular populations in uninjured, acutely or chronically injured skeletal muscle could vary dramatically. Furthermore, it is crucial that *in vivo* analysis of cell populations is performed on freshly-isolated cells, as changes in the physical environment and culture medium during *ex vivo* expansion can greatly impact surface-marker expression, as can different isolation protocols.

The majority of studies describing interstitial muscle stem cells in healthy and pathological tissue have been performed in rodents. Differences in marker expression between species are well documented in satellite cells (reviewed in [7]) and the field is now gradually improving the knowledge on the human satellite cell niche, their markers and properties [91]. Similar characterization efforts are being done for human mesenchymal progenitors [56]. Therefore, it is crucial to identify and characterise the

comparable interstitial muscle stem cell populations in human muscle to the well-known rodent ones, in order to maximise therapeutic relevance.

Finally, a thorough characterisation of the different subpopulations of muscle satellite cells and interstitial progenitors may enable the development of next-generation protocols to derive them from human pluripotent stem cells [92] for drug screening, tissue engineering and cell therapies of skeletal muscle disorders.

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Figure Legends

Figure 1. Illustration of the main cellular populations in adult skeletal muscle.

A skeletal muscle fibre and a blood vessel are shown. Satellite cells, interstitial populations and vessel-associated cell populations and their main protein markers (blue) are indicated. Differentiation of satellite cells, pericytes and mesenchymal progenitors/PICs is showed.

Table 1. Summary of skeletal muscle-resident stem or progenitor cellular populations. Markers used for isolation and characterization, and known functions and therapeutic implications are indicated.

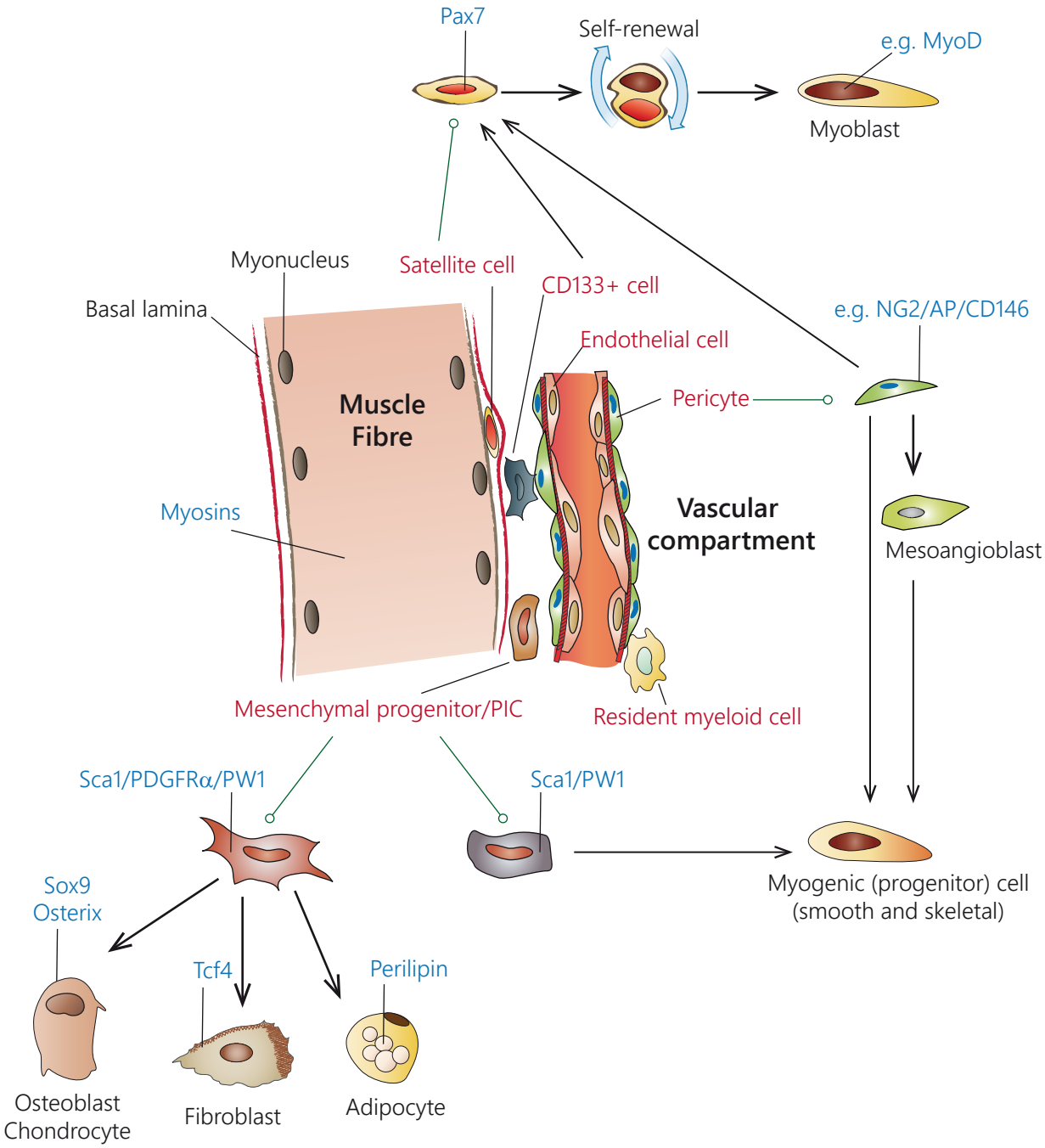
Pax7
Self-renewal
e.g. MyoD
Myoblast
Myonucleus
Satellite cell
CD133+ cell
Basal lamina
e.g. NG2/AP/CD146
Endothelial cell
Muscle Fibre
Pericyte
Myosins
Vascular compartment
Mesoangioblast
Mesenchymal progenitor/PIC
Resident myeloid cell
Sca1/PDGFRα/PW1
Sca1/PW1
Sox9
Osterix
Myogenic (progenitor) cell (smooth and skeletal)
Tcf4
Perilipin
Osteoblast
Chondrocyte
Fibroblast
Adipocyte

Figure 1

Table 1.

Cell populations in non-regenerating murine muscle

Cell name	Positive for	Negative for	Differentiation/Function	Therapeutic implications	References
Stem or progenitor cells					
Satellite cells (SC)	Pax7, SM/C2.6, α7-integrin, M-cadherin, B1-integrin, CD34, Myf5, PW1, Nestin, (Pax3)	CD45, CD31, PDGFRα, (Sca1)	Myogenesis in vivo and ex vivo	Genetically corrected intra-muscular (IM) transplantation	[93,11,94,28,95,47,70,71,96]. Reviewed in [97,4].
Adult pericytes	TNAP, PDGFRβ, NG2, Nestin (type 2), Desmin, CD146, SMA	Nestin (type 1), Pax7, CD56, PDFRα (type 2)	Angiogenesis, myogenesis, pericyte and SC self-renewal, fibrosis/fatty infiltration (type 1)	Genetically corrected IM and intra-arterial transplantation	[24,26,21]. Reviewed in [12].
PICs	PW1/Peg3, Sca1, CD34, (PDGFRα)	Pax7, (PDGFRα)	PDGFRα+ adipogenesis and PDGFRα- myogenesis. Contribute to SC pool	---	[74,71]
FAPs/ Mesenchymal progenitors	Sca1, PDGFRα, PDGFRβ, Vimentin, Adam 12, PW1, tcf4	CD31, CD45, α7-integrin, SM/C-2.6, αSMA, NG2, Pax7	Sca1-: Adipogenesis, osteogenesis and Chondrogenesis ; Sca1+ : Adipo/fibrogenesis	Targets for anti-fibrotic interventions with drugs inhibiting PDGFRα	[47,52,71,48,49]. Reviewed in [17].
Other stem or progenitor populations					
CD133+ cells	CD133, (CD34)	(CD34)	Myogenesis in vivo and ex vivo	Genetically corrected IM and intra-arterial transplantation	[80,81,79]
Muscle side population (SP)	Sca1, ABCG2, (CD45), (CD31),(CD34), (Pax7)	Hoechst-negative, (CD45), (CD31), c-Kit, (CD34)	Unclear: CD45+: haematopoiesis; CD31+ :angiogenesis; negative cells myogenesis	---	[73,98-100]. Reviewed in [4].
Myoendothelial cells	CD34, Sca1, cMet	CD45, CD14, CD31, CD49, CD144, c-Kit, FLK, Pax3, Pax7, MyoD, Myf5, M-cadherin	Unclear: Adipogenesis, angiogenesis and myogenesis	---	[101]
Other resident cells					
Resident myeloid cells	CD45, CD11b, F4/80	CD11c, Ly-6C, CX3CR1	Recruitment of neutrophils, monocytes	---	[102-105]. Reviewed in [106,107].
Endothelial cells	CD31, Sca1, FLK-1, CD144/VE-Cadherin, CD34	CD45, CD11b, α7-integrin, Pax7	Vessel formation	---	[108-110,20]
Fibroblasts	Tcf4, PDGFRα	CD45, CD31, CD11b, α7-integrin, Pax7	Extracellular matrix production	---	[111,112]

Note: As these populations are heterogeneous, not all the cells express all the indicated markers and some markers may have high/medium/low subpopulations, like Pax7 in satellite cells [9]. Markers between brackets affect only a subpopulation or depend on origin/developmental stage, markers between brackets and underlined are controversial.