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Skeletal muscle aging – Stem cells in the spotlight

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ABSTRACT

Aging is characterized by a progressive decline in tissue and organ function often linked to a reduced stem cell functionality, a cell population important for regeneration. Skeletal muscle mass and regenerative capacity decrease with advancing age. Muscle stem cells, also termed satellite cells, are a prerequisite for regeneration of skeletal muscle. Their functionality declines with increasing age, driven by intrinsic changes and changes in the stem cell niche. Here, we discuss the current understanding how muscle stem cells are affected during aging. The aging associated alterations include among others upregulation of developmental pathways in aged muscle stem cells and changes in the extracellular matrix.

1. Introduction

Skeletal muscle is a tissue of high plasticity enabling the adaptation to physiological demands such as training or organismal growth (Bentzinger et al., 2013a; Schmidt et al., 2019). It`s remarkable regenerative capacity largely depends on muscle stem cells (MuSCs), also termed satellite cells after their location close to the myofiber. Consequently, depletion of MuSCs via a diphteria toxin based approach fully inhibited regeneration of skeletal muscle (Lepper et al., 2011; Murphy et al., 2011; Sambasivan et al., 2011).

Under resting conditions MuSCs are located underneath the basal lamina of the myofiber in a quiescent state characterized by the expression of Pax7 and Sprouty-1 (Chang and Rudnicki, 2014; Mauro, 1961; Shea et al., 2010). Recently, it was demonstrated that quiescent MuSCs undergo transcriptomic alterations during the isolation process. Among those changes were early activation, indicated by upregulation of the activation marker MyoD, as well as induction of a stress response suggested by the high expression of heat shock proteins (Hspb1, Hspa1b). Additionally, novel markers for MuSC quiescence were determined as they were highly expressed in quiescent MuSCs but rapidly downregulated during isolation. These markers include members of the Hox gene family (Hoxa9, Hoxd4), metabolic proteins (Ppar-alpha) and a number of zinc finger proteins (Zfp324, Zfp386) (Machado et al., 2017; van Velthoven et al., 2017; van Velthoven and Rando, 2019). *In vivo*, activation of MuSCs can be caused by injury driving MuSCs out of quiescence into proliferation and migration to the site of injury. These myogenic progenitors then differentiate and fuse to form multinucleated myotubes that will mature to myofibers, thereby repairing the site of injury (Bischoff, 1975; Schmidt et al., 2019; Sousa-Victor et al., 2018). This regenerative myogenesis is accompanied by a transcription factor cascade that resembles embryonic development, including myogenic regulatory factors like MyoD, Myf5, Myogenin and MRF4 (Bentzinger et al., 2013a; Chal and Pourquie, 2017; Hernandez-Hernandez et al., 2017; Schmidt et al., 2019).

Skeletal muscle tissue possesses the ability to undergo multiple regeneration cycles in healthy adults. However, with increasing age the regenerative capacity as well as MuSC functionality are declining (Brack and Munoz-Canoves, 2016; Mashinchian et al., 2018; Price et al., 2014; Sousa-Victor et al., 2014). Furthermore, aging of skeletal muscle is characterized by reduced muscle mass and muscle strength, also termed sarcopenia (Delmonico et al., 2007). Interestingly, recent studies suggest that the age-mediated loss of muscle mass is independent of MuSCs since their depletion in adult mice is not critical for maintenance of myofiber size or composition (Fry et al., 2015). Although it was demonstrated that MuSCs contribute to homeostatic myofibers in adult mice by long-term labeling experiments, this contribution is highly dependent on muscle type and age (Keefe et al., 2015). Moreover, a reduction of the MuSC number seems to cause increased fibrosis in the aged (Fry et al., 2015). In a follow up study it was shown that myogenic progenitor cells directly modulate the ECM (extracellular matrix) composition of their environment by an exosome-based mechanism, linking the decline in MuSC number (as seen during aging) to increased

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fibrosis (Fry et al., 2017). Albeit these observations are suggesting a MuSC contribution to skeletal muscle homeostasis further studies are necessary to clarify if and how MuSCs are involved in driving sarcopenia.

In addition to cell-autonomous, intrinsic factors, the functionality of MuSCs is depending on niche derived components (Bentzinger et al., 2013a; Munoz-Canoves et al., 2019; Schmidt et al., 2019). Importantly, both are affected during aging. Here, we will give a brief overview on MuSC intrinsic and extrinsic alterations affecting the muscle stem cell compartment during aging.

2. Changes in signaling pathways in muscle stem cells during aging

Long-term tissue maintenance and regeneration of skeletal muscle are at least partially depending on the ability of adult MuSCs to maintain quiescence through epigenetic silencing of the cell cycle regulator p16INK4a (Sousa-Victor et al., 2014). During aging, they switch from a reversible quiescent state into an irreversible cell cycle arrest (Sousa-Victor et al., 2014). Interestingly, acute shRNA-mediated knockdown of p16INK4a ameliorated the impaired activation capacity of geriatric MuSCs while also life-long removal of p16INK4a resulted in improved muscle function in a progeria mouse model (Baker et al., 2011; Sousa-Victor et al., 2014). Recently, it was shown that p16INK4a transcription in MuSCs was directly repressed by the transcription factor Slug and that Slug overexpression improved MuSC self-renewal and muscle regeneration during aging (Zhu et al., 2019). Moreover, changes in the transcriptional and epigenetic landscape of aging MuSCs have been described recently (Liu et al., 2013; Schworer et al., 2016; Sousa-Victor et al., 2014). Aged quiescent MuSCs display increases of H3K27me3 which is associated with transcriptional repression (Hernando-Herraez et al., 2019; Liu et al., 2013). In old activated MuSCs permissive chromatin states cause the aberrant induction of developmental pathways thereby impairing MuSC functionality. For instance, upregulation of Hoxa9 in activated aged MuSCs triggers developmental pathways compromising their functionality (Schworer et al., 2016). Furthermore, it was shown that p38α/β-MAPK signaling is overly active in aged MuSCs leading to a dramatic decline in self-renewal by hindering asymmetric cell divisions (Bernet et al., 2014; Cosgrove et al., 2014). Additionally, upregulation of developmentally important signaling pathways such as canonical Wnt, JAK/STAT and downregulation of Notch signaling in aged MuSCs diminish their functionality (Brack et al., 2007; Carlson et al., 2009; Conboy et al., 2003; Price et al., 2014; Tierney et al., 2014). Notch signaling was demonstrated to be important for maintaining MuSC quiescence. However, it remains not fully understood yet how Notch signaling regulates MuSC functionality and how this changes with increasing age. Conboy and colleagues showed that Notch signaling regulates quiescence of MuSCs declining with age (Conboy et al., 2003). Baghdadi et al. demonstrated that Notch maintains quiescence by inducing collagen V production connecting it to calcitonin receptor signaling (Baghdadi et al., 2018). Furthermore, it was also shown that inactivation of canonical Notch signaling resulted in spontaneous differentiation of quiescent MuSCs and depletion of the stem cell pool (Bjornson et al., 2012; Mourikis et al., 2012). In another study Liu and colleagues showed that Notch deficiency in aged mice decreased p53 activity and caused MuSC death by mitotic catastrophe, thus compromising muscle regeneration (Liu et al., 2018). Further analyses have to be performed to fully unravel the role of Notch signaling in MuSCs with a special focus on aged MuSCs. Upregulation of JAK/STAT signaling and its effector target genes play an important role during aging and in muscular dystrophy through deregulation of MuSC homeostasis (Price et al., 2014; Tierney et al., 2014). Knockdown of JAK/STAT or pharmacological inhibition of JAK/STAT signaling in aged MuSCs increased their engraftment potential and improved regeneration of skeletal muscle in the aged. Moreover, deregulation of symmetric divisions in the aged was ameliorated by inhibition of JAK/STAT signaling in aged MuSCs (Price et al., 2014). In addition to changes in different signaling pathways aged MuSCs also display alterations in metabolism and autophagy.

3. Changes in metabolism and autophagy in muscle stem cells during aging

The acute cellular energy demands determine the metabolic activity of cells. Consequently, different energy production pathways are likely utilized during proliferation, differentiation and aging although the metabolism of MuSCs has not yet been described in detail. A recent study from Pala et al. compared quiescent MuSCs (freshly isolated) from young and aged mice to activated MuSCs from young mice at day 3 and 5 after injury (Pala et al., 2018). They demonstrated that quiescent MuSCs have low energy demands in general. Furthermore, they show that quiescent MuSCs display perturbed oxidative phosphorylation during aging suggesting that aged MuSCs rely rather on glycolysis. Interestingly, they reported that 3 days after injury MuSCs display strongly increased oxidative phosphorylation and glycolysis with the ratio skewed towards oxidative phosphorylation at day 5 after injury, the time point when myogenic cells start to differentiate (Pala et al., 2018). Another study by Ryall and colleagues investigated the metabolic changes MuSCs undergo during cultivation (Ryall et al., 2015b). They demonstrated that quiescent MuSCs (freshly isolated) undergo a switch from fatty acid oxidation towards glycolysis when cultured for 48 h, concomitant with their activation (Ryall et al., 2015b). The activation of MuSCs and the switch to glycolysis result in lower NAD^+ levels in the cell which is associated with repressed nuclear SIRT1-activity leading to increased acetylation of H4K16. *Vice versa* loss of SIRT1 in MuSCs causes increased H4K16 acetylation and premature differentiation suggesting that SIRT1 is a metabolic sensor connecting metabolic changes to epigenetic regulation of MuSCs (Ryall et al., 2015a). A study by Zhang et al. further demonstrated that mitochondrial dysfunction, controlled by NAD^+, is a hallmark of aged MuSCs and that treatment with the NAD^+ precursor nicotinamide riboside prevents MuSC senescence and improves mitochondrial function in the aged (Sousa-Victor et al., 2014; Zhang et al., 2016). The discrepancies in metabolic changes of MuSCs following activation described above are probably due to the differential experimental setup, particularly the comparison of MuSCs which had undergone activation *in vivo* due to injury of the whole muscle in the study performed by Pala et al. while MuSCs were activated *in vitro* in the study conducted by Ryall and colleagues.

Calorie restriction is a dietary intervention shown to increase lifespan in multiple organisms by inhibition of mTOR (mammalian target of rapamycin), one of the master regulators of nutrient sensing (Johnson et al., 2013, Kenyon, 2010). In MuSCs, short term calorie restriction improved regeneration of aged skeletal muscle, which is accompanied by increased mitochondrial content, oxygen consumption and SIRT1 expression (Cerletti et al., 2012). Additionally, mTOR inhibition in old MuSCs improved their functionality *in vivo*, at least partially through increasing autophagy, thus linking changes in metabolism to changes in autophagy in the aged (Garcia-Prat et al., 2016a, b).

In addition to its role as a nutrient sensor SIRT1 was also shown to regulate the autophagic flux in MuSCs. A study by Tang and Rando demonstrated that deficiency of SIRT1 or inhibition of autophagy caused a delay in MuSC activation probably due to a lack of nutrients (Tang and Rando, 2014). This is in contrast to the results described by Ryall and colleagues who observed premature differentiation following loss of SIRT1. Those differences might be due to different mouse models which were used for the analyses (Ryall et al., 2015a; Tang and Rando, 2014). While Ryall and colleagues used a constitutive Pax7-Cre driver to drive excision of exon 4 of the *sirt1* gene, Tang and Rando used a tamoxifen inducible $Pax7Cre^{ER}$ in adult mice. The differences in time of

excision (embryonal versus adult) could be causative for the apparently opposite results in addition to the different means of measuring activation. In one study MyoD protein expression was used as a measure of activation while the other study used EdU incorporation. Further analyses need to be performed to solve this conundrum.

MuSCs are quiescent under resting conditions and therefore cannot eliminate altered or damaged proteins and organelles, e.g. damaged mitochondria, through division. This makes quiescent MuSCs particularly susceptible for proteostatic stress (Garcia-Prat et al., 2016b). In young MuSCs reversible quiescence is accompanied by basal autophagy keeping proteostasis at bay (Garcia-Prat et al., 2016a, b). With increasing age, the reduced autophagic activity in MuSCs leads to proteostatic stress resulting in the accumulation of damaged mitochondria and increased ROS levels driving the expression of p16INK4a in geriatric MuSCs (Brack and Munoz-Canoves, 2016; Garcia-Prat et al., 2016b). Restoration of autophagy or treatments counteracting ROS restored the regenerative capacity of skeletal muscle in geriatric mice (Garcia-Prat et al., 2016a). Although changes in signaling pathways and metabolic alterations play a key role in determining the MuSC fate, systemic factors have a major impact on skeletal muscle and MuSCs as well. Among those factors indirectly affecting skeletal muscle tissue are components reaching skeletal muscle via the blood stream.

4. Age-dependent systemic changes affecting muscle stem cells

The first evidence of systemic factors affecting MuSC function arose from heterochronic parabiosis experiments (Conboy et al., 2005). Here, Conboy and colleagues demonstrated that exposing aged mice to a more youthful systemic environment promoted regeneration of skeletal muscle shown by increased MuSC proliferation and differentiation concomitant with reduced fibrosis. This study is supported by different *in vitro* studies which demonstrated that MuSC proliferation and cell fate are under control of serum factors (Brack et al., 2007; Carlson et al., 2008; Conboy et al., 2005). Furthermore, it was shown in rats using whole muscle transplantation grafts that the regenerative capacity of old and young muscles is mainly depending on the age of the host under those conditions since transplantation into a young host allowed successful engraftment while engraftments into the old environment – either from young or old individuals – failed (Carlson and Faulkner, 1989). Engraftment studies in mice using isolated MuSCs from young and aged or even geriatric animals into young recipients demonstrated that aged and geriatric MuSCs maintain their regenerative defect (Price et al., 2014; Sousa-Victor et al., 2014). On the first glance, these results seem to be contradictory, but due to experimental differences cannot be directly compared. When isolated MuSCs from old donors are transplanted into a young recipient, they compete with the resident MuSCs from the young host and are in direct contact with the youthful niche. When a whole muscle is transplanted, less - if any - competition of donor and recipient MuSCs takes place. Furthermore, the MuSCs from the transplanted muscle are still in their endogenous niche and therefore effects from the host are mainly restricted to systemic factors. Additionally, the studies investigated different muscles and different time points of regeneration (60 days versus 7 or 21 days) impeding a direct comparison of the results. In recent years, the effect of circulating factors on MuSC function in the aged has been further investigated. One of the factors showing reduced serum levels in aged individuals is the anti-aging hormone klotho (Pedersen et al., 2013) which was shown to counteract impaired MuSC function in the aged (Ahrens et al., 2018; Sahu et al., 2018). Moreover, growth differentiation factor (GDF11) was reported to be differently abundant in the blood of aged individuals. So far, the role of GDF11 on aged MuSCs is still under debate (Egerman et al., 2015; Sinha et al., 2014). MuSCs are not only affected by changes in factors which reach skeletal muscle via the blood stream, also the direct environment of the MuSCs – the niche – shows age-dependent alterations.

5. Changes in the local environment during aging – the NMJ

The neuromuscular junctions (NMJs) are part of the muscle stem cell niche and are formed at the interphase between the myofibers and their innervating motor neurons facilitating signal transmission (Sanes and Lichtman, 1999). Over 20 years ago, Sane and colleagues showed that after nerve injury, the maintenance of reinnervated NMJs is declining if myofibers and their associated MuSCs are absent (Sanes and Lichtman, 1999). However, it remains poorly understood if and how MuSCs interact with motor axon innervation (Aguera et al., 2019; McGeachie and Allbrook, 1978; McGeachie, 1989; Murray and Robbins, 1982; Rodrigues Ade and Schmalbruch, 1995). Neurotransmission at the NMJ and subsequent skeletal muscle contraction are dependent on acetylcholine receptor signaling. Elevated acetylcholine levels induce NMJ phenotypes similar to aging, including degeneration of myofibers (Sugita et al., 2016). However, when acetylcholine levels were reduced in a heterozygous knockout mouse model ($VAChT^{Het}$) NMJ and myofiber size were increased while NMJ fragmentation or denervation in young and aged mice were not affected. Moreover, aged heterozygous mice ($VAChT^{Het}$) showed a higher number of Pax7 positive cells compared to control and young mice, suggesting an amelioration of the age-dependent reduction of MuSC numbers although the mechanism is still elusive (Vaughan et al., 2019).

In chronic denervation models MuSCs leave quiescence and start to proliferate. But strikingly, they fail to undergo normal myogenic differentiation and migrate to interstitial spaces, underlining the importance of adequate innervation for MuSC functionality (Borisov et al., 2005; Bruusgaard and Gundersen, 2008; Dedkov et al., 2001). Upon neuromuscular disruption, not only the skeletal muscle needs to regenerate but NMJs – and therefore innervation – have to be re-established. Only recently, the interdependency between MuSCs and NMJs was elegantly shown by an approach in which MuSCs were depleted (Liu et al., 2017, 2015). The authors demonstrated that combining MuSC depletion with sciatic nerve injury exacerbates myofiber atrophy, increases fibrosis and impairs skeletal muscle reinnervation. However, NMJ functionality was not affected by MuSC depletion alone during homeostasis but only in the context of NMJ regeneration after injury (Liu et al., 2015). Interestingly, depleting MuSCs in the old resulted in an early-onset of sarcopenia-related reductions in NMJ integrity and myofiber size. The authors suggest that MuSCs are a source for post-synaptic myonuclei which are important for NMJ structure integrity. Strikingly, overexpression of Sprouty1 in old MuSCs ameliorated force generation capacity and NMJ integrity (Liu et al., 2017).

The NMJ exhibits a characteristic extracellular matrix (ECM) composition at the post-synaptic site. The main constituents are collagen IV and VI among others as well as specific laminin isoforms (Cescon et al., 2018; Tintignac et al., 2015). Interestingly, loss of collagen VI resulted in fragmentation of the NMJs reminiscent of natural aging (Cescon et al., 2018). This is an elegant example how the different cell types in the muscle stem cell niche affect each other and that already slight changes – as occurring during aging – affect various cell populations in the niche thereby causing remodelling (Fig. 1).

6. Changes in the aged muscle stem cell niche

MuSCs are heavily affected by their local microenvironment. Non-muscle stem cells such as FAPs (Fibro-Adipogenic-Progenitors), PICs (Pw1+ interstitial cells) or macrophages are secreting growth factors and cytokines and modify the extracellular matrix (ECM) thereby making up a big proportion of the muscle stem cell niche (Bentzinger et al., 2013a; Schmidt et al., 2019). During aging, the muscle stem cell niche changes with the ECM being one of the most affected components (Hwang and Brack, 2018). Aged myofibers secrete higher levels of FGF2 (fibroblast growth factor 2) into the muscle stem cell niche leading to a depletion of a subpopulation of MuSCs, namely the low-cycling satellite cell population (Chakkalakal et al., 2012). Furthermore, the study

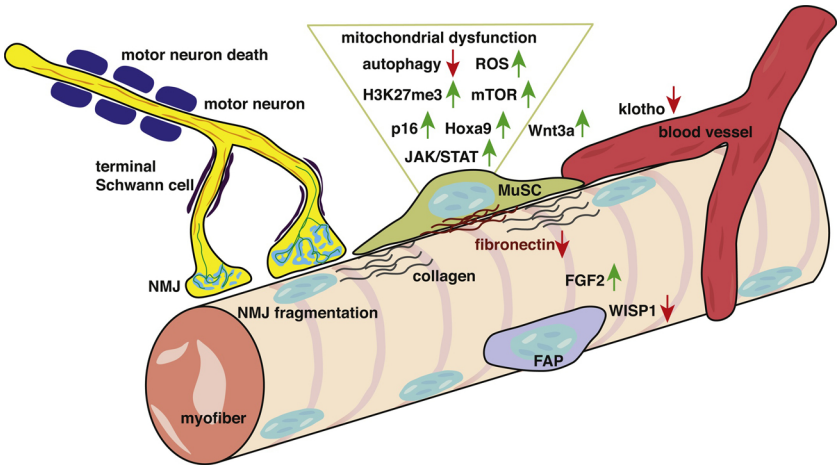

Fig. 1. Extrinsic and intrinsic changes in muscle stem cells during aging. Intrinsic changes in MuSCs and changes in their niche occur during aging thereby affecting MuSC functionality and consequently impair regeneration of skeletal muscle. Changes in MuSCs include activation of developmental signaling pathways while changes in the niche affect local and secreted factors among others. MuSC: muscle stem cell; FAP: fibro-adipogenic progenitor; NMJ: neuromuscular junction.

showed that inhibition of the FGF receptor 1 (FGFR1) is able to rescue the age-dependent defects in MuSC cycling and apoptosis (Chakkalakal et al., 2012). Increasing TGFβ (transforming growth factor) and FGF2 signaling synergize with alterations in Notch signaling and changes in the ECM to impair MuSC functionality (Hwang and Brack, 2018). This is supported by the finding that aged MuSCs demonstrate an abnormal localization of β-1-Integrin. This abnormal β-1-integrin localization then leads to a decreased response to FGF2 resulting in aberrant ERK activation (Rozo et al., 2016). Changes in Notch, Wnt and TGFβ activity contribute to an impairment in the balance between activation and differentiation which is observed in aged MuSCs (Brack et al., 2008, 2007; Carlson et al., 2009, 2008; Conboy et al., 2003). Canonical Wnt signaling is upregulated in skeletal muscle during aging (Brack et al., 2007). Through parabiosis experiments it was demonstrated that the increased activity of canonical Wnt signaling in the aged leads to a myogenic-to-fibrogenic conversion of young MuSCs resulting in impaired regeneration and increased fibrosis (Brack et al., 2007). Of note, inhibition of canonical Wnt signaling either by Dkk1 or the anti-aging hormone klotho could reinstate functionality of aged MuSCs (Ahrens et al., 2018; Brack et al., 2007). Wnt signaling plays an important role in MuSC function, further underscored by a recent study that demonstrated that Wnt4 generated in the myofiber maintains MuSC quiescence through RhoA (Eliazer et al., 2019). If Wnt4 signaling is also affected during aging needs to be determined in the coming years.

In addition to changes in the ECM composition, the stiffness of the MuSC niche is generally increasing during aging, causing impaired proliferation and fibrogenic conversion of MuSCs (Gao and Zhang, 2008; Lacraz et al., 2015; Stearns-Reider et al., 2017). Collagens, among other molecules, are known to impact the stiffness of the ECM, e.g. knockout of collagen VI was described to reduce the stiffness of the MuSC niche (Urciuolo et al., 2013). Interestingly, MuSCs also deposit ECM proteins like fibronectin and collagen V, thereby remodelling their own niche (Baghdadi et al., 2018; Bentzinger et al., 2013b). Fibronectin levels in the MuSC niche were shown to decline with increasing age leading to impaired regeneration of skeletal muscle (Lukjanenko et al., 2016). Notably, this phenotype is reversible by supplementing old regenerating muscle with fibronectin (Lukjanenko et al., 2016). Aging also affects cells which display supportive functions during muscle regeneration, e.g. FAPs fail to induce the expression of WISP1 (Wnt1-inducible signaling pathway protein 1), which is required for MuSC expansion and commitment (Lukjanenko et al., 2019).

The biggest challenge in the next years will be to determine which changes in MuSCs and their niche arise first during aging and how they are interconnected with each other thereby enabling a better understanding how aging affects MuSCs.

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