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Losing stem cells in the aged skeletal muscle niche

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Why stem cell numbers decline with age is a major question in regenerative biology and medicine. Skeletal muscle has emerged as a powerful paradigm to address this issue. Recently, genetic and cell marking strategies were used to uncover a new and causal relationship between muscle stem cells and differentiated fibers that constitute their niche and provoke their loss.

Although rats have several advantages for studying aging as they manifest aging phenotypes sooner before age-related death, the availability of powerful genetic tools for mice and the ability to use multiple readouts, in particular for skeletal muscle, has made mouse an excellent model to study age-related decline of stem cell and tissue function. The progressive loss of skeletal muscle mass, strength and endurance during aging, known as sarcopenia, allows a framework to investigate the alteration of muscle stem cell properties during the decline of tissue function. In addition, the ability of stem cells to affect muscle repair after injury can be assayed using multiple parameters either in the context of the endogenous tissue, or after transplantations.

Whether muscle stem (satellite) cells lose their regenerative capacity during aging, or contribute to the age-related decline in muscle function, are issues that have not been fully resolved. Notably, signs of aging can be manifested at different times depending on the strain of mouse used (some having longer lifespans). The 18-22-month period is particularly critical in this context as some, but sometimes not all phenotypes, could be detectable (see http://research.jax.org/faculty/harrison/ger1vLifespan1.html and [1]).

Mounting evidence points to signaling pathways, either via the circulation or within the niche, that impact muscle stem cell and tissue aging. For example, perturbation in Notch signaling has been reported to be linked to decline in regenerative capacity and stem cell function in aged mice [2, 3], however, the precise role that this pathway plays in quiescent and activated satellite cells remains unclear. Although previously thought to be required to break quiescence of satellite cells, recent reports with genetically modified mice to inactivate Notch signaling during homeostasis have shown that high Notch activity is required to maintain satellite cell quiescence, and that this activity drops significantly as satellite cells enter the cycle [4-6]. Other signaling pathways also impact on stem cell aging ([7] and references therein), however, their source (circulation vs niche) and mechanism of action remain to be elucidated.

Chakkalakal *et al*. [7] addressed these issues using an impressive array of experimental approaches. First, the authors confirmed previous studies showing that satellite cell numbers decline with age [8, 9]. It is notable that the extent of decline was variable in previous studies, as well as in the present report, likely due to the methods and markers employed (on muscle sections or isolated myofibers), muscle group examined, or other factors such as genetic strain of mice. The transcription factor Pax7 is extensively used to identify satellite cells, however, the differential loss of expression of Pax7 and other markers, particularly those on the cell surface that can be damaged by the harsh enzymatic dissociation protocols, can lead to a misrepresentation in satellite cell numbers. In spite of these technical challenges, there is general agreement that satellite cell numbers are significantly diminished in aged mice. This is the starting point of the study by Chakkalakal and colleagues [7].

The authors examined signaling pathways involved in regulating the quiescent state using a powerful genetic marking strategy that permits monitoring of the proliferative index of muscle satellite cells over extended periods, as well as their engraftment potential after transplantation. Specifically, reporter transgenic mice carrying H2B-GFP (Histone2B-green fluorescent protein fusion protein) under the regulatory control of a tetracycline-inducible promoter (TetO-H2B-GFP) were fed with doxycycline (tetracycline analogue) under a variety of conditions, including pulse or pulse-chase labeling. Dilution of this label due to cell division can be monitored (detectable for about 8 divisions in the absence of the inducer) by fluorescence-activated cell sorting (FACS). In these experiments, the satellite cells were isolated from whole muscle by enzymatic digestion, followed by selection using cell surface markers: positive for VCAM1 and integrin-α7, and negative for CD31, CD45 and propidium iodide.

Importantly, when cells were marked with H2B-GFP then chased for 20 months, at least two distinct label-retaining cell (LRC) populations were observed with discernable GFP intensities, suggesting that the satellite cell pool is heterogeneous in division rates,

consistent with a previous study using a nucleotide analogue [10]. Interestingly, label dilution was more pronounced when the pulse was initiated in aged mice then chased for 12 weeks, indicative of a less stable quiescent state. The authors then transplanted young and aged H2B-GFP+ satellite cells into preinjured regenerating tibialis anterior muscle of wild-type recipient mice (Dox-fed to prevent loss of the donor-derived H2B-GFP expression). In this assay, aged H2B-GFP+ satellite cells showed an ~60% reduction in the number of Pax7+ satellite cells and myofiber myonuclei compared to adult H2B-GFP+ satellite cells. In this type of analysis, two points should be taken into account: i) diffusion of the H2B-GFP nuclear marker in the myofiber from one myonucleus to another can result in the overestimation of the differentiation index of the population being examined; ii) the aged H2B-GFP+ satellite cells are more likely to contain a higher fraction of proliferating cells at the time of transplantation compared to adult satellite cells.

Consistent with their observations, aged satellite cells cultured for 4 days had ~30% less Pax7+ and about 3-fold more Myogenin+ (differentiation marker) cells, and 2-fold more apoptotic cells, pointing to a less robust self-renewal capacity and a higher propensity of aged satellite cells to differentiate.

To characterize the more stem-like cells within the heterogeneous satellite cell population in aged mice, the authors then exploited the H2B-GFP marker in a pulse-chase paradigm and fractionated these cells by FACS into LRC (low proliferative index; marker retention) and non-LRC (high proliferative index; marker dilution) subpopulations. Accordingly, the former expressed higher levels of upstream markers such as Pax7 and Spry1, an inhibitor of FGF signaling. As with the comparison between young and aged cultured cells indicated above, non-LRC aged satellite cells generated less Pax7+ and more Myogenin+ cells compared to the LRC fraction, yet no differences were observed in susceptibility to apoptosis in this case. Therefore, in addition to heterogeneity characterizing the satellite cell population, satellite cells in aged mice have a greater propensity to break quiescence.

To address the molecular nature of the instability in quiescence, Chakkalakal and colleagues [7] performed a battery of experiments which collectively highlight a prominent role for FGF signaling, previously shown to act as a mitogen in cultured satellite cells. Of the different ligands tested, FGF2 was the most highly expressed in aged compared to young myofibers. First, they showed that myofibers are the major source of the ligand within the muscle. Second, using an elaborate biochemical fractionation protocol, purified myofiber extracts (PMEs) and interstitial extracts were concentrated and added to cultured satellite cells, or to a model for reversibly quiescent Pax7+ (RSCs) that arise after generation of differentiated myotubes following myoblast fusion. Skeletal muscle extracts were used in the past to identify FGF as a mitogen for satellite cell proliferation [11, 12].

These findings were confirmed in the cell culture model indicated above, using FGF2 blocking antibody, the FGF receptor antagonist SU5402, and adenovirus-Cre-mediated inactivation of the receptor in *FGFR1*$^{floxed/floxed}$ mice. Remarkably, intraperitoneal administration of beads coated with the antagonist SU5402 was sufficient to reduce the number of proliferating satellite cells in the hindlimb of aged mice to the levels found in adult controls. It should be noted that although an increase (up to 3-fold in some assays) in cycling cells in the RSC model was observed in culture with PME or FGF2 and blocking reagents, this only constituted a fraction of the population, suggesting that other pathways (e.g., rupture of Notch signaling) influence aged satellite cell behavior (see Figure 1). To address the issue of a cumulative effect of disruption in signaling, prolonged (18 months) exposure to FGF signaling using *Spry1* null mice resulted in a 50% loss of satellite cells. Given these observations, it is also possible that a subpopulation of aged satellite cells is resistant to breaking quiescence.

Sprouty family members act as targets of FGF signaling, as well as negative regulators of this pathway. Interestingly, Spry1, but not the other family members, was downregulated by FGF2, and its expression was higher in aged LRCs relative to non-LRCs, in keeping with its high expression in quiescent satellite cells. Using an inducible *Pax7*^{CreER} mouse in combination with loss- (*Spry1*^{floxed}; increased FGF signaling) and gain- (transgenic *CAG-GFP*^{floxed}*-Spry1*; decreased FGF signaling) -of-function mice, the role of Spry1 was evaluated. Satellite cells from these mice were cultured in the presence of FGF2 and aged PME (with higher levels of FGF2). Using these different experimental scenarios, the authors showed that FGF signaling was modulated by Spry1 levels to maintain satellite cell quiescence. Consistent with this conclusion, loss of Spry1 function in adult mice did not impact quiescence as FGF2 levels are not elevated in young mice. By contrast, overexpression of *Spry1* in aged mice resulted in fewer satellite cells breaking quiescence.

Elevated FGF signaling in aged mice also affected muscle regeneration as *Spry1* null aged mice had significantly reduced myofiber diameter 30 days after recovery from severe muscle injury due to the diminution of the satellite cell pool. Inhibition of FGFR1 in this model also resulted in reduced myofiber diameter due to impaired repair, however, the satellite cell pool was increased, consistent with the notion that reduced FGF signaling increased self-renewal. Given that the addition of FGF2 to young satellite cells was sufficient to break quiescence, it would be interesting to determine the response of Notch

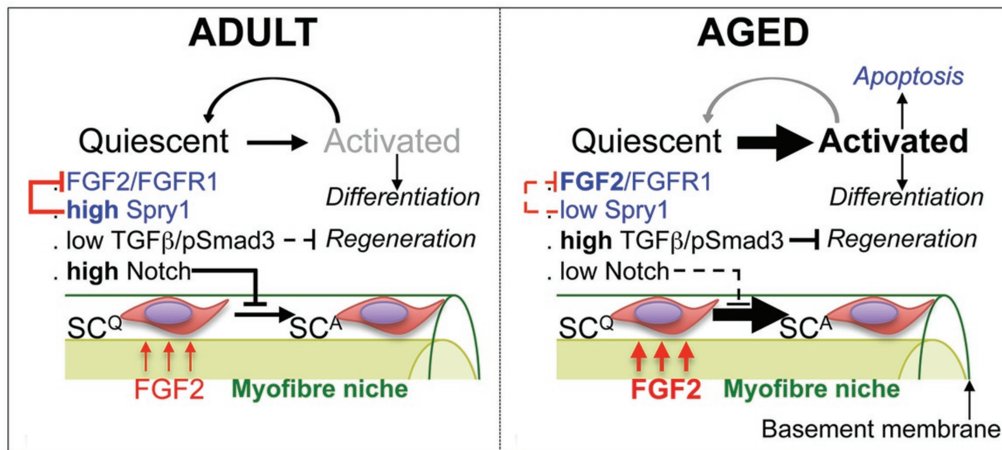

Figure 1 Scheme depicting signaling pathways regulating young and old muscle stem cells. Chakkalakal and colleagues [7] examined the effects of FGF signaling on maintenance of the quiescent (Q) and activated (A) states in muscle stem cells (red arrows, blue text). Previous work implicated other signaling pathways, including TGFβ and Notch, in regulating muscle stem cell quiescence and aging [2-6, 8, 14-16].

signaling in this context as high Notch activity is critical for maintenance of the quiescent cell state [4, 5]. In parabiotic pairing experiments where young and old mice share a common circulatory system, young serum was shown to improve muscle regeneration in the older heterochronic pair [13]. In this context, it would be interesting to determine whether young serum counteracts the effects of increased FGF levels produced from aged myofibers, or whether other mechanisms would prevent the breaking of muscle stem cell quiescence.

Future work should also focus on sorting out the relationships among the different signaling pathways that appear to be deregulated in aged mice (see Figure 1), the causal link from indirect effects, and the hierarchical level at which they act. These issues constitute major challenges in studies in aging of stem and differentiated cells.

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