

1 **Intrinsic and extrinsic factors collaborate to activate pharyngeal satellite cells**

2 **without muscle injury**

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20

21 **Abstract**

22 Satellite cells (SCs), adult muscle stem cells in craniofacial muscles proliferate and
23 differentiate/fuse without injury, unlike quiescent SCs in uninjured limb muscle. However,
24 whether intrinsic or extrinsic factors driving their increased basal activity are largely unknown.
25 We compared SCs from the pharynx, which contains constrictor muscles critical for
26 swallowing, to SCs from limb muscle. Pharyngeal SCs are intrinsically more proliferative and
27 contain higher mitochondrial content relative to limb SCs. Pharyngeal SCs occupy less
28 quiescent microenvironments containing collagen V and pharyngeal muscles provide a
29 distinctive SC niche enriched with neighboring resident macrophages and fibroadipogenic
30 progenitors. Loss of SCs impacts pharyngeal myofiber cross-sectional area and the number of
31 neighboring cells, suggesting that SCs are required to maintain pharyngeal muscle
32 homeostasis and its unique niche. Taken together, this study gives new insights to explain the
33 distinctive SC activity of craniofacial muscles, which may explain their unique susceptibility
34 to various muscular dystrophies.

35 **Keywords:** Skeletal muscle stem cells/ Satellite cells/ Pharyngeal muscle/ Satellite cell
36 activation/ Craniofacial muscle

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38 **1. Introduction**

39 The pharynx is a muscular passageway of the digestive and respiratory tracts extending
40 from the nasal and oral cavity to the larynx and esophagus. The pharynx contains a group of
41 skeletal muscles that play a critical role in many vital processes such as swallowing, breathing,
42 and speaking. Like other craniofacial muscles, pharyngeal muscles originate from non-
43 segmented cranial mesoderm during vertebrate embryogenesis, while trunk and limb muscles
44 are derived from somites (Mootoosamy & Dietrich, 2002; Noden & Francis-West, 2006). These
45 distinctive embryonic origins are associated with unique transcriptional regulatory networks in
46 myogenic progenitor cells as exemplified by PAX3-dependent limb muscle development and
47 PITX2/TBX1-dependent craniofacial muscle development. However, both early muscle
48 development pathways converge to a common myogenic program that requires expression of
49 myogenic regulatory factors such as MYF5, MYOD and myogenin (Goulding, Lumsden, &
50 Paquette, 1994; Relaix, Rocancourt, Mansouri, & Buckingham, 2005; Shahrighim Tajbakhsh,
51 Rocancourt, Cossu, & Buckingham, 1997). While mature craniofacial and limb/trunk muscles
52 are histologically very similar, they are differentially susceptible to muscular dystrophies. For
53 example, extraocular muscles are typically spared in Duchenne muscular dystrophy (Khurana
54 et al., 1995) but are preferentially affected by oculopharyngeal muscular dystrophy (OPMD),
55 a late-onset genetic disorder characterized by progressive dysphagia and ptosis (Victor, Hayes,
56 & Adams, 1962). Thus, the distinct embryonic origins of craniofacial muscles could drive
57 functional consequences in adult muscles.

58 An important common feature of craniofacial and limb/trunk muscles is presence of
59 muscle specific stem cells termed satellite cells (SCs). SCs are a heterogeneous population of
60 progenitor cells underneath the basal lamina of muscle fibers and are crucial for skeletal muscle

61 regeneration (Lepper, Conway, & Fan, 2009; Mauro, 1961; Sambasivan et al., 2011). Like most
62 other adult stem cells, SCs are quiescent under homeostatic basal physiological conditions.
63 When activated by injury or disease, SCs rapidly re-enter the G₁ phase of the cell cycle,
64 proliferate as myoblasts, and progress along a defined differentiation program known as
65 myogenesis (Shi & Garry, 2006). The properties of SCs during skeletal myogenesis have been
66 extensively investigated using easily accessible limb muscles, but some groups have expanded
67 studies to SCs in other muscle types including craniofacial muscles. According to these studies,
68 SCs from pharyngeal muscles (Randolph et al., 2015) and extraocular muscles (EOMs
69 (Stuelsatz et al., 2015)) contain a population of activated SCs that chronically proliferate and
70 differentiate into myofibers in the absence of muscle damage. The increased SC activity in
71 craniofacial muscles raises the question of whether their unique biological properties are
72 influenced by cell intrinsic factors or by the specialized microenvironment, known as the niche.
73 Multiple studies have demonstrated that extracellular components like collagen (Baghdadi et
74 al., 2018), diffusible cytokines, and growth factors released from neighboring cells such as
75 resident or infiltrating macrophages and fibroadipogenic progenitors (FAPs) (Evano &
76 Tajbakhsh, 2018) have a major influence on satellite cell activity in limb muscles
77 (Vishwakarma, Rouwkema, Jones, & Karp, 2017). In contrast, very few studies have probed
78 how the unique niche of craniofacial muscles affects SC activity (Formicola, Marazzi, &
79 Sassoon, 2014).

80 In this study, we compared SCs from pharyngeal and gastrocnemius muscles and
81 discovered distinctive intrinsic attributes of pharyngeal SCs and extrinsic factors of the
82 pharyngeal muscle niche, which may contribute to activate pharyngeal SCs without muscle
83 injury. We demonstrate that pharyngeal SCs are larger, have increased mitochondrial content,

84 and show accelerated *in vitro* proliferation and differentiation compared with gastrocnemius
85 SCs. We also show that pharyngeal SCs secrete factors, which may act in an auto/paracrine
86 manner, to induce increased proliferation of SCs relative to factors secreted from limb SCs. We
87 confirm that pharyngeal muscles are enriched with resident macrophages and FAPs, both
88 known to stimulate proliferation and differentiation of SC *in vivo*, thus providing a unique
89 niche to the resident SC population. Finally, we used SC ablation experiments to determine the
90 contribution of SC to maintenance of pharyngeal muscle and the pharyngeal muscle niche.
91 These studies provide insight into the unique properties of craniofacial muscles, which may
92 explain the differential susceptibility of these muscles to aging and disease.

93

94 **2. Materials and methods**

95 ***Mice***

96 C57BL/6J mice (Jax000664), *Pax7*^{CreERT2/CreERT2} mice (Jax017763), *Rosa*^{tdTomato/tdTomato}
97 (Jax007909), *Rosa-DTA* (Jax009669), *Pax3*^{Cre} (Jax005549), *Rosa*^{mT/mG} (Jax007576) were
98 purchased from Jackson Laboratories (Bar Harbor, ME; www.jax.org). Three or 12 months old
99 mice were used as noted in figure legend. Homozygous *Pax7*^{CreERT2/CreERT2} male mice were
100 crossed with homozygous *Rosa*^{flox-stop-flox-tdTomato} (tdTomato) to obtain *Pax7*^{CreERT2/+}; *Rosa*
101 ^{tdTomato/+} (*Pax7*^{CreERT2}-tdTomato) mice (Sambasivan et al., 2011). To label satellite cells with
102 red fluorescence (tdTomato), tamoxifen, 1 mg (Sigma-Aldrich, St. Louis, MO) per 10 grams
103 body weight, was injected intraperitoneally once daily for 5 days. Flow cytometry was used to
104 determine the recombination efficiency in *Pax7*^{CreERT2}-tdTomato mice. Quantitative
105 polymerase chain reaction (qPCR) was used to determine the recombination efficiency in *Pax7*

106 *Cre^{ERT2}-DTA* mice. Experiments were performed in accordance with approved guidelines and
107 ethical approval from Emory University's Institutional Animal Care and Use Committee and
108 in compliance with the National Institutes of Health.

109

110 ***Dissection of Muscle Tissue***

111 Pharyngeal tissue dissection was performed as described previously (Randolph et al.,
112 2015). Briefly, histologic sections included pharyngeal tissue extending from the soft palate
113 caudally to the cranial aspects of the trachea and esophagus. Cross sections were prepared in
114 both transverse and longitudinally for circular outside and longitudinal inside muscles,
115 respectively. For collection and isolation of myogenic cells, the larynx and trachea were
116 excluded from pharyngeal samples. Gastrocnemius muscles were used as control limb
117 muscles.

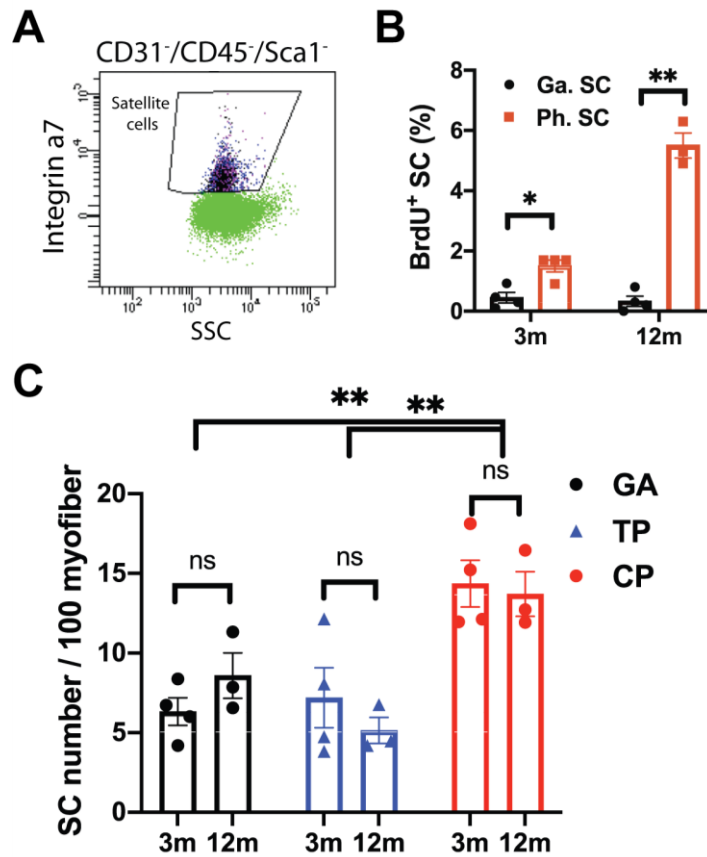
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119 ***Satellite Cell Isolation and Fluorescence Activated Cell Sorting***

120 To obtain purified satellite cells (SCs), primary cells were isolated as described
121 previously with small modifications (Randolph et al., 2015). Briefly, dissected pharyngeal and
122 gastrocnemius muscles were minced and digested using 0.2% collagenase II (Gibco, Carlsbad,
123 California) and 2.5U/ml Dispase II (Gibco, Carlsbad, California) in Dulbecco's modified
124 Eagle's medium (DMEM) at 37°C while shaken at 80 rpm for 90 minutes. Digested muscles
125 were then rinsed with same volume of Ham's F10 media containing 20% FBS and 100 µg/ml
126 penicillin/streptomycin (P/S). Then, mononucleated cells were collected using 70 µm cell

127 strainer (Thermo Fisher Scientific, Waltham, MA). To facilitate rapid isolation of a pure
128 pharyngeal and hind limb SCs, we used an antibody-free fluorescence based lineage labeling
129 strategy, whereby Pax7 positive SCs are marked with a red fluorescence, tdTomato, upon
130 tamoxifen-mediated Cre recombinase activation. Fluorescence-activated cell sorting (FACS)
131 was performed using a BD FACSAria II cell sorter (Becton-Dickinson, <http://www.bd.com>,
132 Franklin Lakes, NJ) at the Emory University School of Medicine Core Facility for Flow
133 Cytometry. Analyses of flow cytometry data were performed using FACSDiva (BD version
134 8.0.1) and FCS Express 6 Flow. FACS-purified SCs were plated at 500 cells per well in 48-
135 well plate coated with Matrigel (Corning Life Sciences, New York, NY, Ca No. 354277) and
136 cultured for five days in Ham's F10 media (Hyclone, Pittsburgh, PA; www.gelifesciences.com)
137 containing 20% FBS and 25 ng/mL FGF2 (PeproTech, Rocky Hill, NJ). In Supplementary
138 Figure 1, we used antibody strategy to isolate pharyngeal and hindlimb SCs. Cells were labeled
139 using the following antibodies: 1:400 CD31-PE (clone 390; eBiosciences, San Diego, CA
140 <http://www.ebioscience.com/>), 1:400 CD45-PE (clone 30-F11; BD Biosciences,
141 wwwbdbiosciences.com, San Jose, CA), 1:4000 Sca-1-PE-Cy7 (BD Biosciences, clone D7;
142 www.ablab.ca, Vancouver, Canada), 1:500 α 7-integrin-APC (Clone R2F2; AbLab). Dead cells
143 were excluded by 5 μ g/ml propidium iodide (PI) staining. SCs were collected according to the
144 following sorting criteria: PI⁻/CD31⁻/CD45⁻/Sca1⁻/Intergrin7 α ⁺.

145



147

148 **Supplementary Figure 1. Active *in vivo* proliferation of pharyngeal satellite cells of 12 months old mice compared**
149 **with ones of 3 months old mice.** A. Flow cytometry gating strategy for satellite cells defined by CD31⁻/CD45⁻/Sca1⁻/Integrin
150 $\alpha 7$ ⁺. B. Percentage of BrdU⁺ SCs in gastrocnemius and pharyngeal muscles of 3 and 12 month old mice. n = 4 for each age group.
151 Statistical significance was determined by 2-way ANOVA. C. Number of SC in gastrocnemius (GA), thyropharyngeus (TP) and
152 cricopharyngeus (CP) muscles of 3 and 12 month old mice. n = 3 or 4 for each age groups. The value represents mean \pm SEM.
153 Statistical significances was determined by 2-way ANOVA. Asterisks indicate statistical significance (*p<0.05 and **p<0.01).

154

155 ***In Vivo Cell Proliferation Assays by Flow Cytometry***

156 To compare the proliferative abilities of SCs in pharyngeal and hindlimb muscles *in vivo*,
157 *Bromo-2'-deoxyuridine (BrdU)* assays were performed. Three-month-old C57BL/6 male mice
158 were injected with 10 μ g BrdU (Sigma-Aldrich, St. Louis, MO; www.sigmaaldrich.com)/gram
159 body weight intraperitoneally every 12 hours for 2 days before sacrifice. Muscles were
160 dissected and digested as described above. To assess proliferation, isolated mononucleated cells
161 from pharyngeal or gastrocnemius muscles were immunostained with the following antibodies:
162 1:400 CD31-PE (clone 390; eBiosciences, San Diego, CA <http://www.ebioscience.com/>),
163 1:400 CD45-PE (clone 30-F11; BD Biosciences, www.bdbiosciences.com, San Jose, CA),
164 1:4000 Sca-1-PE-Cy7 (BD Biosciences, clone D7; www.ablab.ca, Vancouver, Canada), 1:500
165 α 7-integrin-APC (Clone R2F2; AbLab). Subsequently cells were labeled for BrdU using a
166 FITC-BrdU flow kit in accordance with the manufacturer's instructions (BD Biosciences,
167 www.bdbiosciences.com, San Jose, CA). Proliferating SCs were collected according to the
168 following sorting criteria: CD31⁻/CD45⁻/Sca1⁻/Integrin7 α ⁺/BrdU⁺.

169

170 ***Fusion index and nuclei number analysis***

171 For fusion assay, SCs were cultured for 10 days to induce spontaneous differentiation
172 (Stuelsatz et al., 2015). Cells were fixed in 2% formaldehyde in PBS for 10 min at room
173 temperature and stained with Phalloidin-iFluor 594 (abcam, ab176757) for 30 minutes at room
174 temperature. Nuclei were then stained with 4',6-diamidino-2-phenylindole (DAPI) and cells
175 were mounted with Vectashield (Vector Labs, www.vectorlabs.com, Burlingame, CA).
176 Myoblast fusion was quantified by counting myonuclei in myotubes. Fusion index was

177 calculated as the percentage of nuclei of myotubes with two or more nuclei relative to the total
178 number of nuclei in the images. We randomly collected 10 images for each line.

179

180 ***MitoTracker staining***

181 Pharyngeal and gastrocnemius muscles were dissected from *Pax7 Cre^{ERT}-tdTomato* mice,
182 digested into mononuclear cells and sorted using flow cytometry. Isolated cells were incubated
183 with 50 nM MitoTracker® Green FM (Life Technologies, catalog number: M-7514) at 37°C
184 for 30 min. The cells were washed twice prior to analysis using the FACS LSR II flow
185 cytometer or by fluorescence microscopy.

186

187 ***Transwell cultures of satellite cells (SCs) and myogenic progenitor cells (MPCs)***

188 To provide continuous cytokine delivery, we used a co-culture system of sorted
189 tdTomato⁺ (PAX7⁺) SCs from pharyngeal or gastrocnemius muscles as donor cells and
190 expanded gastrocnemius myogenic progenitor cells (MPCs) as recipient cells. PAX7⁺ satellite
191 cells from pharyngeal or gastrocnemius muscles settled in the micro-wells at 500 cells/well in
192 the Matrigel-coated 24 well plate. Gastrocnemius MPCs were labelled with 5 µM
193 CellTracker™ Green CMFDA (Life Technologies, C7025) at 37°C for 45 minutes, seeded at 1
194 × 10⁴ cells/mL in a collagen-coated permeable transwell insert (Corning #3413, Transwell®
195 with 0.4 µm Pore Polyester Membrane Insert), and cultured using MPC culture medium for 1
196 day to completely adhere. Two days after sorting, transwell inserts (containing MPC) were
197 placed on the micro-wells containing sorted SCs for co-culture and were placed on blank

198 micro-wells (without SCs) as a control. Culture medium was exchanged every other day. On
199 day 4 of co-culture, the number of green fluorescence positive MPCs in the insert was counted
200 and the ratio of cell growth was normalized to MPC number in the blank wells.

201

202 ***Gene expression analysis by real-time qPCR***

203 The gastrocnemius and pharyngeal MPCs and muscles were analyzed for the expression
204 of related markers by comparative real-time qPCR. Total RNA from samples was extracted
205 using QIAamp RNA blood mini kit (Qiagen, Hilden, Germany) according to the
206 manufacturer's instructions. Isolated RNA (250 ng) was reverse transcribed into
207 complementary DNA (cDNA) using qScript™ cDNA SuperMix (Quanta Biosciences,
208 Gaithersburg, MD) and then analyzed by real-time qPCR. Amplification of cDNA was
209 performed using Power SYBR® Green Master Mix (Applied Biosystems, Waltham, MA) and
210 2.5 µM of each primer. All primer sequences are listed in Supplementary Table 1. PCR
211 reactions were performed for 35 cycles under the following conditions: denaturation at 95°C
212 for 15 sec and annealing + extension at 60°C for 1 min. Quantitative levels for all genes were
213 normalized to endogenous *Hprt* expression. Fold change of gene expression was determined
214 using the $\Delta\Delta C_t$ method (Livak & Schmittgen, 2001).

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219 **Supplementary Table 1. Primers used for gene expression analysis**

Genes	Primer sequences
<i>Col5a1</i>	5'- GCTACTCCTGTTCCCTGCTGC -3'
	5'- TGAGGGCAAATTGTGAAAATC -3'
<i>Hgf</i>	5'- AAAGGGACGGTATCCATCACT -3'
	5'- GCGATAGCTCGAAGGCCAAAAAG -3'
<i>Fst</i>	5'- CCCCAACTGCATCCCTTGTAAG -3'
	5'- TCCAGGTGATGTTGGAACAGTC -3'
<i>Pax7</i>	5'- CTGTGCTGGGACTTCTTCCT -3'
	5'- AGACTCAGGGCTTGGGAAGG -3'
<i>Acta1</i>	5'- CCCAAAGCTAACC GGGAGAAG -3'
	5'-CCAGAATCCAACACGATGCC -3'
<i>Hprt</i>	5'-TCAGTCAACGGGGGACATAAA -3'
	5'- GGGGCTGTACTGCTTAACCAG -3'

220

221 ***Immunohistochemistry/Immunofluorescence***

222 Immunohistochemistry/Immunofluorescence was performed as follows: sections were
223 incubated with blocking buffer (5% goat serum, 5% donkey serum, 0.5% BSA, 0.25% Triton-
224 X 100 in PBS) for 1 hour and then labeled with primary antibodies (Supplementary Table 2) or
225 isotype controls overnight at 4°C in blocking buffer. The following day, sections were washed
226 three times with washing buffer (0.2% Tween-20 in PBS) and incubated with fluorescence
227 probe-conjugated secondary antibodies for 1 hour at room temperature. We used mannose
228 receptor-1 (CD206) as a marker for resident M2 macrophages and platelet-derived growth
229 factor receptor α (PDGFR α) as a marker for FAPs. The TSA Green kit (Tyramide Signal
230 Amplification; Perkin Elmer, www.perkinelmer.com, Waltham, MA) was used for CD206 and
231 PDGFR α staining to enhance the immunostaining signal, after 1 hour incubation with

232 biotinylated goat-anti-mouse F(ab')₂ IgG fragments (2.5 µg/ml). Nuclei were then stained with
233 DAPI and mounted using Vectashield (Vector Labs, www.vectorlabs.com, Burlingame, CA).

234 **Supplementary Table 2. Antibodies used for immunofluorescence staining**

Detection of	Name	Host species	Dilution or Concentration	Manufacturer (Cat #)
Resident M2 macrophage	Mannose Receptor (CD206)	Rabbit IgG	1:200	Abcam (ab125028)
Fibroblast progenitors (FAPs)	PDGFR α	Rabbit IgG	1:200	Cell Signaling Tech. (3174S)
Collagen V A1	COLVA1	Rabbit IgG	1:200	Sigma (SAB1306996)
Basement membranes	Laminin	Rabbit IgG	1:400	Sigma (L9393)
Basement membranes	Wheat Germ Agglutinin (WGA)	Alexa Fluor™ 647 Conjugate	1:400	Invitrogen (W32466)

235

236 ***Statistical Analyses***

237 Statistical analysis was performed using Prism 8.0. Results are expressed as the mean \pm
238 standard error of the mean (SEM). Experiments were repeated at least three times unless a
239 different number of repeats is stated in the legend. Statistical testing was performed using the
240 unpaired two-tailed Student's t-test or ANOVA as stated in the figure legends. $p < 0.05$ was
241 considered statistically significant. Statistical method, p-values, and sample numbers are
242 indicated in the figure legends.

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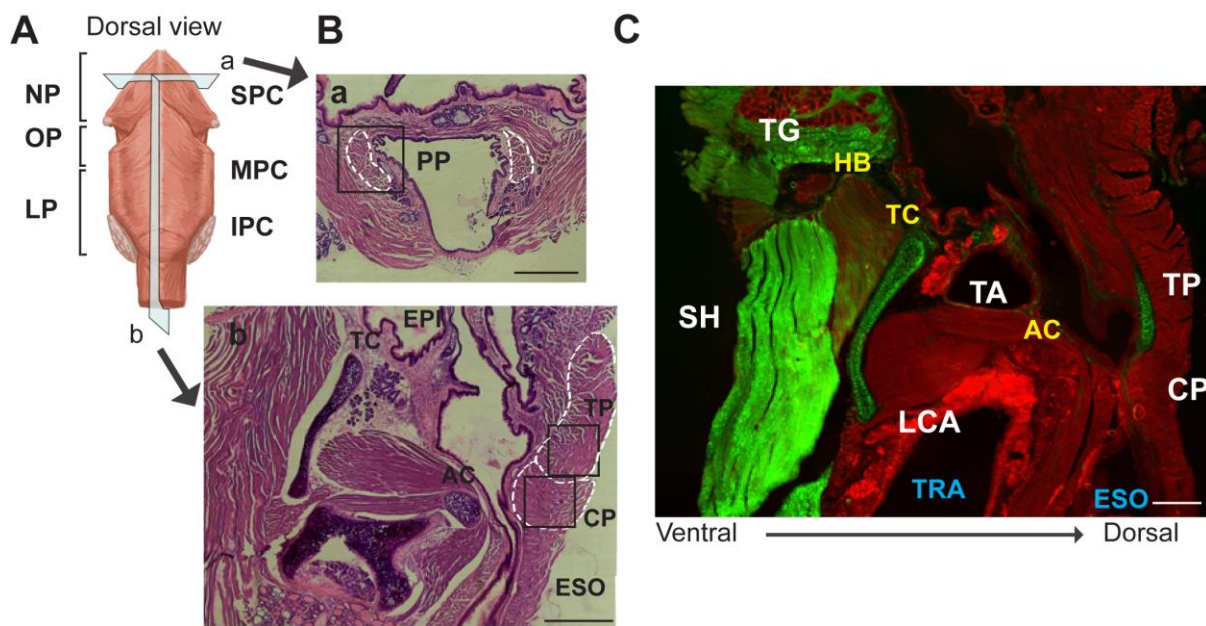
247 **3. Results**

248 ***3.1. Anatomical structure and embryonic origins of pharyngeal muscles***

249 Based on location, the pharynx is separated into three major sections: the nasopharynx
250 (NP), oropharynx (OP), and laryngopharynx (LP). The muscles of pharynx consist of a
251 circular outer layer and a longitudinal inner layer (Fig 1A) (Randolph et al., 2014). The inner
252 layer of the pharyngeal wall is comprised of three paired muscles known as the
253 palatopharyngeus (PP, (Fig 1B-a)), the stylopharyngeus, and the salpingopharyngeus. The
254 outer layer of the pharynx is comprised of three pharyngeal constrictor (PC) muscles: superior
255 (SPC), middle (MPC), and inferior (IPC) (Fig 1A). The IPC is particularly important for
256 swallowing and consists of two muscles, the thyropharyngeus (TP) and cricopharyngeus (CP),
257 which form a sphincter at the transition from the pharynx to the esophagus (Fig 1B-b). During
258 swallowing, the successive contraction of pharyngeal constrictor muscles is required to
259 constrict the pharyngeal lumen to propel the bolus downward to the CP, which is vital to the
260 efficient transfer of the bolus to the esophagus (Cook, 1993). Here, we focused on IPC muscles
261 as CP muscles are involved in several types of pharyngeal pathologies including
262 cricopharyngeal spasm (Búa, Olsson, Westin, Rydell, & Ekberg, 2015) and oculopharyngeal
263 muscular dystrophy (Gómez-Torres et al., 2012).

264 The PAX3 transcription factor is a critical upstream regulator of somitic myogenesis
265 during skeletal muscle development, but it is not expressed in developing craniofacial muscles
266 including pharyngeal muscles (McLoon, Thorstenson, Solomon, & Lewis, 2007; Shahrighim
267 Tajbakhsh et al., 1997). To confirm that the PAX3 lineage does not contribute to pharyngeal
268 muscle development, we performed PAX3 lineage tracing using *Pax3^{Cre/+}-mTmG* mice, which

269 label all PAX3 lineage-derived cells with membrane targeted green fluorescent protein (GFP,
270 mG) and non-PAX3 lineage-derived cells with membrane targeted red fluorescent protein,
271 tdTomato (mT) (W. Liu et al., 2013). Similar to extraocular muscles of *Pax3^{Cre/+}-mTmG* mice
272 (Stuelsatz et al., 2015), the TP and CP muscles showed red fluorescence without GFP
273 expression, confirming that they do not originate from PAX3 expressing embryonal
274 progenitors (Fig 1C). Intrinsic laryngeal muscles including thyroarytenoid (TA) and lateral
275 cricoarythenoid (LCA) also expressed tdTomato, indicating that they are non-PAX3 derived
276 muscles. On the other hand, extrinsic laryngeal muscles, such as sternohyoid (SH), and tongue
277 muscles (TG) showed green fluorescence expression, suggesting that the PAX3 lineages
278 contribute to both muscles (Dong et al., 2006; Harel et al., 2009).



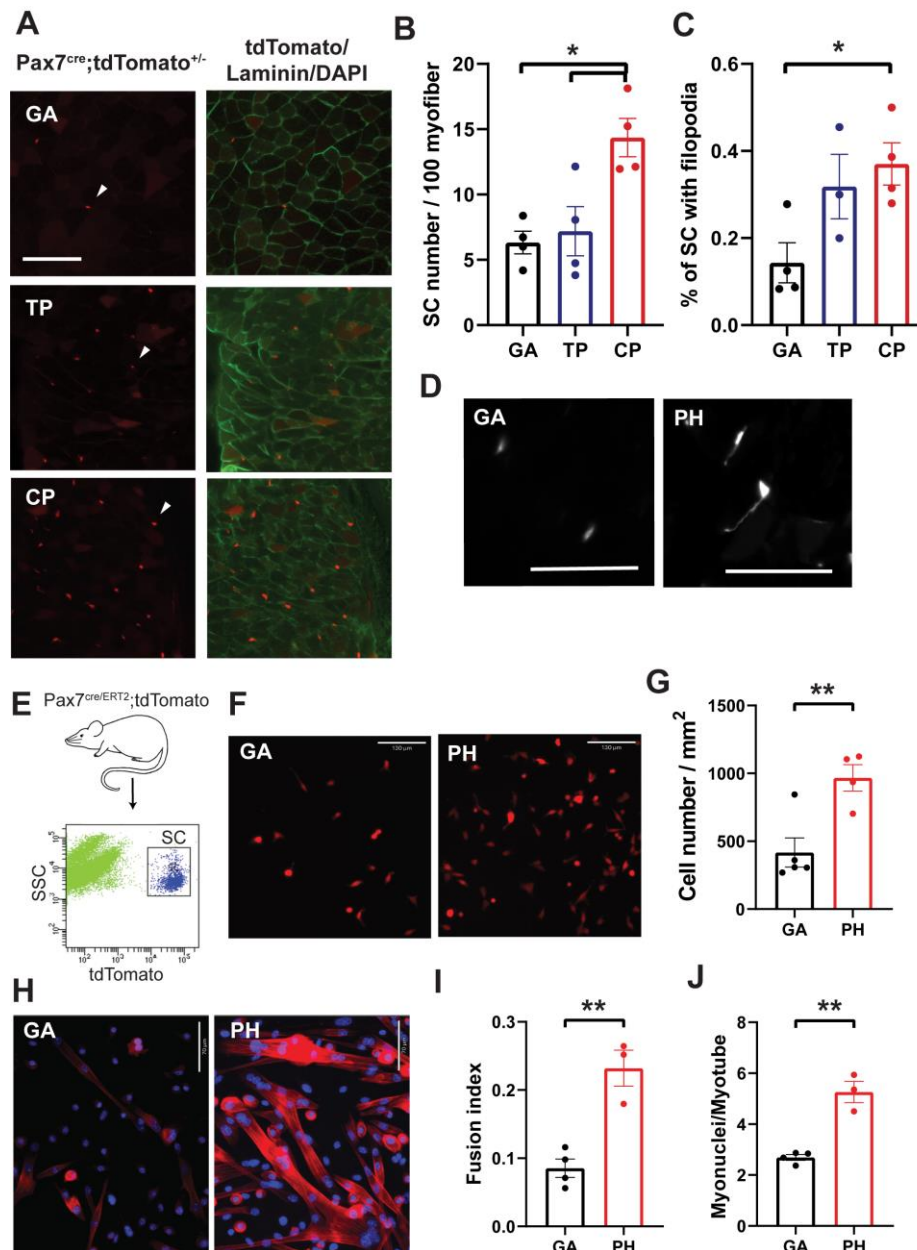
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280 **Figure 1. Anatomy and embryonic origins of pharyngeal muscles.** A. Illustration of the outer skeletal muscles surrounding the
281 nasopharynx (NP), oropharynx (OP) and laryngopharynx (LP). B. Representative histological images of transverse (a, upper) and
282 longitudinal sections (b, bottom) in pharyngeal muscles. Scale bars = 330 μ m. C. Representative longitudinal section of larynx and
283 pharynx expressing PAX3 lineage-derived muscles (green) and non-PAX3 lineage-derived muscles (red) from 20 week old
284 *Pax3^{Cre/+}/mTmG* mice. Abbreviations: superior pharyngeal constrictor (SPC); middle pharyngeal constrictor (MPC); inferior
285 pharyngeal constrictor (IPC); thyropharyngeus (TP); cricopharyngeus (CP); palatopharyngeus (PP); epiglottis (EPI); thyroid cartilage
286 (THY); esophagus (ESO); trachea (TRA); tongue (TG); sternohyoid (SH); hyoid bone (HB); thyroid cartilage (TC); arytenoid
287 cartilage (AC); lateral cricoarythenoid (LCA).

288

289 Adult SCs are distinguished by expression of the paired-box/homeodomain
290 transcription factor PAX7, which is expressed during quiescence and early activation of SCs
291 and plays a key role in maintenance of self-renewed SCs (Bosnakovski et al., 2008). To
292 investigate the SCs in craniofacial muscles, we used a genetically engineered, tamoxifen-
293 inducible *Pax7 Cre^{ERT2}-tdTomato* mouse, which labels all PAX7 lineage-derived cells with
294 red fluorescent protein (tdTomato). After tamoxifen injection, we observed tdTomato-labeled
295 SCs in sectioned TP, CP, and gastrocnemius (GA, limb) muscles (Fig 2A). The number of SCs
296 in CP muscles was significantly higher than the number of SCs in GA and TP muscles (Fig
297 2B). Unexpectedly, we also detected significantly increased number of cellular protrusions
298 (filopodia) in SCs from CP muscles relative to SCs in GA muscles (Fig 2C and D). This result
299 is consistent with a previously published study that showed extensive filopodia in extraocular
300 muscles (Verma, Fitzpatrick, & McLoon, 2017), but the role of filopodia in SC function is
301 unknown.

302



303

304 **Figure 2. Sorted pharyngeal satellite cells show a high level of proliferation and differentiation.** A. Representative cross-
 305 section expressing PAX7⁺ SCs (red) in gastrocnemius (GA), thyropharyngeus (TP) and cricopharyngeus (CP) muscles from 3 months
 306 old Pax7 Cre^{ERT2}-tdTomato mouse. White arrow heads indicate examples of PAX7⁺ tdTomato expressing SCs. Basal lamina was
 307 immunostained with anti-Laminin antibody (green). B, C. Quantified numbers of SCs per 100 myofibers (B) and quantified
 308 percentages of SCs with filopodia (C) in gastrocnemius and pharyngeal muscles from 3 month old Pax7 Cre^{ERT2}-tdTomato mouse. n
 309 = 3 or 4. Data were analyzed by 1-way ANOVA. D. Representative image of filopodia in gastrocnemius (GA) and pharyngeal (PH)
 310 SCs. Scale bars = 50 μm. E. Scheme of flow cytometry gating strategy for SC isolation using Pax7 Cre^{ERT2}-tdTomato mouse. F.
 311 Representative image of 5-day cultured myogenic progenitor cells derived from gastrocnemius and pharyngeal SCs of Pax7 Cre^{ERT2}-
 312 tdTomato mouse. Scale bars = 130 μm. G. Analysis of cell number/mm² in gastrocnemius and pharyngeal myogenic progenitor cells
 313 derived from SCs of 3 month old mice. n = 4. Statistical significance was determined by Student's t-test. H. Representative image
 314 of gastrocnemius (GA) and pharyngeal (PH) myogenic progenitor cells derived from sorted SCs of Pax7 Cre^{ERT2}-tdTomato mice
 315 after 10 days of culture. Scale bars = 70 μm. I. Quantified fusion index at 10 days after culture. Fusion index was calculated as the
 316 percentage of total nuclei that resided in cells containing 2 or more nuclei. n = 3 or 4. Statistical significance was determined by
 317 Student's t-test. J. Number of myonuclei per myotube at 10 days after culture. n = 3 or 4. Statistical significance was determined by
 318 Student's t-test. For all graphs, the value represents mean ± SEM. Asterisks indicate statistical significance (*p<0.05 and **p<0.01).

319

320 **3.2. Pharyngeal satellite cells are more proliferative and differentiative than**
321 ***gastrocnemius* satellite cells *in vitro*.**

322 Pharyngeal SCs are highly proliferative *in vivo* relative to hindlimb SCs as
323 demonstrated by bromodeoxyuridine labeling and flow cytometric analysis using a known
324 satellite cell gating strategy (CD31⁻/CD45⁻/Sca1⁻/Integrin α 7⁺/BrdU⁺) (Fig EV1A)
325 (Randolph et al., 2015). To exclude *in vivo* niche effects on satellite cell proliferation, we
326 sorted by tdTomato signal equal numbers of pharyngeal and gastrocnemius SCs from *Pax7*
327 *Cre^{ERT2}-tdTomato* mice and cultured them for 5 days (Fig 2E and 2F). After 5 days, we
328 detected twice the number of cells in wells containing pharyngeal SCs than those containing
329 SCs from gastrocnemius (Fig 2G). To investigate the differentiation potential of pharyngeal
330 SC, we cultured sorted satellite cells for 10 days to induce spontaneous differentiation
331 (Stuelsatz et al., 2015). The cultured pharyngeal SCs consistently exhibited an increased
332 differentiation at day 10 (Fig 2H) along with an increased fusion index and increased number
333 of myonuclei per myotube compared to the gastrocnemius SCs (Fig 2I and 2J). These results
334 indicate that pharyngeal SCs retain highly proliferative and differentiative properties
335 compared to the limb muscles in the absence of *in vivo* niche factors, suggesting that cell-
336 intrinsic factors contribute to the unique properties of pharyngeal SCs.

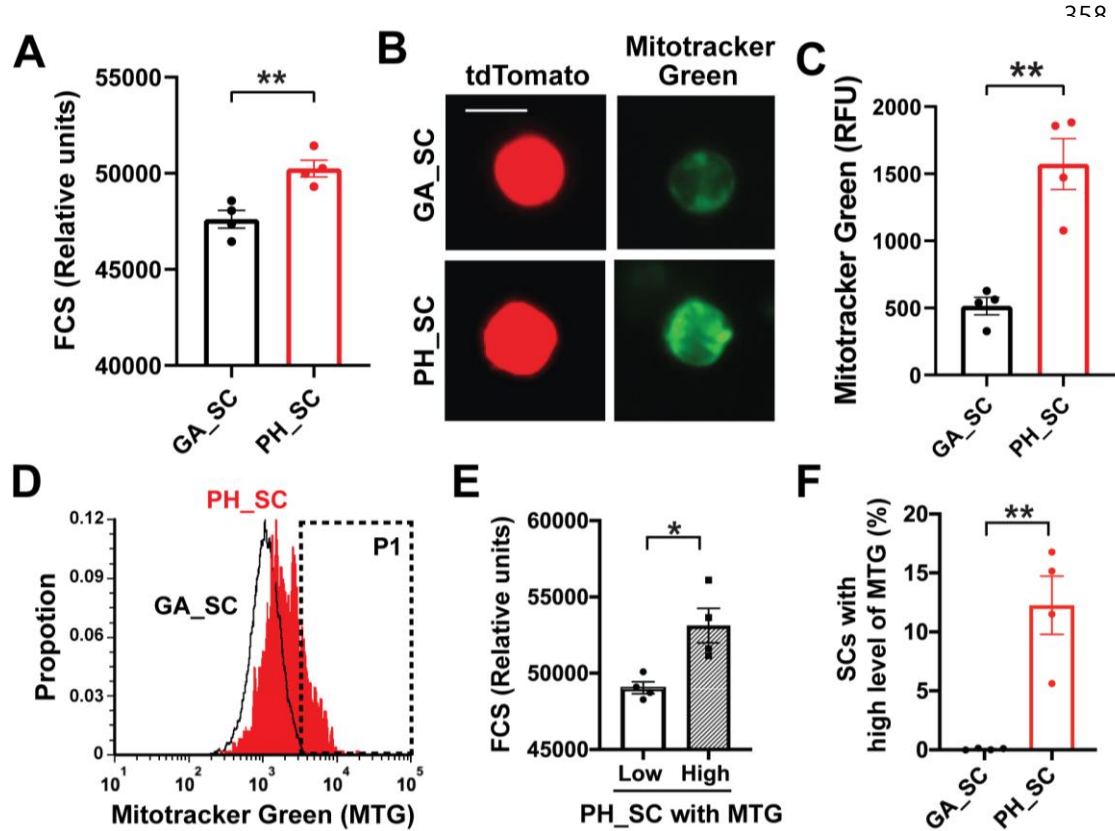
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338 **3.3. Pharyngeal satellite cells are larger and contain elevated mitochondrial content.**

339 When analyzing gastrocnemius and pharyngeal SCs by flow cytometry, two light-
340 scattering parameters, the forward scatter (FSC; cell size) and side scatter (SSC; intracellular
341 granularity and complexity), were also measured. We noticed that FSC values for pharyngeal

342 SCs were 10% higher as compared to gastrocnemius SCs (Fig 3A), suggesting that pharyngeal
343 SCs are larger. The increased cell size and rapid proliferation of pharyngeal SCs resemble
344 properties of the G_{Alert} state that exists in SCs of the contralateral muscles after induced muscle
345 injury mice (Rodgers et al., 2014). Given that G_{Alert} SCs also have increased mitochondrial
346 content and activity, we hypothesized that pharyngeal SCs also have more mitochondria. We
347 stained mitochondria in freshly-isolated SCs and detected increased green fluorescence by
348 microscopy (Fig 3B) and increased relative fluorescence units (RFU) of MitoTracker Green
349 (MTG) by flow cytometry (Fig 3C and 3D) in pharyngeal SCs relative to SCs from
350 gastrocnemius muscle. We observed that the pharyngeal SCs with higher MTG signal (P1 gate
351 of Fig 3D) were larger (increased FCS values) than the pharyngeal SCs with lower MTG signal
352 (Fig 3E). Approximately 12% of pharyngeal SCs had increased cell size and higher
353 mitochondria contents compared to less than 1% of gastrocnemius SCs (Fig 3F). This result
354 indicates that pharyngeal SCs contain increased mitochondrial mass compared with
355 gastrocnemius SCs, which may provide energy and metabolites for early activation and higher
356 proliferation of pharyngeal SCs.

357



360 **Figure 3. Pharyngeal satellite cells contain more mitochondrial contents.** A. Quantified forward scatter (FCS) values for
 361 pharyngeal (PH_SC) and gastrocnemius SCs (GA_SC) as determined by flow cytometry. n=4. B. Microscopic fluorescence images
 362 showing tdTomato expressing SCs from gastrocnemius or pharyngeal muscles mitochondria stained with MitoTracker Green. Scale
 363 bars = 10 μ m. C. Representative flow cytometry histogram of MitoTracker Green fluorescence levels of both gastrocnemius (black
 364 line) and pharyngeal (red line) SCs. The P1 gate indicates the SC population with high levels of MitoTracker Green. D. Quantitated
 365 MitoTracker Green (MTG) relative fluorescence units (y-axis, RFU) of gastrocnemius (black line) or pharyngeal (red line) SCs. n=4.
 366 E. Quantified forward scatter (FCS) values for pharyngeal SCs (PH_SC) with low and high MitoTracker Green (MTG) as determined
 367 by flow cytometry. n=4. F. Percentage of SCs with high levels of MitoTracker Green (MTG) in pharyngeal (PH_SC) and
 368 gastrocnemius SCs (GA_SC). n=4. For all graphs, the value represents mean \pm SEM. Statistical significance was determined by
 369 Student's t-test. Asterisks indicate statistical significance (**P<0.01).

370

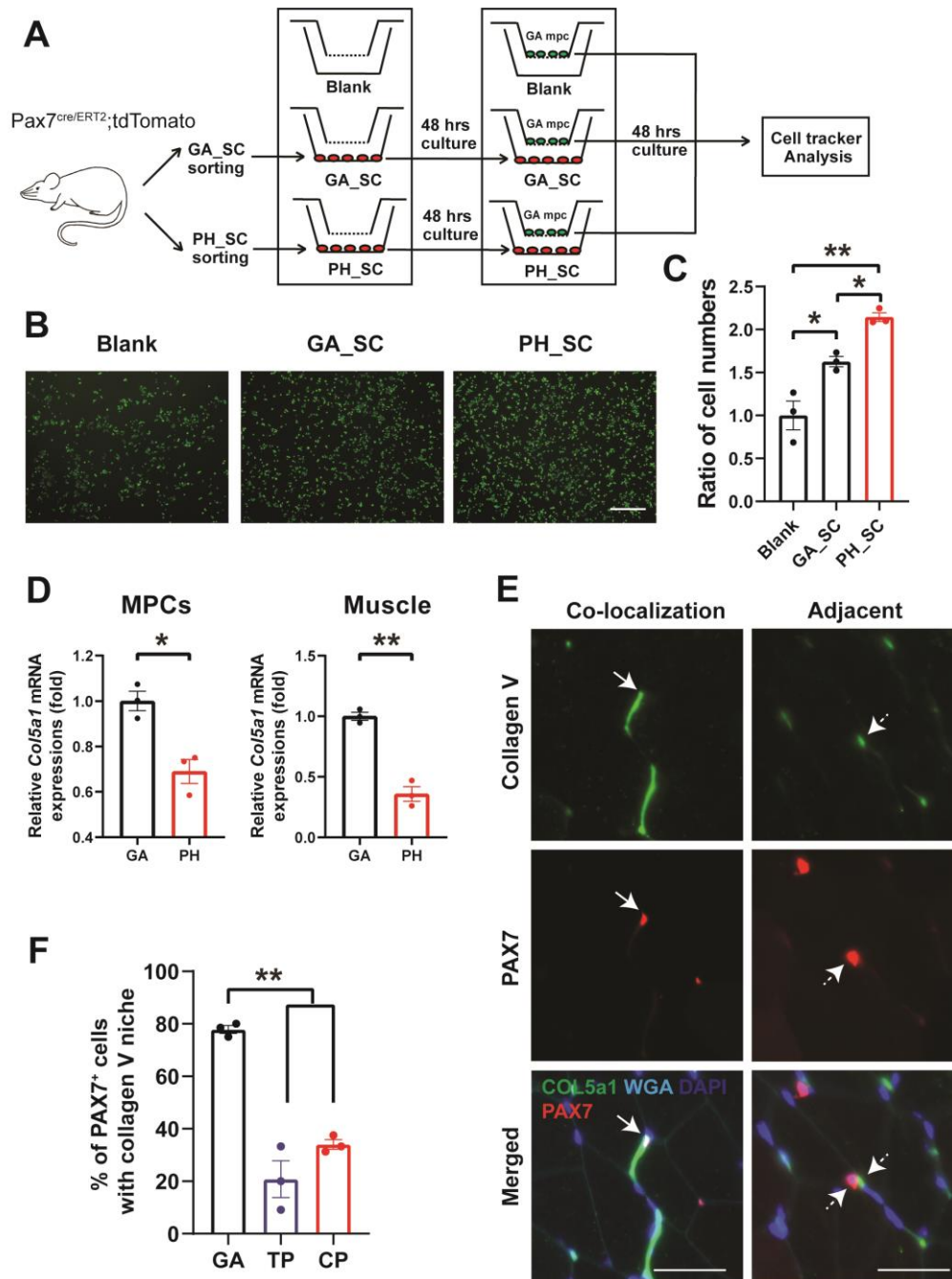
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373 **3.4. Pharyngeal satellite cells secrete proliferation enhancing factors.**

374 The increased proliferation of pharyngeal SCs *in vitro* (Fig 2G) suggests that pharyngeal
375 SCs may secrete pro-proliferative factors. Previous microarray analysis revealed that
376 pharyngeal SCs contain higher levels of RNAs encoding secreted factors that induce cellular
377 proliferation and immune cell infiltration as compared limb SCs (Randolph et al., 2015). To
378 investigate the effect of secreted auto/paracrine factors from gastrocnemius and pharyngeal
379 SCs on proliferation, we sorted PAX7⁺ SCs from *Pax7 Cre^{ERT2}-tdTomato* mice and seeded
380 SCs onto the bottom wells of transwell system. Identical batches of gastrocnemius SCs were
381 seeded onto the upper transwell inserts above either gastrocnemius or pharyngeal SCs and
382 cultured for 48 hours (Fig 4A). We quantified proliferation in the top transwells by counting
383 the number of gastrocnemius SCs dyed with Cell Tracker Green CMFDA (Fig 4B). Compared
384 to controls (blank bottom wells), the number of cells was dramatically increased in both
385 gastrocnemius and pharyngeal SC co-cultured groups (Fig 4C). Importantly, proliferation in
386 the pharyngeal SC group was significantly increased relative to the gastrocnemius SC group.
387 This result suggests that pharyngeal SCs secrete more pro-proliferative factors or fewer anti-
388 proliferative factors during *in vitro* culture compared to gastrocnemius SCs.

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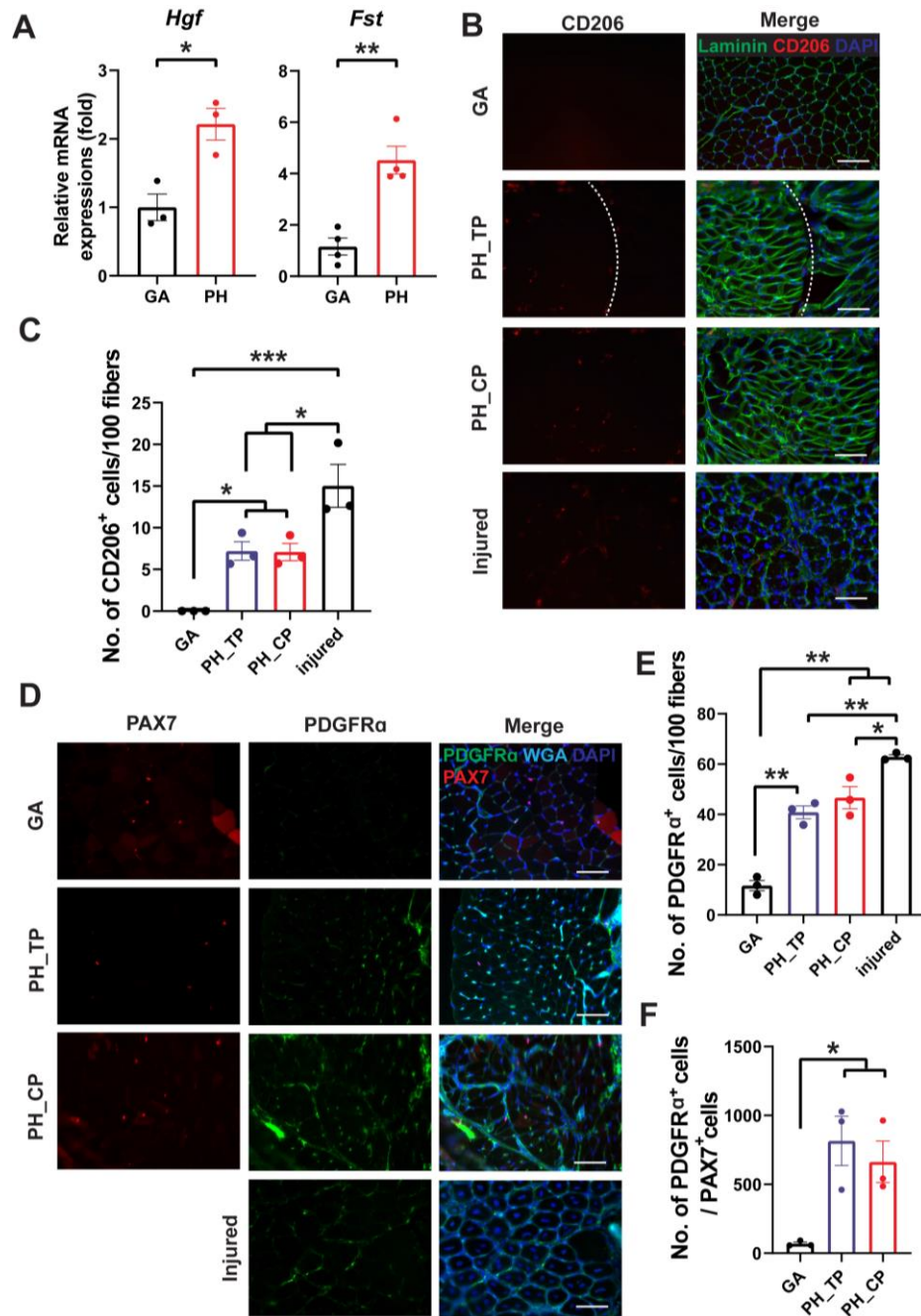
391 **Figure 4. Pharyngeal satellite cells secrete pro-proliferating factors and partially connect with quiescent niche.** A.
 392 Schematic illustration of transwell-coculture to investigate the effects of cytokines secreted from gastrocnemius and pharyngeal
 393 satellite cells. An empty bottom well (Blank) was used as a negative control. B. Representative images of Cell Tracker Green stained
 394 gastrocnemius myogenic progenitor cells cocultured with gastrocnemius (GA) or pharyngeal (PH) satellite cells. Scale bars = 330 μ m.
 395 C. The normalized (ratio to transwell of Blank bottom) cell numbers of gastrocnemius myogenic progenitor cells after Cell Tracker
 396 image analysis using cytokine interphase system. n = 3. Statistical significance was determined by 1-way ANOVA. D. Relative mRNA
 397 expression level of *Col5a1* in gastrocnemius (GA) and pharyngeal (PH) myogenic progenitor cells (MPCs) and muscles obtained
 398 from 3 month old mice. n = 3. Statistical significance was determined by Student's t-test. E. Representative image of co-localization
 399 or adjacent expression of COLV and PAX7 in gastrocnemius muscle section. Scale bars = 35 μ m. F. Quantified graph showing the
 400 percentage of COLV⁺ niche per PAX7⁺ cells in gastrocnemius (GA), thyropharyngeus (TP) and cricopharyngeus (CP) muscles of
 401 *Pax7 Cre^{ERT2}-tdTomato* mouse. Statistical significance was determined by 1-way ANOVA. For all graphs, the value represents mean \pm
 402 SEM. Asterisks indicate statistical significance (*p<0.05, **p<0.01).

403 **3.5. Pharyngeal satellite cells are less associated with collagen V, a marker of the quiescent**
404 ***niche.***

405 Although we demonstrated that cell-intrinsic factors are at least partly responsible for
406 the increased proliferation and differentiation of pharyngeal SCs, we hypothesized that niche
407 factors including the extracellular matrix (ECM) and other cell types also contribute to the
408 unique phenotypes of pharyngeal SCs. The skeletal muscle extracellular matrix includes
409 several types of collagen and contributes to muscle contraction and maintenance as well as
410 the satellite cell niche (Gillies & Lieber, 2011). A recent study reported that extracellular
411 matrix collagen V (COLV) is secreted by SCs and is important for maintenance of the
412 quiescent state (Baghdadi et al., 2018). We detected decreased levels of the *Col5a1* mRNA,
413 which encodes COLV, in pharyngeal MPCs and muscles relative to gastrocnemius
414 counterparts (Fig 4D). To confirm that reduced *Col5a1* mRNA in pharyngeal muscles is
415 indeed associated with a less quiescent niche, we examined the presence of Collagen V protein
416 relative to PAX7⁺ SCs in pharyngeal and gastrocnemius muscle sections from *Pax7 Cre^{ERT2}-*
417 *tdTomato* mice. We quantified PAX7⁺ cells within a collagen V⁺ niche, which is defined by
418 co-localization or adjacent location of COLV and PAX7 (Fig 4E). The percentage of PAX7⁺
419 SCs in the collagen V⁺ niche is significantly higher in GA muscles than in TP and CP muscles
420 (Fig 4F), indicating that pharyngeal SC niches contain less collagen V than limb SC niches.
421 Taken together, these data suggest that the SC niche in pharyngeal muscles is less supportive
422 of the quiescent state than the SC niche in limb muscles.

423
424 **3.5. The pharyngeal muscle niche contains multiple cell types and secreted factors to**
425 ***enhance satellite cell activation.***

426 To explore the contribution of extrinsic secreted factors to pharyngeal SC activation,
427 we measured the level of known SC activating factors in pharyngeal muscles. We focused on
428 secreted factors known to modulate SC proliferation including hepatocyte growth factor
429 (HGF), a well-known activator of quiescent SCs upon muscle injury (Allen, Sheehan, Taylor,
430 Kendall, & Rice, 1995), and follistatin (FST), also known as a myostatin inhibitor (Amthor et
431 al., 2004), which induces SC activation and fusion (Gilson et al., 2009; Jones et al., 2015).
432 Interestingly, the mRNA levels of *Hgf* and *Fst* were increased in pharyngeal muscles
433 compared to gastrocnemius muscles (Fig 5A). This result led us to hypothesize that other cell
434 types within pharyngeal muscles contribute to SC activation. Resident macrophages and FAPs
435 have been known to be a primary source of HGF (Sisson et al., 2009) and follistatin (Madarò,
436 Mozzetta, Biferali, & Proietti, 2019), respectively. Thus, we chose these cell types for further
437 analysis. To identify resident macrophages and FAPs in pharyngeal muscles, we stained
438 sections for CD206, a surface marker of resident macrophages (Kosmac et al., 2018) and
439 PDGFR α , a surface marker of FAPs (Joe et al., 2010) (Fig 5B and 5D). The number of CD206⁺
440 cells per 100 fibers in pharyngeal muscles was significantly higher than in uninjured
441 gastrocnemius (Fig 5C). We also detected a significant increase in PDGFR α ⁺ cells per 100
442 fibers or per PAX7⁺ cells in pharyngeal muscles relative to uninjured gastrocnemius muscles
443 (Fig 5E and 5F). Although the number of CD206⁺ cells or PDGFR α ⁺ cells in pharyngeal
444 muscles was higher than in uninjured muscles, these numbers were less than the number of
445 CD206⁺ cells or PDGFR α ⁺ cells in 3-day injured limb muscles. Based on these findings, we
446 suggest that the increased numbers of macrophages and FAPs in pharyngeal muscles may
447 activate pharyngeal SCs via secretion of HGF and follistatin.



448

449 **Figure 5. Pharyngeal muscles contain an increased number of resident macrophages and fibroadipogenic progenitor**
 450 **cells (FAPs).** A. Relative mRNA expression level of hepatocyte growth factor (*Hgf*) and follistatin (*Fst*) in gastrocnemius (GA) and
 451 pharyngeal (PH) muscles obtained from 3 month mice. n = 3. Statistical significance was determined by Student's t-test. B.
 452 Representative images of CD206⁺ cells in gastrocnemius (GA) and pharyngeal (PH) muscles. Merged images show immunostaining
 453 with anti-CD206 (Red) and anti-laminin (green) antibodies and DAPI (blue). The asterisk in the right side of dashed white line
 454 indicate thyropharyngeus (TP) muscle area. Scale bars = 330 μ m. C. Quantified number of CD206⁺ cells per 100 myofibers. BaCl₂-
 455 injured tibialis anterior (TA) muscles are used as a positive control. n = 3. Statistical significance was determined by ANOVA. D.
 456 Representative images of fibroadipogenic progenitors (FAPs) in gastrocnemius (GA) and pharyngeal (PH) muscles. Merged images
 457 show immunostaining with anti-PDGFR α (Cyan) and anti-laminin (green) antibodies and DAPI (blue). Scale bars = 330 μ m. E.
 458 Quantified number of PDGFR α ⁺ cells per 100 myofibers. BaCl₂-injured tibialis anterior (TA) muscles are included as a positive
 459 control. n = 3. Statistical significance was determined by 1-way ANOVA. F. Quantified number of PDGFR α ⁺ cells per PAX7⁺ cells.
 460 n = 3. For all graphs, the value represents mean \pm SEM. Asterisks indicate statistical significance (*p<0.05 and **p<0.01).

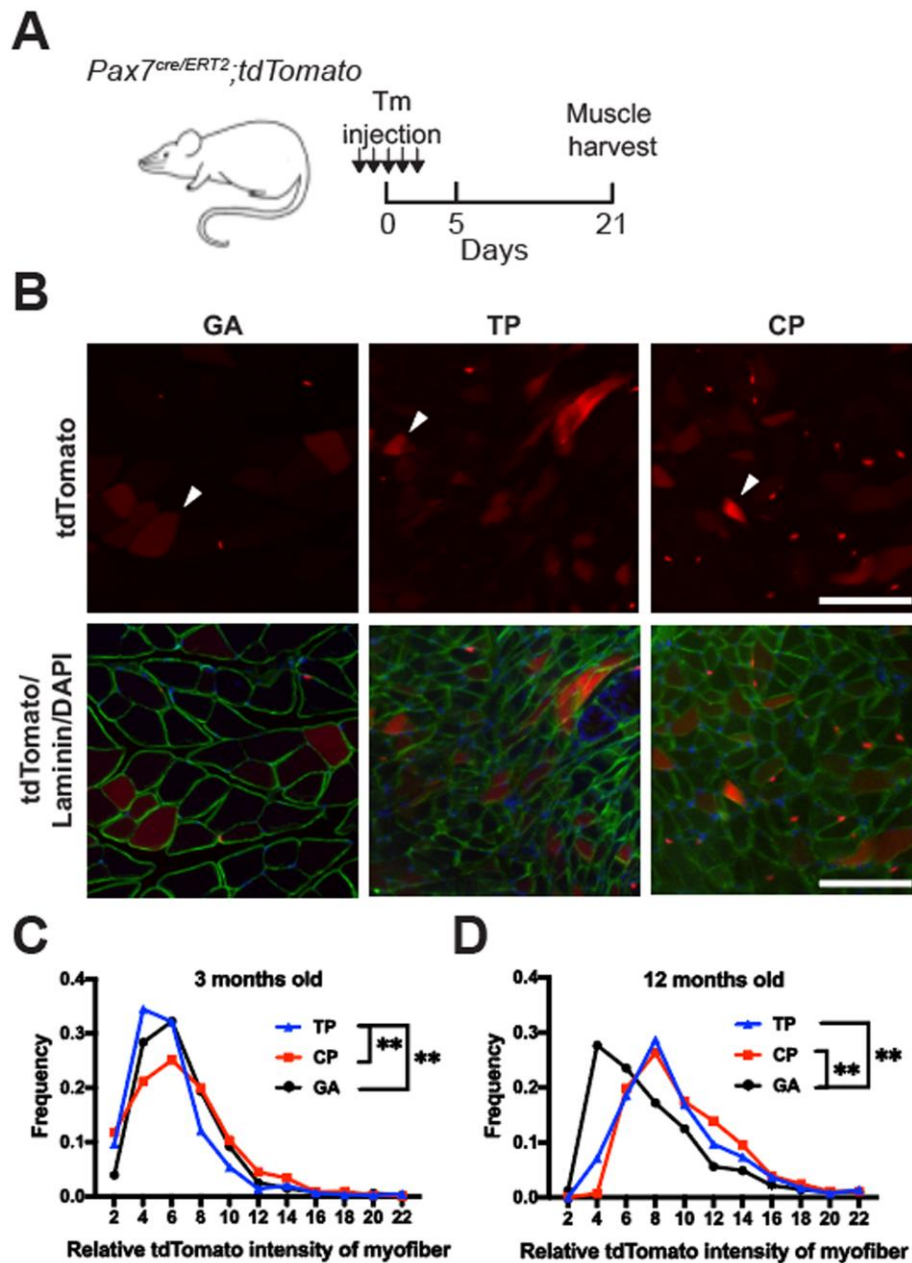
461 **3.6. Pharyngeal satellite cells contribute to muscle homeostasis and niche maintenance**
462 **of pharyngeal muscle.**

463 Given the increased proliferation and differentiation of pharyngeal SCs, we
464 hypothesized that pharyngeal SCs are important for maintaining pharyngeal muscle
465 homeostasis. In a previous study, ablation of SCs in mice from 2 to 6 months of age led to no
466 change of laryngeal pharyngeal muscle fiber cross-sectional area (CSA) but a small significant
467 change of nasal pharyngeal muscle (Randolph et al., 2015). However, in 12 month-old mice,
468 pharyngeal SCs showed significantly higher proliferation (Supplementary Figure 1,
469 (Randolph et al., 2015)) and fusion with consistent SC number compared to pharyngeal SCs
470 of 3 month-old mice (Supplementary Figure 2). Therefore, we investigated the functional
471 importance of pharyngeal SCs around 12 month-old mice by utilizing *Pax7 Cre^{ERT2} -DTA*
472 mice, which express tamoxifen-inducible, SC-specific diphtheria toxin and thus induce
473 ablation of PAX7⁺ SCs cells *in vivo*. To deplete SC in middle aged mice, we treated with
474 tamoxifen to induce SC-specific DTA expression in 6 month-old mice and harvested
475 pharyngeal muscles after 9 months of SC ablation (Fig 6A). As shown in Fig 6B, we detected
476 a significant decrease in the level of *Pax7* mRNA in the SC-ablated *Pax7 Cre^{+/-} -DTA^{+/+}* TM
477 (Tamoxifen) group compared to the control *Pax7 Cre^{+/-} -DTA^{+/+}* CO (Corn oil vehicle control)
478 group. There was no significant difference between the *Pax7 Cre^{+/-} -DTA^{+/+}* TM (Tamoxifen)
479 group and *Pax7 Cre^{-/-} -DTA^{+/+}* TM (Cre control) group. To test whether ablation of PAX7⁺ SCs
480 impacts pharyngeal myofiber size, we stained sectioned TP and CP muscles sections with
481 hematoxylin/eosin and measured the CSA of myofibers. There was no significant change in
482 myofiber CSA in TP muscles of SC-ablated *Pax7 Cre^{+/-} -DTA^{+/+}* TM mice relative to controls
483 (Fig 6C). Unexpectedly, the frequency distribution of CSA shifted to the right in CP muscles

484 of *Pax7 Cre^{+/-}-DTA^{+/+}* TM mice, indicating a preponderance of larger myofibers after ablation
485 of SCs (Fig 6C). This result implies that SCs in the CP muscle are involved in the maintenance
486 of pharyngeal muscle size.

487 Considering that SCs are known to signal to other cell types in the skeletal muscle niche
488 (Fry, Kirby, Kosmac, McCarthy, & Peterson, 2017), we hypothesized that loss of secreted
489 factors from pharyngeal SCs affect the neighboring cells close to the SC niche. We sectioned
490 pharyngeal muscles from SC-ablated *Pax7 Cre^{ERT2}-DTA* mice and quantified CD206⁺ cells
491 (resident macrophages) and PDGFR α ⁺ cells (FAPs) using immunofluorescent staining and
492 microscopy (Fig 6D-E). We observed significantly decreased numbers of CD206⁺ cells in the
493 TP muscle of *Pax7 Cre^{ERT2}-DTA* mice (Fig 6D). Conversely, we detected a significant increase
494 in the number of PDGFR α ⁺ cells in the TP and CP muscles of *Pax7 Cre^{ERT2}-DTA* mice (Fig
495 6E). Although the difference was not statistically significant, *Cre^{-/-}-DTA^{+/+}* TM (Cre control)
496 mice showed a trend of reduced *Pax7* expression (Fig 6B) as well as similar trends of
497 neighboring cell pool change with *Pax7 Cre^{ERT2}-DTA* mice (Fig 6D-E). Taken together, these
498 data suggest that pharyngeal SCs are required not only to maintain the size of myofibers in
499 cricopharyngeus muscles but also to control the number of neighboring macrophages and
500 FAPs that contribute to the microenvironment of the pharyngeal SC niche.

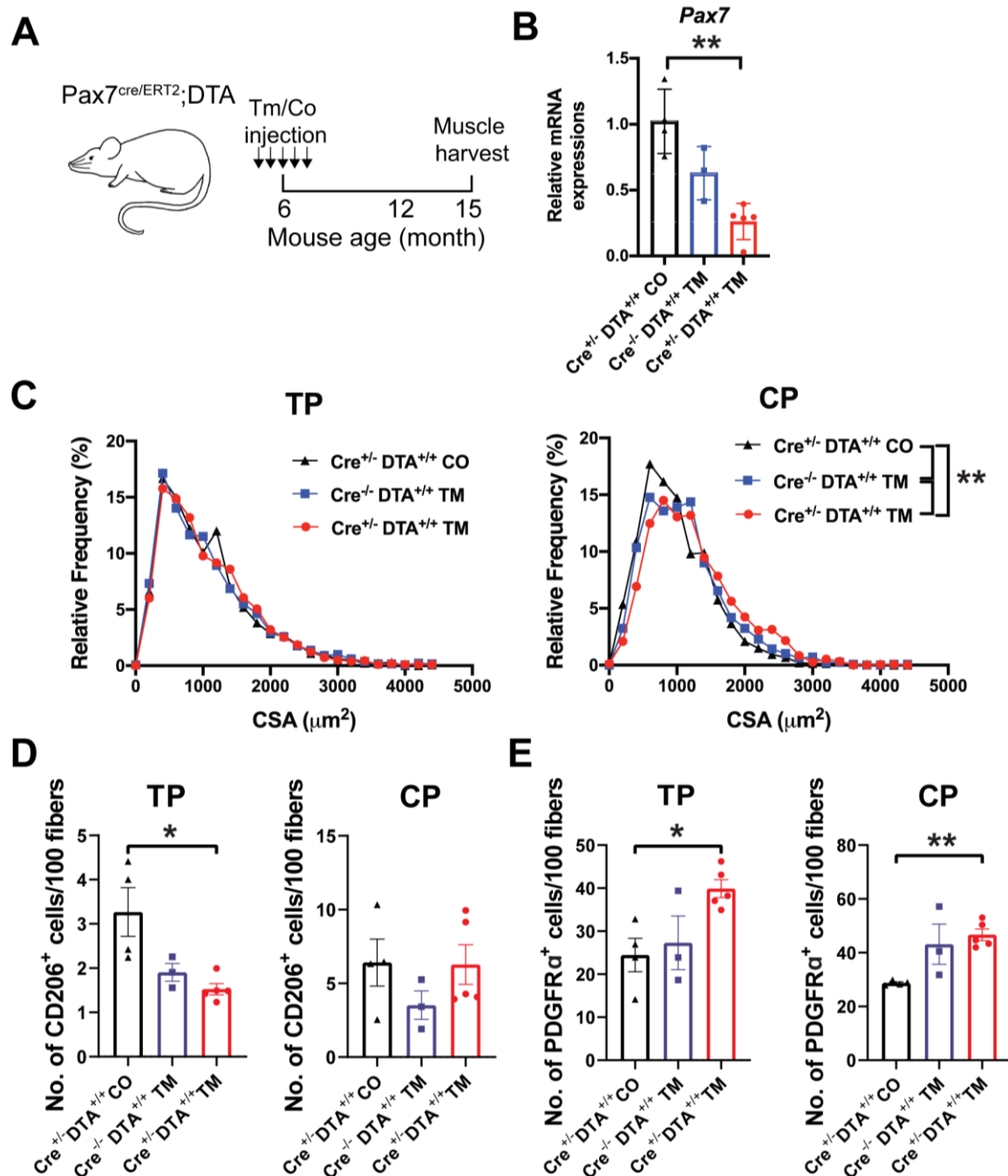
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503 **Supplementary Figure 2. Increased fusion of pharyngeal satellite cells in 12 month old mice compared with 3 month**
 504 **old mice.** A. Scheme of experiments using *Pax7 Cre^{ERT2}-tdTomato* mice. Abbreviations: Tamoxifen (TM); Control (CO). B.
 505 Representative cross-section expressing PAX7⁺ (red) satellite cells in gastrocnemius (GA), thyropharyngeus (TP) and
 506 cricopharyngeus (CP) muscles from 12 month old *Pax7 Cre^{ERT2}-tdTomato* mice. White arrow heads indicate examples of PAX7⁺ SC
 507 fused myofibers, which show diffused tdTomato expression inside the myofiber. Basal lamina was immune-stained with anti-
 508 Laminin antibody (green), and DAPI (blue) was used to stain nuclei. C, D. Frequency distribution of tdTomato intensity in myofibers
 509 from gastrocnemius (GA), thyropharyngeus (TP) and cricopharyngeus (CP) muscles of 3 month (C) and 12 month (D) old *Pax7*
 510 *Cre^{ERT2}-tdTomato* mice. Statistical significance was determined by Kruskal-Wallis test. Asterisks indicate statistical significance
 511 (**p<0.01).

512



513

514 **Figure 6. Myofiber cross-sectional area and niche factors are impacted by satellite cell ablation in pharyngeal muscle.**
 515 A. Scheme of experiments using *Pax7 Cre^{ERT2}-DTA* mice. Abbreviations: Tamoxifen (TM); Control (CO). B. Relative mRNA
 516 expression level of *Pax7* in muscle samples (*Pax7 Cre^{+/-};DTA^{+/+} TM*, *Pax7 Cre^{-/-};DTA^{+/+} TM*, *Pax7 Cre^{+/-};DTA^{+/+} CO*) obtained from
 517 *Pax7 Cre^{ERT2}-DTA* mice with or without tamoxifen (TM) treatment. n = 4 or 5. Statistical significance was determined by 1-way
 518 ANOVA. C. Frequency distribution plots of myofiber cross-sectional area (CSA) from the thyropharyngeus (TP) and cricopharyngeus
 519 (CP) muscle regions are shown. n = 4, with 1400-1800 myofibers combined per condition. Statistical significance was determined by
 520 Kruskal-Wallis test. D, E. Quantified number of CD206⁺ cells (D) and PDGFR α ⁺ cells (E) per 100 myofibers in TP and CP of *Pax7*
 521 *Cre^{ERT2}-DTA* mice. n = 4 or 5. Statistical significance was determined by 1-way ANOVA. For all graphs, the value represents mean \pm
 522 SEM. Asterisks indicate statistical significance (*p<0.05 and **p<0.01).

523

524 **4. Discussion**

525 Although craniofacial muscles including pharyngeal muscles differ from body muscles
526 in embryonic origin and core genetic programs (S Tajbakhsh, 2009), the majority of satellite
527 cell (SC) studies have focused on those in the limb muscles. Studies of SCs in craniofacial
528 muscle are difficult due to its small size, the difficulty dissection, and the lack of functional
529 assays. To overcome these difficulties, we employed a series of genetic mouse models to either
530 label or ablate SCs and reveal the distinct characteristics of pharyngeal muscle SCs. To explain
531 the highly proliferative and differentiative properties of pharyngeal SCs, we investigated both
532 intrinsic factors, such as mitochondria and autocrine factors, and extrinsic niche including
533 ECM and secreted factors from other cell types within pharyngeal muscles. Our study provides
534 new evidence to explain how both intrinsic mechanisms and extrinsic factors govern the unique
535 state of craniofacial SCs.

536 **G-alerted features of pharyngeal SCs: cell size and mitochondria contents.**

537 Dividing SCs typically follow one of two fates, either a return to the quiescent G_0 state
538 to renew the SC pool (Li & Clevers, 2010) or entry into an actively cycling G_1 state to proceed
539 along the myogenic lineage (Yin, Price, & Rudnicki, 2013). A third state, G_{Alert} , has also been
540 described, which is intermediate to quiescence and activation (Rodgers et al., 2014). Our data
541 indicate that SCs in uninjured pharyngeal muscles exhibit multiple aspects of the G_{Alert} state
542 including increased cell size, enhanced mitochondrial mass, and increased propensity to
543 proliferate and differentiate (Rodgers et al., 2014). Given that SCs in the G_{Alert} state are 'primed'
544 to rapidly respond to injury stimuli, the G_{Alert} -like state of pharyngeal SCs suggests a poor
545 ability to maintain quiescence and, once activated, their cell fate is more forced toward

546 commitment when compared to quiescent limb SCs. Indeed, we detected enhanced
547 proliferation and differentiation of cultured SCs isolated from pharyngeal muscle relative to
548 those isolated from the gastrocnemius. This unique “alert”-like state is thought to be tightly
549 regulated by distinct intrinsic or extrinsic factors of pharyngeal muscles itself: cellular
550 metabolism, cell autonomous signaling, cell-cell signaling, the extracellular environment,
551 inflammatory mediators, and so on (Aurora & Olson, 2014; Quarta et al., 2016).

552 One of the core intrinsic factors governing SC state is mitochondria-mediated metabolic
553 regulation, which is considered critical for cell fate decisions, activation, and myoblast
554 proliferation (Duguez, Sabido, & Freyssenet, 2004; Zhang, Menzies, & Auwerx, 2018). We
555 found that pharyngeal SCs showed relatively higher mitochondrial content relative to quiescent
556 limb SCs, which contain relatively few mitochondria. This result is consistent with a previously
557 reported increase in mitochondrial content upon SC activation in limb muscle, despite a shift
558 to glycolytic metabolism (Montarras, L'honoré, & Buckingham, 2013; Ryall et al., 2015).
559 Interestingly, impaired mitochondrial function has been associated with the pathology of
560 oculopharyngeal muscular dystrophy, which preferentially affects craniofacial muscles
561 (Chartier et al., 2015; Vest et al., 2017). Further investigation is necessary to investigate the
562 importance of mitochondrial metabolism in pharyngeal SC biology and in pharyngeal muscle
563 pathologies.

564 **Pharyngeal SC-derived niches: auto/paracrine factors and collagen V.**

565 The skeletal muscle niche is critically important in regulating SC state. We tested two
566 important components of the SC-derived niche including autocrine signaling via secreted
567 factors and ECM components. In co-culture experiments, we determined that pharyngeal SCs

568 enhance proliferation of limb MPCs via secreted factors, though further studies are needed to
569 determine the identity of the soluble signals secreted by pharyngeal SCs. Our study also shows
570 that the pharyngeal muscle niche contains less collagen V protein, which is produced by SCs
571 and is considered to be a dominant regulator of the quiescent niche in skeletal muscles
572 (Baghdadi et al., 2018). This result is consistent with the differentially expressed ECM genes
573 pharyngeal SCs reported in a previous microarray study (Randolph et al., 2015). It is possible
574 that other proteoglycans or ECM proteins contribute to regulation of quiescence in a pharyngeal
575 SC-specific manner. Taken together, these data add weight to the hypothesis that pharyngeal
576 SCs are intrinsically less quiescent.

577 **Neighboring cells to activate pharyngeal SCs: resident macrophages and FAPs.**

578 Neighboring cells contribute to the microenvironment of SCs via soluble factors or direct
579 cell-to-cell contact. HGF is one such auto/paracrine factor involved in SC activation in
580 response to muscle injury, overuse, or mechanical stretches (Miller, Thaloor, Matteson, &
581 Pavlath, 2000; Sheehan, Tatsumi, Temm-Grove, & Allen, 2000; Tatsumi, 2010; Tatsumi,
582 Anderson, Nevoret, Halevy, & Allen, 1998). HGF is secreted into the extracellular matrix of
583 uninjured muscles as pro-HGF and, upon injury, proteolysis by urokinase-type plasminogen
584 activator (uPA) (Sisson et al., 2009) or HGF activator (Rodgers, Schroeder, Ma, & Rando, 2017)
585 generates active HGF that in turn activates SCs (Bernet-Camard, Coconnier, Hudault, & Servin,
586 1996; Sisson et al., 2009; Stoker, Gherardi, Perryman, & Gray, 1987). Although we did not
587 determine which form of HGF is found in pharyngeal muscle, previous microarray data
588 comparing pharyngeal SCs with limb SCs (Randolph *et al.*, 2015) revealed increased levels of
589 the *Plat* gene encoding tissue-type plasminogen activator (tPA), which is similar (identity 32.8%
590 and similarity 43%) to uPA and cleaves pro-HGF (Mars, Zarnegar, & Michalopoulos, 1993).

591 Thus, pharyngeal SCs may contribute to processing pro-HGF to the active form without injury.
592 Interestingly, extraocular muscle SCs, which also proliferate and differentiate without injury,
593 contain high levels of *Plat* mRNA relative to limb SCs (Pacheco-Pinedo et al., 2009). Thus,
594 HGF is likely an important signal modulating pharyngeal and extraocular SC activity, but
595 additional studies are needed to better define the mechanism.

596 Although HGF signaling can occur in an autocrine manner in SCs, the majority of HGF
597 is secreted by macrophages (Sisson et al., 2009) that infiltrate the skeletal muscle niche after
598 injury (Pillon, Bilan, Fink, & Klip, 2013). Previous microarray analysis revealed that
599 pharyngeal SCs contain elevated levels of mRNAs encoding cytokines known to attract
600 macrophages (*Ccl2*, *Ccl12*, *Ccl7*) or induce macrophage polarization (*Lif* and *Il-6*) (Duluc et
601 al., 2007; Xuan, Qu, Zheng, Xiong, & Fan, 2015). As expected, we detected relatively high
602 numbers resident macrophage without injury in pharyngeal muscles compared to uninjured
603 limb muscles. To further address the role of SCs in macrophage recruitment to pharyngeal
604 muscles, we utilized a genetic mouse model (*Pax7 Cre^{ERT2} -DTA* mice) to ablate satellite cells.
605 We found that SC ablation led to a decrease in the number of M2 macrophages in the
606 thyropharyngeal muscle. This result confirms that pharyngeal SCs are important for recruiting
607 resident M2 cells under basal conditions. However, the roles of M2 for pharyngeal SC
608 activation as well as function of pharyngeal muscles remain to be determined.

609 Another niche signal important for modulating SC activity is follistatin, which is a TGF-
610 β antagonist (Amthor et al., 2004) suggested to prime myoblasts for myogenic differentiation
611 and promote myofiber hyperplasia (Jones et al., 2015; Medeiros, Phelps, Fuentes, & Bradley,
612 2009). We provide evidence that pharyngeal muscles have the increased levels of follistatin
613 mRNA and its cellular source, FAPs. (Reggio et al., 2020). These results are consistent with

614 previous reports in extraocular muscles (Formicola et al., 2014) and in contralateral limb
615 muscles, which contains G_{Alert} SCs (Rodgers et al., 2014). Interestingly, we found that SC may
616 regulate the pool of FAPs, as we detected elevated numbers of FAPs in SC-depleted muscles,
617 which was consistent with previous report using limb muscles (Formicola et al., 2018).
618 However, fibrosis and fatty infiltration were not detected in SC-depleted pharyngeal muscles
619 unlike chronic injured muscles (X. Liu et al., 2016). In contrast, mice in which the FAPs
620 population has been ablated showed reduced numbers of SC in limb muscles indicating that
621 FAPs are important for maintaining SC pools (Ancel, Mashinchian, & Feige, 2019; Wosczyzna
622 et al., 2019). Taken together, our results and previously published studies indicate that
623 correlations exists between SC and FAPs pools in skeletal muscle.

624 **Role of pharyngeal SC for muscle maintenance.**

625 To understand how SCs participate in pharyngeal muscle maintenance, we measured the
626 myofiber CSA in cricopharyngeal and thyropharyngeal muscles in SC-ablated mice. Unlike
627 other SC-depletion studies (Keefe et al., 2015; Pawlikowski, Pulliam, Dalla Betta, Kardon, &
628 Olwin, 2015; Randolph & Pavlath, 2015), the conditional depletion of pharyngeal SCs from
629 adult mice led to an increase in the CSA of cricopharyngeal myofibers. These results indicate
630 that SCs are required for maintenance of cricopharyngeus muscle size, though the mechanism
631 allowing for this adjustment remains completely unknown. However, it is inconsistent with our
632 expectation and the previous report showing that satellite cell ablation led to reduced CSA in
633 12-month SC-depleted EOM (Keefe et al., 2015) and in 4-month SC-depleted nasopharyngeal
634 muscles (no CSA change in laryngopharynx, which contains our target muscles, CP and TP)
635 (Randolph & Pavlath, 2015). One possible explanation for our findings is that the ablation of
636 SCs is incomplete and the remaining diminished population of SCs in pharyngeal muscles

637 produces a hypertrophic response related to the increase in the number of FAPs. Alternatively,
638 hypertrophy of pharyngeal muscle may be a result of small fiber fusion in SC-depleted
639 conditions or SC-independent hypertrophic growth (McCarthy et al., 2011).

640 In conclusion, this study is the first comprehensive analysis of both intrinsic and extrinsic
641 factors associated with the highly proliferative and differentiative features of pharyngeal SC.
642 While it is not clear whether the unique embryonic origins of pharyngeal SCs leads intrinsic
643 differences in proliferative and myogenic properties, this study demonstrates that both
644 pharyngeal SC-secreted factors and pharyngeal muscle niches are capable of activating
645 pharyngeal SCs without injury. Although the role of highly active SCs in pharyngeal muscle
646 function is still ambiguous, we propose that unique properties of pharyngeal SCs are a
647 promising area of study to better understand pharyngeal muscle specific pathologies, such as
648 oculopharyngeal muscular dystrophy.

649

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654

655 **Author contributions**

656 EK designed the study, performed experiments, image analysis, analyzed data, prepared
657 figures and wrote the manuscript. YZ performed experiments and image analysis. FW performed
658 experiments and analyzed data. JA prepared samples. KEV performed experiments, analyzed data and
659 wrote the manuscript. HJC designed the study, performed experiments, image analysis, analyzed data,

660 prepared figures and wrote the manuscript.

661

662 **Conflict of interest**

663 The authors declare that they have no conflict of interest.

664

665 **Data availability**

666 All data to support the conclusions of this manuscript are included in the main text and
667 supplementary materials.

668

669 **Reference**

- 670 Allen, R. E., Sheehan, S. M., Taylor, R. G., Kendall, T. L., & Rice, G. M. (1995). Hepatocyte growth
671 factor activates quiescent skeletal muscle satellite cells in vitro. *Journal of cellular physiology*,
672 165(2), 307-312.
- 673 Amthor, H., Nicholas, G., McKinnell, I., Kemp, C. F., Sharma, M., Kambadur, R., & Patel, K. (2004).
674 Follistatin complexes Myostatin and antagonises Myostatin-mediated inhibition of myogenesis.
675 *Developmental Biology*, 270(1), 19-30.
- 676 Ancel, S., Mashinchian, O., & Feige, J. N. (2019). Adipogenic progenitors keep muscle stem cells
677 young. *Aging (Albany NY)*, 11(18), 7331-7333.
- 678 Aurora, A. B., & Olson, E. N. (2014). Immune modulation of stem cells and regeneration. *Cell stem*
679 *cell*, 15(1), 14-25.
- 680 Búa, B. A., Olsson, R., Westin, U., Rydell, R., & Ekberg, O. (2015). Treatment of cricopharyngeal
681 dysfunction: a comparative pilot study. *BMC research notes*, 8(1), 301.
- 682 Baghdadi, M. B., Castel, D., Machado, L., Fukada, S.-i., Birk, D. E., Relaix, F., . . . Mourikis, P. (2018).
683 Reciprocal signalling by Notch–Collagen V–CALCR retains muscle stem cells in their niche.
684 *Nature*, 557(7707), 714-718.
- 685 Bernet-Camard, M., Coconnier, M., Hudault, S., & Servin, A. (1996). Differential expression of
686 complement proteins and regulatory decay accelerating factor in relation to differentiation of
687 cultured human colon adenocarcinoma cell lines. *Gut*, 38(2), 248-253.
- 688 Bosnakovski, D., Xu, Z., Li, W., Thet, S., Cleaver, O., Perlingeiro, R. C., & Kyba, M. (2008).
689 Prospective isolation of skeletal muscle stem cells with a Pax7 reporter. *Stem Cells*, 26(12),
690 3194-3204.
- 691 Chartier, A., Klein, P., Pierson, S., Barbezier, N., Gidaro, T., Casas, F., . . . Bellec, M. (2015).
692 Mitochondrial dysfunction reveals the role of mRNA poly (A) tail regulation in
693 oculopharyngeal muscular dystrophy pathogenesis. *PLoS Genetics*, 11(3), e1005092.
- 694 Cook, I. J. (1993). Cricopharyngeal function and dysfunction. *Dysphagia*, 8(3), 244-251.
- 695 Dong, F., Sun, X., Liu, W., Ai, D., Klysik, E., Lu, M.-F., . . . Baldini, A. (2006). Pitx2 promotes
696 development of splanchnic mesoderm-derived branchiomic muscle. *Development*, 133(24),
697 4891-4899.
- 698 Duguez, S., Sabido, O., & Freyssenet, D. (2004). Mitochondrial-dependent regulation of myoblast
699 proliferation. *Experimental Cell Research*, 299(1), 27-35.
- 700 Duluc, D., Delneste, Y., Tan, F., Moles, M.-P., Grimaud, L., Lenoir, J., . . . Ifrah, N. (2007). Tumor-
701 associated leukemia inhibitory factor and IL-6 skew monocyte differentiation into tumor-
702 associated macrophage-like cells. *Blood, The Journal of the American Society of Hematology*,

- 703 *110*(13), 4319-4330.
- 704 Evano, B., & Tajbakhsh, S. (2018). Skeletal muscle stem cells in comfort and stress. *NPJ Regenerative*
705 *medicine*, *3*(1), 1-13.
- 706 Formicola, L., Marazzi, G., & Sassoon, D. A. (2014). The extraocular muscle stem cell niche is resistant
707 to ageing and disease. *Frontiers in aging neuroscience*, *6*, 328.
- 708 Formicola, L., Pannérec, A., Correra, R. M., Gayraud-Morel, B., Ollitrault, D., Besson, V., . . . Marazzi,
709 G. (2018). Inhibition of the activin receptor type-2B pathway restores regenerative capacity in
710 satellite cell-depleted skeletal muscle. *Frontiers in physiology*, *9*, 515.
- 711 Fry, C. S., Kirby, T. J., Kosmac, K., McCarthy, J. J., & Peterson, C. A. (2017). Myogenic progenitor
712 cells control extracellular matrix production by fibroblasts during skeletal muscle hypertrophy.
713 *Cell stem cell*, *20*(1), 56-69.
- 714 Gómez-Torres, A., Jiménez, A. A., Infante, E. R., Bueno, A. M., Zamora, I. T., & Ortega, F. E. (2012).
715 Cricopharyngeal myotomy in the treatment of oculopharyngeal muscular dystrophy. *Acta*
716 *Otorrinolaringologica (English Edition)*, *63*(6), 465-469.
- 717 Gillies, A. R., & Lieber, R. L. (2011). Structure and function of the skeletal muscle extracellular matrix.
718 *Muscle & Nerve*, *44*(3), 318-331.
- 719 Gilson, H., Schakman, O., Kalista, S., Lause, P., Tsuchida, K., & Thissen, J.-P. (2009). Follistatin
720 induces muscle hypertrophy through satellite cell proliferation and inhibition of both myostatin
721 and activin. *American Journal of Physiology-Endocrinology and Metabolism*, *297*(1), E157-
722 E164.
- 723 Goulding, M., Lumsden, A., & Paquette, A. J. (1994). Regulation of Pax-3 expression in the
724 dermomyotome and its role in muscle development. *Development*, *120*(4), 957-971.
- 725 Harel, I., Nathan, E., Tirosh-Finkel, L., Zigdon, H., Guimarães-Camboa, N., Evans, S. M., & Tzahor,
726 E. (2009). Distinct origins and genetic programs of head muscle satellite cells. *Developmental*
727 *Cell*, *16*(6), 822-832.
- 728 Joe, A. W., Yi, L., Natarajan, A., Le Grand, F., So, L., Wang, J., . . . Rossi, F. M. (2010). Muscle injury
729 activates resident fibro/adipogenic progenitors that facilitate myogenesis. *Nature Cell Biology*,
730 *12*(2), 153-163.
- 731 Jones, A. E., Price, F. D., Le Grand, F., Soleimani, V. D., Dick, S. A., Megeney, L. A., & Rudnicki, M.
732 A. (2015). Wnt/ β -catenin controls follistatin signalling to regulate satellite cell myogenic
733 potential. *Skeletal muscle*, *5*(1), 14.
- 734 Keefe, A. C., Lawson, J. A., Flygare, S. D., Fox, Z. D., Colasanto, M. P., Mathew, S. J., . . . Kardon, G.
735 (2015). Muscle stem cells contribute to myofibres in sedentary adult mice. *Nature*
736 *communications*, *6*(1), 1-11.

- 737 Khurana, T. S., Prendergast, R. A., Alameddine, H. S., Tome, F., Fardeau, M., Arahata, K., . . . Kunkel,
738 L. M. (1995). Absence of extraocular muscle pathology in Duchenne's muscular dystrophy:
739 role for calcium homeostasis in extraocular muscle sparing. *The Journal of experimental*
740 *medicine*, 182(2), 467-475.
- 741 Kosmac, K., Peck, B. D., Walton, R. G., Mula, J., Kern, P. A., Bamman, M. M., . . . Johnson, D. L.
742 (2018). Immunohistochemical identification of human skeletal muscle macrophages. *Bio-*
743 *protocol*, 8(12), e2883.
- 744 Lepper, C., Conway, S. J., & Fan, C.-M. (2009). Adult satellite cells and embryonic muscle progenitors
745 have distinct genetic requirements. *Nature*, 460(7255), 627-631.
- 746 Li, L., & Clevers, H. (2010). Coexistence of quiescent and active adult stem cells in mammals. *Science*,
747 327(5965), 542-545.
- 748 Liu, W., Shan, T., Yang, X., Liang, S., Zhang, P., Liu, Y., . . . Kuang, S. (2013). A heterogeneous lineage
749 origin underlies the phenotypic and molecular differences of white and beige adipocytes.
750 *Journal of Cell Science*, 126(16), 3527-3532.
- 751 Liu, X., Ning, A. Y., Chang, N. C., Kim, H., Nissenson, R., Wang, L., & Feeley, B. T. (2016).
752 Investigating the cellular origin of rotator cuff muscle fatty infiltration and fibrosis after injury.
753 *Muscles, ligaments and tendons journal*, 6(1), 6–15.
- 754 Livak, K. J., & Schmittgen, T. D. (2001). Analysis of relative gene expression data using real-time
755 quantitative PCR and the 2⁻ΔΔCT method. *Methods*, 25(4), 402-408.
- 756 Madaro, L., Mozzetta, C., Biferali, B., & Proietti, D. (2019). Fibro-Adipogenic Progenitors (FAPs)
757 cross-talk in skeletal muscle: the social network. *Frontiers in physiology*, 10, 1074.
- 758 Mars, W. M., Zarnegar, R., & Michalopoulos, G. K. (1993). Activation of hepatocyte growth factor by
759 the plasminogen activators uPA and tPA. *The American journal of pathology*, 143(3), 949–958.
- 760 Mauro, A. (1961). Satellite cell of skeletal muscle fibers. *The Journal of biophysical and biochemical*
761 *cytology*, 9(2), 493-495.
- 762 McCarthy, J. J., Mula, J., Miyazaki, M., Erfani, R., Garrison, K., Farooqui, A. B., . . . Keller, C. (2011).
763 Effective fiber hypertrophy in satellite cell-depleted skeletal muscle. *Development*, 138(17),
764 3657-3666.
- 765 McLoon, L. K., Thorstenson, K., Solomon, A., & Lewis, M. (2007). Myogenic precursor cells in
766 craniofacial muscles. *Oral diseases*, 13(2), 134-140.
- 767 Medeiros, E. F., Phelps, M. P., Fuentes, F. D., & Bradley, T. M. (2009). Overexpression of follistatin in
768 trout stimulates increased muscling. *American Journal of Physiology-Regulatory, Integrative*
769 *and Comparative Physiology*, 297(1), R235-R242.
- 770 Miller, K. J., Thalloor, D., Matteson, S., & Pavlath, G. K. (2000). Hepatocyte growth factor affects

- 771 satellite cell activation and differentiation in regenerating skeletal muscle. *American Journal of*
772 *Physiology-Cell Physiology*, 278(1), C174-C181.
- 773 Montarras, D., L'honoré, A., & Buckingham, M. (2013). Lying low but ready for action: the quiescent
774 muscle satellite cell. *The FEBS journal*, 280(17), 4036-4050.
- 775 Mootoosamy, R. C., & Dietrich, S. (2002). Distinct regulatory cascades for head and trunk myogenesis.
776 *Development*, 129(3), 573-583.
- 777 Noden, D. M., & Francis-West, P. (2006). The differentiation and morphogenesis of craniofacial
778 muscles. *Developmental dynamics: an official publication of the American Association of*
779 *Anatomists*, 235(5), 1194-1218.
- 780 Pacheco-Pinedo, E. C., Budak, M. T., Zeiger, U., Jørgensen, L. H., Bogdanovich, S., Schrøder, H. D., . . .
781 Khurana, T. S. (2009). Transcriptional and functional differences in stem cell populations
782 isolated from extraocular and limb muscles. *Physiological Genomics*, 37(1), 35-42.
- 783 Pawlikowski, B., Pulliam, C., Dalla Betta, N., Kardon, G., & Olwin, B. B. (2015). Pervasive satellite
784 cell contribution to uninjured adult muscle fibers. *Skeletal muscle*, 5(1), 42.
- 785 Pillon, N. J., Bilan, P. J., Fink, L. N., & Klip, A. (2013). Cross-talk between skeletal muscle and immune
786 cells: muscle-derived mediators and metabolic implications. *American Journal of Physiology-*
787 *Endocrinology and Metabolism*, 304(5), E453-E465.
- 788 Quarta, M., Brett, J. O., DiMarco, R., De Morree, A., Boutet, S. C., Chacon, R., . . . Shrager, J. B. (2016).
789 An artificial niche preserves the quiescence of muscle stem cells and enhances their therapeutic
790 efficacy. *Nature Biotechnology*, 34(7), 752-759.
- 791 Randolph, M. E., Luo, Q., Ho, J., Vest, K. E., Sokoloff, A. J., & Pavlath, G. K. (2014). Ageing and
792 muscular dystrophy differentially affect murine pharyngeal muscles in a region-dependent
793 manner. *The Journal of physiology*, 592(23), 5301-5315.
- 794 Randolph, M. E., & Pavlath, G. K. (2015). A muscle stem cell for every muscle: variability of satellite
795 cell biology among different muscle groups. *Frontiers in aging neuroscience*, 7, 190.
- 796 Randolph, M. E., Phillips, B. L., Choo, H. J., Vest, K. E., Vera, Y., & Pavlath, G. K. (2015). Pharyngeal
797 satellite cells undergo myogenesis under basal conditions and are required for pharyngeal
798 muscle maintenance. *Stem Cells*, 33(12), 3581-3595.
- 799 Reggio, A., Rosina, M., Krahmer, N., Palma, A., Petrilli, L. L., Maiolatesi, G., . . . Testa, S. (2020).
800 Metabolic reprogramming of fibro/adipogenic progenitors facilitates muscle regeneration. *Life*
801 *science alliance*, 3(3), e202000646.
- 802 Relaix, F., Rocancourt, D., Mansouri, A., & Buckingham, M. (2005). A Pax3/Pax7-dependent
803 population of skeletal muscle progenitor cells. *Nature*, 435(7044), 948-953.
- 804 Rodgers, J. T., King, K. Y., Brett, J. O., Cromie, M. J., Charville, G. W., Maguire, K. K., . . . Tsai, C.-R.

- 805 (2014). mTORC1 controls the adaptive transition of quiescent stem cells from G₀ to G₁ Alert.
806 *Nature*, 510(7505), 393-396.
- 807 Rodgers, J. T., Schroeder, M. D., Ma, C., & Rando, T. A. (2017). HGFA is an injury-regulated systemic
808 factor that induces the transition of stem cells into G₁ Alert. *Cell reports*, 19(3), 479-486.
- 809 Ryall, J. G., Dell'Orso, S., Derfoul, A., Juan, A., Zare, H., Feng, X., . . . Fulco, M. (2015). The NAD⁺-
810 dependent SIRT1 deacetylase translates a metabolic switch into regulatory epigenetics in
811 skeletal muscle stem cells. *Cell stem cell*, 16(2), 171-183.
- 812 Sambasivan, R., Yao, R., Kissenpfennig, A., Van Wittenberghe, L., Paldi, A., Gayraud-Morel, B., . . .
813 Galy, A. (2011). Pax7-expressing satellite cells are indispensable for adult skeletal muscle
814 regeneration. *Development*, 138(17), 3647-3656.
- 815 Sheehan, S. M., Tatsumi, R., Temm-Grove, C. J., & Allen, R. E. (2000). HGF is an autocrine growth
816 factor for skeletal muscle satellite cells in vitro. *Muscle & Nerve: Official Journal of the*
817 *American Association of Electrodiagnostic Medicine*, 23(2), 239-245.
- 818 Shi, X., & Garry, D. J. (2006). Muscle stem cells in development, regeneration, and disease. *Genes &*
819 *Development*, 20(13), 1692-1708.
- 820 Sisson, T. H., Nguyen, M.-H., Yu, B., Novak, M. L., Simon, R. H., & Koh, T. J. (2009). Urokinase-type
821 plasminogen activator increases hepatocyte growth factor activity required for skeletal muscle
822 regeneration. *Blood*, 114(24), 5052-5061.
- 823 Stoker, M., Gherardi, E., Perryman, M., & Gray, J. (1987). Scatter factor is a fibroblast-derived
824 modulator of epithelial cell mobility. *Nature*, 327(6119), 239-242.
- 825 Stuelsatz, P., Shearer, A., Li, Y., Muir, L. A., Ieronimakis, N., Shen, Q. W., . . . Yablonka-Reuveni, Z.
826 (2015). Extraocular muscle satellite cells are high performance myo-engines retaining efficient
827 regenerative capacity in dystrophin deficiency. *Developmental Biology*, 397(1), 31-44.
- 828 Tajbakhsh, S. (2009). Skeletal muscle stem cells in developmental versus regenerative myogenesis.
829 *Journal of internal medicine*, 266(4), 372-389.
- 830 Tajbakhsh, S., Rocancourt, D., Cossu, G., & Buckingham, M. (1997). Redefining the genetic hierarchies
831 controlling skeletal myogenesis: Pax-3 and Myf-5 act upstream of MyoD. *Cell*, 89(1), 127-138.
- 832 Tatsumi, R. (2010). Mechano-biology of skeletal muscle hypertrophy and regeneration: Possible
833 mechanism of stretch-induced activation of resident myogenic stem cells. *Animal Science*
834 *Journal*, 81(1), 11-20.
- 835 Tatsumi, R., Anderson, J. E., Nevoret, C. J., Halevy, O., & Allen, R. E. (1998). HGF/SF is present in
836 normal adult skeletal muscle and is capable of activating satellite cells. *Developmental Biology*,
837 194(1), 114-128.
- 838 Verma, M., Fitzpatrick, K. R., & McLoon, L. K. (2017). Extraocular muscle repair and regeneration.

- 839 *Current ophthalmology reports*, 5(3), 207-215.
- 840 Vest, K. E., Phillips, B. L., Banerjee, A., Apponi, L. H., Dammer, E. B., Xu, W., . . . Pavlath, G. K.
841 (2017). Novel mouse models of oculopharyngeal muscular dystrophy (OPMD) reveal early
842 onset mitochondrial defects and suggest loss of PABPN1 may contribute to pathology. *Human*
843 *Molecular Genetics*, 26(17), 3235-3252.
- 844 Victor, M., Hayes, R., & Adams, R. D. (1962). Oculopharyngeal muscular dystrophy: a familial disease
845 of late life characterized by dysphagia and progressive ptosis of the eyelids. *New England*
846 *Journal of Medicine*, 267(25), 1267-1272.
- 847 Vishwakarma, A., Rouwkema, J., Jones, P. A., & Karp, J. M. (2017). The Need to Study, Mimic, and
848 Target Stem Cell Niches *Biology and Engineering of Stem Cell Niches* (pp. 3-13): Academic
849 Press.
- 850 Wosczyzna, M. N., Konishi, C. T., Carbajal, E. E. P., Wang, T. T., Walsh, R. A., Gan, Q., . . . Rando, T.
851 A. (2019). Mesenchymal stromal cells are required for regeneration and homeostatic
852 maintenance of skeletal muscle. *Cell reports*, 27(7), 2029-2035.
- 853 Xuan, W., Qu, Q., Zheng, B., Xiong, S., & Fan, G. H. (2015). The chemotaxis of M1 and M2
854 macrophages is regulated by different chemokines. *Journal of Leukocyte Biology*, 97(1), 61-69.
- 855 Yin, H., Price, F., & Rudnicki, M. A. (2013). Satellite cells and the muscle stem cell niche. *Physiological*
856 *Reviews*, 93(1), 23-67.
- 857 Zhang, H., Menzies, K. J., & Auwerx, J. (2018). The role of mitochondria in stem cell fate and aging.
858 *Development*, 145(8), dev143420.