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1 **Brain-Derived Neurotrophic Factor is Associated with Human Muscle Satellite Cell**
2 **Differentiation in Response to Muscle Damaging Exercise**

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22 Running Title: BDNF is associated with early differentiation in human satellite cells

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42 **Abbreviations:**

43 BDNF, brain-derived neurotrophic factor; MRF, myogenic regulatory factor; mRNA, messenger
44 RNA; qPCR, quantitative real-time polymerase chain reaction; SC, muscle stem cell/satellite cell

45 **Abstract:**

46 Muscle satellite cell (SC) regulation is a complex process involving many key signaling
47 molecules. Recently, the neurotrophin - brain-derived neurotrophic factor (BDNF) has implicated
48 in SC regulation in animals. To date, little is known regarding the role of BDNF in human SC
49 function *in vivo*. Twenty-nine males (21±0.5y) participated in the study. Muscle biopsies from
50 the thigh were obtained prior to a bout of 300 maximal eccentric contractions (PRE), and 6h,
51 24h, 72h, and 96h of post-exercise. BDNF was not detected in any quiescent (MyoD⁻/Pax7⁺) SCs
52 across the time-course. BDNF co-localized to 39±5% of proliferating (MyoD⁺/Pax7⁺) cells at
53 PRE, which increased to 84±3% by 96h (P<0.05). BDNF was only detected in 13±5% of
54 differentiating (MyoD⁺/Pax7⁻) cells at PRE, which increased to 67±4% by 96h (P<0.05). The
55 number of Myogenin⁺ cells increased 95% from PRE (1.6±0.2 cells/100MF) at 24h (3.1±0.3
56 cells/100MF) and remained elevated until 96h (cells/100MF), p<0.05. The proportion of
57 BDNF⁺/Myogenin⁺ cells was 26±0.3% at PRE, peaking at 24h (49±3%, p<0.05) and remained
58 elevated at 96h, (p<0.05). These data are the first to demonstrate an association between SC
59 proliferation and differentiation and BDNF expression in humans *in vivo*, with BDNF co-
60 localization to SCs increasing during the later stages of proliferation and early differentiation.

61 **Novelty:**

- 62 • **BDNF is associated with SC response to muscle injury**
- 63 • **BDNF was not detected in non-activated (quiescent) SCs**

- 64 • **BDNF is associated with late proliferation and early differentiation of SCs *in vivo* in**
65 **humans**

66

67 Key Words: Pax7, satellite cells, brain-derived neurotrophic factor, muscle, damage, myogenic
68 regulatory factors

69 **Introduction:**

70 The myogenic response to a physiological stressor such as muscle damage involves the
71 coordinated response of several cell types including the tissue-resident stem cells, the muscle
72 satellite cells (SCs). In adult muscle, SCs represent on a small proportion of the total content of
73 myonuclei (~2–5%) yet they possess a remarkable potential for proliferation, capable of
74 expanding their relative proportion over 30-40% as early as 24h after injury to aid in muscle
75 repair and hypertrophy (Hawke and Garry 2001, Snijders et al. 2015). Activation, proliferation,
76 and subsequent differentiation of SCs are controlled by a conserved and unique set of
77 transcriptional networks aptly named: the myogenic regulatory factors (MRFs). The MRFs are
78 basic helix-loop-helix (bHLH) transcription factors that form heterodimeric DNA binding
79 complexes for genes that encode cell-cycle machinery, factors that direct terminal differentiation,
80 and contractile function (Hawke and Garry 2001, Charge and Rudnicki 2004). The MRFs are
81 composed of four main transcription factors: Myf5 and MyoD, which are important in
82 proliferation and early differentiation, and MRF4 and myogenin, which direct terminal
83 differentiation. The master regulator important in controlling the transcription of the MRFs is the
84 paired box transcription factor Pax7. Under the regulation of Pax7 and the MRFs, the process of
85 SCs progressing through the developmental pathway from a quiescent undifferentiated cell to a

86 myotube or fusing with a myofiber has been termed the myogenic program (Relaix and Zammit
87 2012, von et al. 2013, Brack 2014, Oustanina, Hause, and Braun 2004).

88 The myogenic program is influenced by a host of local growth factors and cytokines such
89 as androgens, interleukin-6 (IL-6), myostatin (negative regulation) and insulin growth factor-I
90 (IGF-1) (Snijders et al. 2015) as well as circulating factors and the influence of supporting
91 tissues such as blood vessels (capillaries) and neural tissue (motor neurons) (Joanisse et al. 2017,
92 Snijders et al. 2015, Hawke and Garry 2001). Although the precise mechanisms regulating SCs
93 in humans are not fully elucidated, a large body of literature has begun to classify the factors
94 influencing SC regulation. **Importantly, the microenvironment of the SC – the so called ‘SC**
95 **niche’ is an area of interest for several lines of research. The niche consists of the myofiber and**
96 **the basal lamina between the myofiber and the SC itself, as well as the supporting extracellular**
97 **matrix, capillaries, neuromuscular junctions, and surrounding myofibers. The niche integrates**
98 **the local signaling factors as well as the interactions between the SCs and the adjacent cells and**
99 **structural elements of its associated fiber as well as systemic influence from the circulation.** In
100 the last 10 years we have only really begun to understand the influence and importance of the
101 local environment on SC regulation *in vivo*. Data is emerging that demonstrates the importance
102 of the proximity of the SC to the microcirculation in the muscle to the overall regulation of SC
103 function (Snijders et al. 2017, Joanisse et al. 2016, Joanisse et al. 2017). One area of the SC
104 niche that remains underserved in the current literature is the influence of the motor neuron on
105 human SC regulation. There are significant interactions between skeletal muscle and motor
106 neurons and there are several key molecules involved in these interactions and the neurotrophins
107 are an emerging area of study.

108 Neurotrophins are a class of molecules implicated in neuronal plasticity and survival and
109 are important modulators of neuronal development (Hamburger and Yip 1984, Kablar and
110 Belliveau 2005). The neurotrophin: brain-derived neurotrophic factor (BDNF) has been the
111 basis of intense study and is of particular interest as it has been shown to be produced by skeletal
112 muscle and motor neurons in animals and humans (Pitts et al. 2006). Moreover, skeletal muscle
113 also expresses receptors for BDNF in addition to other neurotrophins (Pitts et al. 2006, Chevrel,
114 Hohlfeld, and Sendtner 2006). BDNF is important for neuromuscular health by enhancing motor
115 neuron survival (Zhang and Poo 2002, Lohof, Ip, and Poo 1993) and a key regulator for normal
116 embryonic development of the neuromuscular system (Mousavi, Parry, and Jasmin 2004,
117 Griesbeck et al. 1995). Data from animal models suggest that BDNF is upregulated in skeletal
118 muscle following muscle injury and the timing of the peak BDNF expression coincides with SC
119 proliferation suggesting a potential role for BDNF with SC regulation. In addition, BDNF has
120 been shown to be expressed in rat SCs (Mousavi and Jasmin 2006), and more recent data from
121 Clow and Jasmin (2010), demonstrates BDNF influences SC regulation and may be required for
122 the early phases of SC and/or myoblast differentiation in a mouse model of regeneration (Clow
123 and Jasmin 2010). In mice with a muscle specific knock out of BDNF, myoblasts failed to
124 transition into differentiation and cultures failed to induce key markers of differentiation such as
125 myogenin, embryonic myosin heavy chain and p21 compared to controls (Clow and Jasmin
126 2010). Although the exact mechanisms remain unknown, data from animal models demonstrate
127 a role for BDNF in the regulation of normal myogenic differentiation *in vivo* and *in vitro* (Clow
128 and Jasmin 2010, Miura et al. 2012, Mousavi and Jasmin 2006). More recently, BDNF and its
129 receptor p75NTR have been localized to human SCs *in vivo* and *in vitro* (Colombo et al. 2011,
130 Colombo et al. 2013). Data from Colombo and colleagues (2013) demonstrate that the *in vitro*

131 silencing of the BDNF gene in human myoblasts interfere with normal myogenesis (Colombo et
132 al. 2013). However, the mechanisms responsible for this impairment are not understood. To
133 date, there is a paucity of data to describe the role BDNF in human muscle SC regulation *in vivo*.
134 Whether BDNF is implicated in normal human muscle SC proliferation or differentiation
135 remains to be elucidated. We hypothesize that BDNF would localize to human muscle SC and
136 that peak elevation in muscle SC BDNF would be associated with MyoD expression and
137 correspond to the onset of differentiation in response to muscle damage *in vivo*.

138

139 **Materials and Methods:**

140 ***Subjects:***

141 Twenty nine healthy young male subjects aged 22 ± 1 y, 81 ± 2 kg were recruited for
142 participation in the study. Exclusion criteria included the use of supplements and performance
143 enhancing medications, smoking, diabetes, a history of respiratory disease and/or any major
144 orthopaedic disability and the use of non-steroidal anti-inflammatory drugs (NSAIDs) and/or
145 statins, and a history of respiratory disease and/or any major orthopaedic disability. Woman
146 were not recruited for the present study due to potential and unknown variations in neurotrophin
147 expression based on changes associated changes in hormone profile. However, future work in
148 this area is necessary to understand the influence of hormone changes on neurotrophins. All
149 subjects were informed of the procedures and potential risks associated with the study and gave
150 their written informed consent to participate. Subjects were told to refrain from consuming
151 alcohol, caffeine, anti-inflammatory medications, nutritional supplements and exercising
152 throughout the time-course of the study. This study was approved by the Hamilton Health

153 Sciences Research Ethics Board and conformed to all declarations on the use of human subjects
154 as research participants.

155 ***Muscle Damage Protocol:***

156 To ensure consistency of the stimulus in relation to previous studies from our lab, we employed a
157 protocol involving maximal isokinetic unilateral muscle lengthening contractions of the
158 *quadriceps femoris* performed on a Biodex dynamometer (Biodex-System 3, Biodex Medical
159 Systems, Inc., USA) at 180 degrees/s⁻¹. For each subject, the exercised leg was selected
160 randomly. Subjects underwent a brief familiarization trial involving 2 sets of 10 submaximal
161 lengthening contractions. The experimental protocol has been extensively validated and it is
162 well documented that this protocol induces a significant level of skeletal muscle damage and is
163 sufficient to elicit a significant SC response (Toth et al. 2011, McKay et al. 2010, Beaton,
164 Tarnopolsky, and Phillips 2002). The protocol is well described elsewhere (Beaton,
165 Tarnopolsky, and Phillips 2002), briefly, the participants were required to perform 30 sets of 10
166 maximal muscle lengthening contractions with one minute rest between sets, for a total of 300
167 lengthening contractions. Investigators provided verbal encouragement to elicit a maximal effort
168 from the participants. These data were recently part of a larger study examining the impact of
169 capillarization and muscle repair (Nederveen et al. 2018).

170 ***Muscle Biopsies:***

171 A total of five percutaneous needle biopsies were obtained from the mid-portion of the *vastus*
172 *lateralis* under local anesthetic (1% lidocaine) using manual suction (Tarnopolsky et al. 2011).
173 One muscle biopsy was obtained prior to exercise for baseline analysis (PRE). Other biopsies
174 were obtained 6h, 24h, 72h, and 96 h post-intervention with the position of the incision
175 randomized to reduce any effect of the previous biopsy on the outcome variables.

176 Approximately 50 mg of muscle tissue from each biopsy was mounted in Optimum Cutting
177 Temperature (OCT) compound and frozen in isopentane cooled in liquid nitrogen for histological
178 analysis. The remaining portion was prepared for mRNA analysis.

179 ***RNA isolation:***

180 RNA was isolated from 25mg of homogenized muscle samples in 1.0 mL of TRIzol reagent
181 using the TRIzol/RNeasy method (Invitrogen Corporation, Canada) and Lysing Maxtrix D tubes
182 (MP Biomedicals, Solon, OH, USA), with the FastPrep-24Tissue and Cell Homogenizer (MP
183 Biomedicals) for a duration of 40 s at a setting of 6 m/sec (5). The RNA (aqueous) phase was
184 purified using the EZNA Total RNA Kit 1 (OmegaBio-Tek, Norcross, GA, USA) as per the
185 manufacturer's instructions. RNA concentration (ng mL^{-1}) and purity (260/280) was
186 determined with the Nano-Drop 1000 Spectrophotometer (Thermo Fisher Scientific, Waltham,
187 MA, USA).

188 ***Reverse Transcription (RT):***

189 Individual samples were reverse transcribed in 20 μL reactions using a commercially available
190 kit (Applied Biosystems High Capacity cDNA Reverse Transcription Kit; Applied Biosystems,
191 USA) according to the manufacturer's instructions. The cDNA synthesis reaction was carried
192 out on an Eppendorf Mastercycle *eppgradient* thermal cycler (Eppendorf, Canada).

193 ***Quantitative Polymerase Chain-Reaction (qPCR):***

194 Individual 25 μL reactions were prepared in 0.2 mL Eppendorf twin.tec PCR plates (Eppendorf,
195 Canada) and run in duplicate for each time-point as described previously (Nederveen et al.
196 2018). Primers for *BDNF* were (forward: 5'-AGCCCTGTATCAACCCAGAA-3' and reverse:
197 5'-CAATGCCAACTCCACATAGC-3'; NCBI ID: 627) and for *Glyceraldehyde Phosphate*
198 *Dehydrogenase (GAPDH)* were 5'-CCTCCTGCACCACCAACTGCTT-3' and reverse 5'-

199 GAGGGGCCATCCACAGTCTTCT-3'; NCBI ID: 2597). qRT-PCR reactions were carried out
200 using a Eppendorf Mastercycler ep realplex² real-time PCR System (Eppendorf, Canada).
201 Relative mRNA expression was calculated using the delta Ct method ($2^{-\Delta Ct}$) (Raue et al. 2006)
202 and fold change from baseline was calculated using the delta-delta Ct method ($2^{-\Delta\Delta Ct}$) (Livak and
203 Schmittgen 2001). A panel of housekeeping genes were run across all time-points to determine
204 the most suitable housekeeping gene. Glutaraldehyde phosphate dehydrogenase (GAPDH)
205 mRNA was not different over time and thus chosen as the appropriate housekeeping gene. Gene
206 expression was normalized to the *GAPDH*. mRNA values were expressed as either total mRNA
207 expression (for between groups comparisons) and/or fold change from PRE for within group
208 comparisons (mean \pm SEM). *GAPDH* was not different from PRE at any of the time points.

209 ***Immunofluorescence:***

210 For Pax7 and MyoD the methods have been described in detail previously (McKay et al. 2012,
211 McKay et al. 2010). Briefly, 7 μ m muscle cross-sections were stained with antibodies against
212 Pax7 (neat; cell supernatant from cells obtained from the DSHB, USA); BDNF (1:100,
213 ab108319, Abcam, USA); MyoD1 (Anti-MyoD1, Clone 5.8A, Dako Canada Inc., Canada);
214 Laminin (1:1000, L8271, Sigma-Aldrich, Canada and Abcam ab11575, Abcam, USA);
215 Myogenin (1:10; F5D clone cell supernatant from cells obtained from the DSHB, USA) and
216 wheat germ agglutinin (WGA, 1:300, W11261) conjugated to AlexaFluor 647 (ThermoFisher
217 Scientific, USA). Secondary antibodies used were: Pax7 (AlexaFluor 594, 1:500, Invitrogen,
218 Molecular Probes Inc., USA); MyoD1, immunoglobulin biotinylated secondary antibody, 1:200,
219 Dako Canada, Inc.; followed by a streptavidin-FITC fluorochrome, 1:100, Biosource. USA);
220 BDNF (Dylight 488, 1:500, Thermo Scientific, Canada); Laminin (AlexaFluor 647, 1:500, or

221 AlexaFluor 488, 1:200, Invitrogen, Molecular Probes Inc., USA); Myogenin (AlexaFluor 647,
222 1:500, Invitrogen, Molecular Probes Inc., USA).

223 Immunofluorescence methods were adapted from previously published methods from our
224 lab (McKay et al. 2012, Joannis et al. 2015). Briefly, for co-immunofluorescent staining,
225 sections were fixed with 2% paraformaldehyde (PFA, Sigma-Aldrich, Canada) for 10 min
226 followed by several washes in PBS. Sections were then incubated for 60 min in an optimized
227 blocking solution containing, 2% bovine serum albumin (BSA; Sigma-Aldrich, Canada), 5%
228 fetal bovine serum (FBS; Gibco, USA), 0.2% Triton-X 100 (Sigma-Aldrich, Canada), 0.1%
229 sodium azide (Sigma-Aldrich, Canada), 5% goat serum (GS, Sigma-Aldrich, Canada).
230 Following blocking, sections were incubated in the primary antibody cocktail (i.e. F5D and
231 laminin diluted in 1% BSA) at 4°C overnight. After several washes, sections were then
232 incubated in the appropriate secondary antibodies. To prevent any migration of secondary
233 antibodies, sections were then re-fixed in 2% and re-blocked in 10% GS in 0.01% Triton-X 100.
234 The sections were then incubated in the second primary antibody cocktail, followed by
235 incubation in the appropriate secondary antibodies. Sections were then washed with PBS
236 followed by 4',6-diamidino-2-phenylindole (DAPI, 1:20000) (Sigma-Aldrich, Canada) for
237 nuclear staining. Staining was verified using the appropriate positive and negative controls to
238 ensure specificity of staining. Multiple experiments with multiple combinations of primaries
239 with all secondaries were used to ensure no cross-reactivity between antigens and no unintended
240 migration of secondaries. Positive controls for Pax7 and MyoD were conducted previously on
241 isolated murine myoblasts to ensure specificity. Multiple secondary only controls were used for
242 stains where multiple antigens were probed on the same slide. Stained slides were viewed with
243 the Nikon Eclipse *Ti* Microscope (Nikon Instruments, Inc., USA) and images were captured and

244 analyzed using the Nikon NIS Elements AR 3.2 software (Nikon Instruments, Inc., USA). For
245 all analyses, the investigator was blinded to both the group and the time-points. 250 ± 15 muscle
246 fibers were analyzed per section for each time point for each subject for all analyses. Although
247 previous work from (Mackey et al. 2009) demonstrated approximately 125 muscle fibers per
248 sample was required to get an accurate SC quantification, we used 250 muscle fibers as a
249 minimum because the sub-populations of SCs based on MyoD are more rare, we felt it necessary
250 to obtain a more accurate count of these cells, especially Pax7/MyoD⁺ -so-called differentiating
251 cells. Based on previous work with MyoD and other rare sub-populations (McKay et al. 2012,
252 Joannisse et al. 2015), we feel quantification of 250 muscle fibers is sufficient for accurate
253 analysis. Immunofluorescent techniques for myogenin (F5D) were adapted from our previous
254 published methods (Joannisse et al. 2015) and optimized to allow for co-immunostaining with
255 BDNF.

256 ***Blood Measures:***

257 Blood samples from the antecubital vein were taken immediately prior to the intervention and at
258 6h, 24h, 72h, and 96h post. Samples were collected in EDTA-containing tubes and centrifuged
259 at 1500 rpm for 10 min at 4°C. Samples were separated into 50 μ L aliquots and stored at -80 °C
260 for analysis at a later date. Plasma samples were thawed on ice. Plasma BDNF was analyzed
261 using a commercially available high sensitivity Quantikine Enzyme-Linked ImmunoSorbent
262 Assay kit according to the manufacturer's instructions (ab99978, Abcam, USA).

263 ***Statistical Analysis:***

264 Statistical analysis was performed using SigmaStat 3.1.0 analysis software (Systat Software).
265 One-way repeated measures ANOVA was performed with time (Pre, 6, 24, 72 and 96 h) as the
266 main effect and Tukey HSD corrections were applied to account for multiple comparisons. In the

267 case of comparisons of MyoD and Myogenin (figure 4e,f) main effects for group (i.e. MyoD vs.
268 Myogenin) were assessed with paired t-tests. The effect of time was already assessed (as above)
269 and only the difference of group at each time point was tested. All results are presented as
270 means \pm SEM. Statistical significance was accepted at $P < 0.05$.

271

272 **Results:**

273 *The mixed muscle SC response to muscle damaging exercise:*

274 In the present study, we quantified ‘early differentiating’ SCs, or cells that were Pax7⁻/MyoD⁺.
275 The ‘quiescent’ (Pax7⁺/MyoD⁻) cells and the ‘active’ (Pax7⁺/MyoD⁺) cells were quantified as part
276 of a larger study examining the impact of capillarization and muscle repair (Nederveen et al.
277 2018). At PRE, ‘quiescent’ cells represented 76% of the total SC pool, and ‘active’ cells
278 represented 21% and ‘early differentiating’ cells represented 4% (Fig. 1a). In the present study,
279 we found that the proportion of cells negative for Pax7 and positive for MyoD was rare at PRE
280 (0.3 \pm 0.1 Pax7⁻/MyoD⁺ cells/100MF) and increased from 4% of the total SC pool at PRE to 18%
281 at 72h, peaking at 25% of the total SC pool at 96h, which represented an increase of 1137% in
282 these cells from PRE (from PRE: 0.3 \pm 0.1 to 3.6 \pm 0.3 Pax7⁻/MyoD⁺ cells/100MF at 96h, $p < 0.05$,
283 Fig. 1b). This cell population was very rare at baseline, representing only 1.1 \pm 0.2 cells in our
284 250 \pm 15 myofiber muscle sections collected at the baseline time-point. Although these cells are
285 rare the quantification was consistent across the baseline time point and is similar to values
286 previously reported (McKay et al. 2012, Joannisse et al. 2015). Taken together, we found that our
287 model of eccentric-contraction induced muscle damage was sufficient in stimulating an increase
288 in ‘early differentiating’ Pax7⁻/MyoD⁺ cells (Fig. 1a), similarly to quiescent and active SCs, as
289 previously published (27). Centrally located myonuclei (CLMN) were rare at baseline

290 (0.29±0.08 CLMN per 100MF). There was a significant increase in CLMN as early as 6h post-
291 intervention (158% increase, 0.75±0.19 CLMN/100MF, p<0.05). The number for CLMN
292 remained elevated throughout the post-intervention time-course peaking at 96h (214% increase
293 from PRE, 0.91±0.18 CLMN/100MF, p<0.05).

294

295 *Muscle SC activity and BDNF colocalization response to muscle damage:*

296 There is a large body of data to support the observation that BDNF co-localizes with satellite
297 cells in animals; however, a paucity of data exists in humans (Mousavi and Jasmin 2006). To
298 test the hypothesis that BDNF is involved in SC regulation we first attempted to localize BDNF
299 to human muscle SCs in muscle cross sections. We show here that BDNF co-localizes with Pax7
300 (Figure 2). To investigate the hypothesized association of BDNF with SC proliferation and
301 differentiation we co-stained muscle cross-sections with Pax7, MyoD and BDNF. At baseline
302 (PRE), despite the majority of SCs being Pax7⁺/MyoD⁻ (76% ‘quiescent’), none of these cells co-
303 stained positive for BDNF. Throughout the post-intervention time-course we were unable to
304 detect any Pax7⁺/MyoD⁻ cells that stained positive for BDNF. Figure 3b illustrates the time
305 course of the co-localization of BDNF with ‘activated’ SCs (Pax7⁺/MyoD⁺/BDNF⁺ cells) as a
306 proportion of the total Pax7⁺/MyoD⁺ cells. At baseline 39±6% of Pax7⁺/MyoD⁺ cells co-
307 localized with BDNF (0.6±0.1 BDNF⁺/ Pax7⁺/MyoD⁺ cells/100MF). This proportion increased
308 significantly from PRE at 24h (71±3%, 4.1±0.2 BDNF⁺/ Pax7⁺/MyoD⁺ cells/100MF; P<0.05),
309 72h (76±2 %, 5.6±0.1 BDNF⁺/ Pax7⁺/MyoD⁺ cells/100MF; P<0.05) and 96h (84±2 %, 5.5±0.2
310 BDNF⁺/ Pax7⁺/MyoD⁺ cells/100MF; P<0.05) (fig. 3b). Interestingly, the co-localization with
311 Pax7⁻/MyoD⁺ cells (early differentiation) increased 238% from PRE at 24h (13±5 % PRE to
312 44±7 % 24h; 0.04±0.01 BDNF⁺/ Pax7⁻/MyoD⁺ cells/100MF at PRE to 0.50±0.07 BDNF⁺/

313 Pax7⁺/MyoD⁺ cells/100MF at 24h; P<0.05) and continued to increase at 72h (66±3 %;
314 1.81±0.09 BDNF⁺/ Pax7⁺/MyoD⁺ cells/100MF; P<0.05) and 96h (68±4 %; 2.30±0.10 BDNF⁺/
315 Pax7⁺/MyoD⁺ cells/100MF; P<0.05) (fig. 3c).

316

317 *Myogenin and BDNF colocalization in response to muscle damage:*

318 In order to confirm the association of BDNF with SC differentiation we analyzed the co-
319 localization of BDNF with the myogenic regulatory factor myogenin. The number of Myogenin⁺
320 cells increased 95% from PRE at 24h (PRE: 1.6±0.2 myogenin⁺ cells/100MF, 24h: 3.3±0.3
321 myogenin⁺ cells/100MF, P<0.001 vs. PRE; P=0.002 24h vs. 6h, Fig. 4c) and remained elevated
322 until 96h, p<0.05. The proportion of BDNF⁺/Myogenin⁺ cells was 27±3% of total myogenin⁺
323 cells at PRE, increasing 76% at 24h (49±3% of total myogenin⁺ cells, p<0.001, Fig. 4d) and
324 remained elevated at 96h (44±3% of total myogenin⁺ cells, p<0.002, Fig. 4d).

325 Figure 4e and 4f demonstrate the temporal relation between the total number of myogenin⁺ cells
326 per 100MF and the total number of MyoD⁺/Pax7⁻ cells per 100MF (Fig 4e). There are
327 significantly more myogenin⁺ cells per 100MF at 24h compared to MyoD⁺/Pax7⁻ cells (3.3±0.3
328 myogenin⁺ cells/100MF vs. 1.1±0.1 MyoD⁺/Pax7⁻ cells/100MF, p<0.05) compared to
329 MyoD⁺/Pax7⁻ cells. The number of BDNF⁺/Myogenin⁺ cells were not different from the number
330 of BDNF⁺/MyoD⁺/Pax7⁻ cells until 72h (Fig 4f: 47.3±3.5% BDNF⁺/Myogenin⁺ cells vs.
331 67.1±3.7% BDNF⁺/MyoD⁺/Pax7⁻ cells at 72h, p<0.05).

332 *Whole-muscle and systemic response of BDNF to muscle damage:*

333 We observe a ~2.4-fold increase in BDNF mRNA expression as early as 6h post intervention
334 (P<0.05, Fig 3a) that remained elevated throughout the post-intervention time-course (P<0.05,
335 Fig 5a). Similarly, BDNF protein in the plasma was detected at baseline and increased 26% at

336 24h post intervention (Pre: 19.3 ± 1.9 pg/mL to 26.3 ± 3.0 pg/mL at 24h, $p < 0.05$, Fig 5b). Plasma
337 BDNF returned to PRE levels by 72h (Fig. 5b).

338

339 **Discussion:**

340 Muscle SCs regulation is a complicated interplay between the systemic environment, the local
341 microenvironment (skeletal muscle derived factors, etc.) and the direct interaction of the SC
342 niche (skeletal muscle, capillaries, motor neurons and interstitium) (Snijders et al. 2015, Joannis
343 et al. 2017, Chakkalakal et al. 2012). The neurotrophin BDNF has been identified as a potential
344 regulator of SC function in animal models (Clow and Jasmin 2010). It appears that BDNF acts
345 via the receptor p75NTR, found on the cell surface of humans SCs (Colombo et al. 2011), to
346 influence SC proliferation and differentiation (Colombo et al. 2011, Colombo et al. 2013).

347 Although the precise cellular signaling pathways responsible for this action remain to be
348 elucidated, previous studies have demonstrated a potential role for BDNF in the regulation of
349 myogenic stem cell proliferation and differentiation, this has never been described *in vivo* in
350 humans. In order to investigate whether BDNF is associated with the SC response in healthy
351 humans we first set out to induce an expansion of the SC pool using an exercise-induced muscle
352 damaging protocol that has been previously shown to cause a robust and reproducible SC
353 response (McKay et al. 2010, Toth et al. 2011, Beaton, Tarnopolsky, and Phillips 2002). In the
354 present study, a significant increase in CLMN was noted from baseline. Although the number of
355 CLMN remained significantly elevated throughout the 96h post-intervention time-course, it is
356 difficult to know if the CLMN represent injured myofibers per se or rather are nuclei undergoing
357 karyolysis or normal myonuclear turnover. In the present study we did not note enough CLMN
358 to conduct co-immunostaining to attempt to identify lineage based on MRF expression, and these

359 cells did not appear to stain with BDNF; however the numbers were very low and thus we were
360 not able to interpret any possible trends. Here we demonstrate a significant increase in Pax7⁻
361 /MyoD⁺ cells per 100MF in mixed whole muscle that peaked 72h post myotrauma, which
362 parallels the fiber-type specific SC response reported on the same subjects in a previous analysis,
363 examining Pax7⁺/MyoD⁻ and Pax7⁺/MyoD⁺ cells (Nederveen et al. 2018). This is consistent
364 with multiple studies from our lab and others using similar muscle damaging protocols (Snijders
365 et al. 2015, Nederveen et al. 2018). It should be noted that this protocol evokes significant
366 muscle injury compared to a normal workout and thus making generalizations to the response to
367 resistance exercise may be difficult. However, it is important to investigate a robust stimulus in
368 order to stimulate enough of a response to be quantifiable based on current techniques, as such
369 the use of 300 eccentric contractions is an effective and standardized model for our human trial.

370 To further characterize the SC response to muscle injury, we identified SCs based on
371 MyoD and Pax7 expression. It is generally accepted that SCs in the quiescent state express some
372 level of Pax7 in post-natal muscle tissue and upon activation, upregulate Myf5 and MyoD
373 (Zammit, Partridge, and Yablonka-Reuveni 2006, Lepper, Partridge, and Fan 2011).
374 Furthermore, as these cells begin to differentiate they downregulate Pax7 and a sub-set of cells
375 down regulate MyoD and remain Pax7 positive, returning to a quiescent state to repopulate the
376 SC pool (Zammit et al. 2004, Zammit, Partridge, and Yablonka-Reuveni 2006). Based on this
377 principle we investigated the sub-populations of SCs that were Pax7⁺/MyoD⁻ (so called
378 ‘quiescent’ SCs), Pax7⁺/MyoD⁺ cells (‘active / proliferating’), and Pax7⁻/MyoD⁺ cells
379 (‘differentiating’ or preparing to differentiate). Similar to previous studies (McKay et al. 2012,
380 Joannisse et al. 2015, Nederveen et al. 2018), we found the majority of SCs were MyoD⁻ at
381 baseline with relatively few Pax7⁻/MyoD⁺ cells (<4% of the total SC pool). These ‘early

382 differentiating' SC (Pax7-/MyoD+) increased 1137% peaking at 96h. Importantly, the
383 differential response of these three sub-populations of SCs allow for the quantification of BDNF
384 in these cells at the different time points following muscle damage.

385 In order to help elucidate the relationship of BDNF with the SC response, we first set out
386 to confirm the localization of BDNF with Pax7⁺ SCs using immunofluorescent techniques (fig.
387 2). Here we identified both Pax7⁺/BDNF⁺ cells and Pax7⁺/BDNF⁻ cells in the same muscle
388 sections (fig. 2). Previous studies in murine muscle have demonstrated BDNF co-localized with
389 SCs *in vitro* and *in vivo* (Mousavi and Jasmin 2006, Clow and Jasmin 2010, Miura et al. 2012).
390 To date, only one other study has attempted to localize BDNF with human SCs (Colombo et al.
391 2013). In that study, SCs were analyzed in muscle cross-sections using immunohistochemical
392 detection and myoblasts were also isolated based on CD56/NCAM expression for *in vitro*
393 experiments (Colombo et al. 2013). They not only localized BDNF protein to the SCs using co-
394 immunohistochemical detection with BDNF and NCAM, but they also used *in situ* hybridization
395 to localize BDNF to SCs in cross-section (Colombo et al. 2013) further lending strength to the
396 potential role of BDNF in SC regulation. In that study, in the basal state, >80% of SCs from
397 patients with inflammatory myopathies were positive for BDNF (BDNF⁺/NCAM⁺). This is in
398 contrast to data from the present study, where in young healthy controls approximately 75% of
399 SCs were negative for MyoD (i.e. SC remain in quiescent state) and were also negative for
400 BDNF. In that study, muscle biopsies were obtained from 17 patients with a known diagnosis of
401 inflammatory myopathy (polymyositis, dermatomyositis, or inclusion body myositis) with a
402 mean age of 65y with 10 patients being male and 7 being female. Taken together, it is difficult to
403 make direct comparison between that study and our data. **It is important to note that BDNF**
404 **protein is also expressed by the muscle fiber and that BDNF expression increases with exercise**

405 (Matthews et al. 2009), but the exact functions of BDNF within muscle fibers is not clear. There
406 may be autocrine/paracrine cross-talk between the fiber and SCs, however this is yet to be
407 established in humans.

408 In the present study, the percentage of SCs co-staining for BDNF increased the most in
409 cells expressing MyoD and importantly, was not detected in cells expressing only Pax7
410 (Pax7⁺/MyoD⁻). Thus, data from the three sub-populations of SCs (based on Pax7 and MyoD
411 co-staining) across the 96h time course may indicate that BDNF expression is involved in the
412 later stages of the myogenic program. Our findings are strengthened by the work of Colombo
413 and colleagues (2013) who also demonstrated using siRNA against BDNF, that myoblast
414 differentiation was impaired when BDNF levels were either silenced or blocked with a BDNF
415 antibody in cell culture (Colombo et al. 2013). Although data from the present study and
416 Colombo et al. (2013) suggest there is an association with BDNF and SC differentiation, and that
417 differentiation is impaired when BDNF is absent (Colombo et al. 2013), data from animal studies
418 suggest that BDNF is associated with SC proliferation and high levels of BDNF impair
419 myogenic differentiation (Clow and Jasmin 2010, Miura et al. 2012). The difference in the exact
420 role of BDNF with regards to regulation of SC proliferation or differentiation between humans
421 and mice may be due to significant differences in study design and possibly due to species
422 differences in signaling. In addition, the lack studies published in this area is also a key factor
423 which must be considered in explaining discrepancies between published data.

424

425 To further determine the association of BDNF with SC differentiation, we investigated
426 the co-expression of BDNF with the myogenic differentiation factor myogenin. Fig. 4 illustrates
427 that an increase in myogenin⁺ cells is seen as early as 24h following myotrauma and that these

428 cells remain elevated at 96h. Furthermore, as the number of these cells increases, the proportion
429 co-expressing BDNF increased from ~27% at baseline to ~49% at 24h, and remained elevated at
430 96h. Therefore, these data suggest there is an association of BDNF with SC differentiation.

431 When we look at both the response of myogenin⁺ cells with BDNF and MyoD⁺/Pax7⁻ cells with
432 BDNF together (Fig. 4e, f) we see that the total proportion of these cell populations increase with
433 time and that BDNF⁺/MyoD⁺/Pax7⁻ cells are increasing at a greater rate and reach a higher
434 proportion of total Myogenin⁺ cells or total MyoD⁺/Pax7⁻ cells (Fig. 4f). This may represent a
435 critical importance of BDNF with late proliferation or early differentiation/transition to
436 differentiation. A limitation of this interpretation is that it is unclear whether myogenin⁺ cells
437 beneath the basal lamina represent SCs that have progressed towards myogenic differentiation or
438 are proliferating cells that are up-regulating myogenin in preparation for differentiation or
439 alternately, are cells that have fully differentiated and fused with the myofiber. Due to technical
440 constraints of using multiple mouse monoclonal antibodies we are currently unable to co-stain
441 myogenin with Pax7 or directly with MyoD. In addition, interpreting the precise mechanisms
442 involved in the in vitro human model is limited by the amount of muscle we can extract and thus
443 the relatively low populations of these cells. The fact we are able to consistently quantify
444 population expansion in the model does lend credence to the biological significance of the
445 interactions of signaling molecules examined here. In addition, multiple labelling of
446 histopathological sections is difficult, especially with rare cell types in whole tissue sections.
447 The use of flow cytometry or fluorescence activated cell sorting may further enable more precise
448 quantification of cell populations with respect to activation status and expression of other
449 proteins such as BDNF. Unfortunately, at the present time the isolation procedure for this

450 technique still requires a lot of tissue, which is a challenge for human studies where tissue is
451 sparse. At present our lab is working on optimizing this technique for future studies.

452 In the present study, circulating BDNF increased from basal levels approximately 26%
453 by 24h and returned to baseline by 72h following unilateral eccentric contractions. In skeletal
454 muscle, whole-muscle BDNF mRNA was increased as early as 6h following muscle damage and
455 remained elevated throughout the 96h post-intervention time-course. The differential time-
456 course of circulating BDNF compared to whole muscle transcripts suggests that it is unlikely that
457 muscle accounts solely for the transient increase in circulating BDNF. Furthermore, the
458 differential timeline of BDNF within the SC compartment suggests that BDNF may have
459 differential effects based on the temporal and spatial factors. Exercise is known to induce an
460 increase in circulating BDNF (Dinoff et al. 2017); however, it is unclear if the source of BDNF
461 is neural tissue, skeletal muscle, other peripheral tissues (such as circulating cells or other
462 organs) or if it is combination of some or all of these (Dinoff et al. 2017, Colombo et al. 2013).
463 Recent data from human skeletal muscle and human primary myoblasts demonstrated that the
464 skeletal muscle, the SCs and local inflammatory cells all produce BDNF in regenerating
465 myofibers, suggesting a key role for autocrine/paracrine and immune effects of BDNF in the
466 local SC microenvironment (i.e., the niche) on myogenesis (Colombo et al. 2013). However, the
467 exact mechanisms responsible for the local and systemic increase in BDNF remain uncertain.
468 Another limitation of this relation is the theoretical cross-reactivity of the BDNF antibody used
469 vs. the specificity of the primer sequences for RNA analysis. Many commercially available
470 BDNF antibodies may cross-react with other neurotrophins such as NGF4, neurotrophin 3 and 4.
471 Unfortunately, based on similarities in protein structures between these compounds we cannot
472 say for certain that 100% of the BDNF antibody is bound solely to BDNF

473 BDNF signaling between skeletal muscle and SCs appears to be an important and
474 understudied area of SC biology (Colombo et al. 2013, Clow and Jasmin 2010). Several basic
475 science studies in animals and humans have begun to classify a role for BDNF in SC
476 proliferation and differentiation (Clow and Jasmin 2010, Miura et al. 2012, Colombo et al.
477 2013). Importantly, BDNF has been shown here to be associated with SC proliferation and
478 differentiation in human muscle *in vivo*, following exercise-induced muscle damage, with BDNF
479 increasing in SCs during the later stages of proliferation and early differentiation. This study
480 provides key evidence that BDNF is associated with the robust SC response to physiological
481 muscle damage in healthy young adults.

482

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611

612 **Figure Legend:**

613 **Figure 1:** *The Satellite cell (SC) response to acute exercise-induced muscle damage.*
614 The SC-specific MyoD response – **(1a)** Graphic representation of the proportion of
615 Pax7⁺/MyoD⁻ ('quiescent' – **black bars**); Pax7⁺/MyoD⁺ ('active' – **white bars**); Pax7⁻/MyoD⁺
616 ('early differentiation' – **grey bars**) cells as a proportion of the total SC population (per 100
617 myofibers) over the time-course. **(1b)** Line graph of this response demonstrating the individual
618 changes in Pax7⁻/MyoD⁺ cells per 100 myofibers ('early differentiation') *p<0.05 vs. Pre and 6h.
619 †p<0.05 vs. Pre, 6h, and 24h. Note: All three groups are significantly different from each other at
620 each time point (group main effect p<0.05).

621

622 **Figure 2:** *Immunohistochemical detection of BDNF within the SC pool.*

623 **(2a)** A representative muscle cross-section demonstrating a BDNF negative SC. The arrow
624 denotes a BDNF negative (green), Pax7 positive (red), cell (nuclei, DAPI) beneath the basal
625 lamina (Laminin, purple).

626 **(2b)** A representative muscle cross section demonstrating a BDNF positive SC. The arrow
627 denotes a BDNF positive (green), Pax7 positive (red), cell (nuclei, DAPI) beneath the basal
628 lamina (Laminin, purple).

629

630 **Figure 3:** *The response of BDNF with the subpopulations of SCs based on MyoD and Pax7 co-* 631 *expression to exercise-induced muscle damage.*

632 **(3a)** A representative muscle cross-section stained with Pax7 (green), MyoD (Red), BDNF
633 (purple) and DAPI (blue). The upper left box is expanded below and illustrates a Pax7⁺/MyoD⁺
634 cells that is positive for BDNF. The box in the lower right corner is expanded to the right and
635 illustrates a Pax7⁺/MyoD⁻ cell that is BDNF negative.

636 (3b) A graphic representation of the quantification of the proportion of Pax7⁺/MyoD⁺ cells that
637 are also positive for BDNF (expressed as a percentage of total Pax7⁺ /MyoD⁺ cells). *p<0.05
638 vs. Pre. †p<0.05 vs. 6h.

639 (3c) A graphic representation of the quantification of the proportion of Pax7⁻MyoD⁺ cells that are
640 also positive for BDNF (expressed as a percentage of total Pax7⁻ /MyoD⁺ cells). *p<0.05 vs.
641 Pre. †p<0.05 vs. 6h.

642

643 **Figure 4:** *The response of myogenin and the co-expression of myogenin and BDNF to exercise-*
644 *induced muscle damage.*

645 (4a) A representative muscle cross-section stained with BDNF (green), myogenin (Red), laminin
646 (orange) and DAPI (blue). The arrow denotes a myogenin positive cell under the basal lamina
647 negative for BDNF.

648 (4b) A representative muscle cross-section stained with BDNF (green), myogenin (Red), laminin
649 (orange) and DAPI (blue). The arrow denotes a myogenin positive cell under the basal lamina
650 that is positive for BDNF.

651 (4c) A graphic representation of the quantification of the response of myogenin positive cells per
652 100 myofibers. *p<0.05 vs. Pre. †p<0.05 vs. 6h.

653 (4d) A graphic representation of the quantification of the proportion of myogenin positive cells
654 that are also positive for BDNF (expressed as a percentage of total myogenin positive cells).

655 *p<0.05 vs. Pre.

656 (4e) A graphic representation of relationship between the total Myogenin positive cell population
657 with the MyoD⁺/Pax7⁻ cell population. Both populations increase in the later part of the recovery
658 period following exercise-induced muscle damage. *p<0.05 vs. Pax7⁻ /MyoD⁺ (main effect of
659 group per time point). Note: effect of time for each group is reported in the previous graphs.

660 (4f) A graphic representation of the Myogenin positive cell population that is BDNF⁺ with the
661 Pax7⁻/MyoD⁺ cell population that is BDNF⁺. *p<0.05 vs. Myogenin (main effect of group per
662 time point). Note: effect of time for each group is reported in the previous graphs.

663

664

665 **Figure 5:** *The whole-muscle and systemic BDNF response to exercise-induced muscle damage.*

666 (5a) *BDNF* mRNA from whole-muscle expressed as fold change from Pre. *p<0.05 from Pre

667 (5b) Plasma BDNF concentration. *p<0.05 from Pre

668

669