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

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

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Abbreviations:

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

Abstract:

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

Novelty:

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

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

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

Introduction:

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

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

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

Neurotrophins are a class of molecules implicated in neuronal plasticity and survival and are important modulators of neuronal development (Hamburger and Yip 1984, Kablar and Belliveau 2005). The neurotrophin: brain-derived neurotrophic factor (BDNF) has been the basis of intense study and is of particular interest as it has been shown to be produced by skeletal muscle and motor neurons in animals and humans (Pitts et al. 2006). Moreover, skeletal muscle also expresses receptors for BDNF in addition to other neurotrophins (Pitts et al. 2006, Chevrel, Hohlfeld, and Sendtner 2006). BDNF is important for neuromuscular health by enhancing motor neuron survival (Zhang and Poo 2002, Lohof, Ip, and Poo 1993) and a key regulator for normal embryonic development of the neuromuscular system (Mousavi, Parry, and Jasmin 2004, Griesbeck et al. 1995). Data from animal models suggest that BDNF is upregulated in skeletal muscle following muscle injury and the timing of the peak BDNF expression coincides with SC proliferation suggesting a potential role for BDNF with SC regulation. In addition, BDNF has been shown to be expressed in rat SCs (Mousavi and Jasmin 2006), and more recent data from Clow and Jasmin (2010), demonstrates BDNF influences SC regulation and may be required for the early phases of SC and/or myoblast differentiation in a mouse model of regeneration (Clow and Jasmin 2010). In mice with a muscle specific knock out of BDNF, myoblasts failed to transition into differentiation and cultures failed to induce key markers of differentiation such as myogenin, embryonic myosin heavy chain and p21 compared to controls (Clow and Jasmin 2010). Although the exact mechanisms remain unknown, data from animal models demonstrate a role for BDNF in the regulation of normal myogenic differentiation *in vivo* and *in vitro* (Clow and Jasmin 2010, Miura et al. 2012, Mousavi and Jasmin 2006). More recently, BDNF and its receptor p75NTR have been localized to human SCs in vivo and in vitro (Colombo et al. 2011, 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 ± 1y, 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

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

Muscle Damage Protocol:

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

Muscle Biopsies:

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

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

RNA isolation:

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

Reverse Transcription (RT):

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

Quantitative Polymerase Chain-Reaction (qPCR):

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

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

Immunofluorescence:

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

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

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

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

Blood Measures:

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

Statistical Analysis:

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

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

Results:

The mixed muscle SC response to muscle damaging exercise:

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

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

Muscle SC activity and BDNF colocalization response to muscle damage:

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

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 BDNF returned to PRE levels by 72h (Fig. 5b).

Discussion:

Muscle SCs regulation is a complicated interplay between the systemic environment, the local microenvironment (skeletal muscle derived factors, etc.) and the direct interaction of the SC niche (skeletal muscle, capillaries, motor neurons and interstitium) (Snijders et al. 2015, Joanisse et al. 2017, Chakkalakal et al. 2012). The neurotrophin BDNF has been identified as a potential regulator of SC function in animal models (Clow and Jasmin 2010). It appears that BDNF acts via the receptor p75NTR, found on the cell surface of humans SCs (Colombo et al. 2011), to influence SC proliferation and differentiation (Colombo et al. 2011, Colombo et al. 2013). Although the precise cellular signaling pathways responsible for this action remain to be elucidated, previous studies have demonstrated a potential role for BDNF in the regulation of myogenic stem cell proliferation and differentiation, this has never been described *in vivo* in humans. In order to investigate whether BDNF is associated with the SC response in healthy humans we first set out to induce an expansion of the SC pool using an exercise-induced muscle damaging protocol that has been previously shown to cause a robust and reproducible SC response (McKay et al. 2010, Toth et al. 2011, Beaton, Tarnopolsky, and Phillips 2002). In the present study, a significant increase in CLMN was noted from baseline. Although the number of CLMN remained significantly elevated throughout the 96h post-intervention time-course, it is difficult to know if the CLMN represent injured myofibers per se or rather are nuclei undergoing karyolysis or normal myonuclear turnover. In the present study we did not note enough CLMN to conduct co-immunostaining to attempt to identify lineage based on MRF expression, and these

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

To further characterize the SC response to muscle injury, we identified SCs based on MyoD and Pax7 expression. It is generally accepted that SCs in the quiescent state express some level of Pax7 in post-natal muscle tissue and upon activation, upregulate Myf5 and MyoD (Zammit, Partridge, and Yablonka-Reuveni 2006, Lepper, Partridge, and Fan 2011). Furthermore, as these cells begin to differentiate they downregulate Pax7 and a sub-set of cells down regulate MyoD and remain Pax7 positive, returning to a quiescent state to repopulate the SC pool (Zammit et al. 2004, Zammit, Partridge, and Yablonka-Reuveni 2006). Based on this principle we investigated the sub-populations of SCs that were $Pax7^{+}$/$MyoD^{-}$ (so called 'quiescent' SCs), $Pax7^{+}$/$MyoD^{+}$ cells ('active / proliferating'), and $Pax7^{-}$/$MyoD^{+}$ cells ('differentiating' or preparing to differentiate). Similar to previous studies (McKay et al. 2012, Joanisse et al. 2015, Nederveen et al. 2018), we found the majority of SCs were $MyoD^{-}$ at 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

cells remain elevated at 96h. Furthermore, as the number of these cells increases, the proportion co-expressing BDNF increased from ~27% at baseline to ~49% at 24h, and remained elevated at 96h. Therefore, these data suggest there is an association of BDNF with SC differentiation. When we look at both the response of myogenin^{+} cells with BDNF and MyoD^{+}/Pax7^{-} cells with BDNF together (Fig. 4e, f) we see that the total proportion of these cell populations increase with time and that BDNF^{+}/MyoD^{+}/Pax7^{-} cells are increasing at a greater rate and reach a higher proportion of total Myogenin^{+} cells or total MyoD+/Pax7^{-} cells (Fig. 4f). This may represent a critical importance of BDNF with late proliferation or early differentiation/transition to differentiation. A limitation of this interpretation is that it is unclear whether myogenin^{+} cells beneath the basal lamina represent SCs that have progressed towards myogenic differentiation or are proliferating cells that are up-regulating myogenin in preparation for differentiation or alternately, are cells that have fully differentiated and fused with the myofiber. Due to technical constraints of using multiple mouse monoclonal antibodies we are currently unable to co-stain myogenin with Pax7 or directly with MyoD. In addition, interpreting the precise mechanisms involved in the in vitro human model is limited by the amount of muscle we can extract and thus the relatively low populations of these cells. The fact we are able to consistently quantify population expansion in the model does lend credence to the biological significance of the interactions of signaling molecules examined here. In addition, multiple labelling of histopathological sections is difficult, especially with rare cell types in whole tissue sections. The use of flow cytometry or fluorescence activated cell sorting may further enable more presice quantification of cell populations with respect to activation status and expression of other proteins such as BDNF. Unfortunately, at the present time the isolation procedure for this

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

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

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

Acknowledgements:

We would like to acknowledge Dr. Jonathan P Little (University of British Columbia, Kelowna, BC, Canada) for his assistance with the BDNF mRNA primers. The Pax7 hybridoma cells developed by Dr. A. Kawakami, and F5D cells developed by Dr. W. E. Wright were obtained from the Developmental Studies Hybridoma Bank, created by the National Institute of Child Health and Human Development and maintained at Department of Biology of The University of Iowa (Iowa City, IA USA).

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Figure Legend:

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

Figure 2: *Immunohistochemical detection of BDNF within the SC pool.*
(**2a**) A representative muscle cross-section demonstrating a BDNF negative SC. The arrow denotes a BDNF negative (green), Pax7 positive (red), cell (nuclei, DAPI) beneath the basal lamina (Laminin, purple).
(**2b**) A representative muscle cross section demonstrating a BDNF positive SC. The arrow denotes a BDNF positive (green), Pax7 positive (red), cell (nuclei, DAPI) beneath the basal lamina (Laminin, purple).

Figure 3: *The response of BDNF with the subpopulations of SCs based on MyoD and Pax7 co-expression to exercise-induced muscle damage*.
(3a) A representative muscle cross-section stained with Pax7 (green), MyoD (Red), BDNF (purple) and DAPI (blue). The upper left box is expanded below and illustrates a $Pax7^{-}/MyoD^{+}$ cells that is positive for BDNF. The box in the lower right corner is expanded to the right and illustrates a $Pax7^{+}/MyoD^{-}$ cell that is BDNF negative.

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

Figure 4: *The response of myogenin and the co-expression of myogenin and BDNF to exercise-induced muscle damage.*
(**4a**) A representative muscle cross-section stained with BDNF (green), myogenin (Red), laminin (orange) and DAPI (blue). The arrow denotes a myogenin positive cell under the basal lamina negative for BDNF.
(**4b**) A representative muscle cross-section stained with BDNF (green), myogenin (Red), laminin (orange) and DAPI (blue). The arrow denotes a myogenin positive cell under the basal lamina that is positive for BDNF.
(**4c**) A graphic representation of the quantification of the response of myogenin positive cells per 100 myofibers. *p<0.05 vs. Pre. †p<0.05 vs. 6h.
(**4d**) A graphic representation of the quantification of the proportion of myogenin positive cells that are also positive for BDNF (expressed as a percentage of total myogenin positive cells). *p<0.05 vs. Pre.
(**4e**) A graphic representation of relationship between the total Myogenin positive cell population with the $MyoD^{+}/Pax7^{-}$ cell population. Both populations increase in the later part of the recovery period following exercise-induced muscle damage. *p<0.05 vs. $Pax7^{-}$ /$MyoD^{+}$ (main effect of group per time point). Note: effect of time for each group is reported in the previous graphs.
(**4f**) A graphic representation of the Myogenin positive cell population that is $BDNF^{+}$ with the $Pax7^{-}/MyoD^{+}$ cell population that is $BDNF^{+}$. *p<0.05 vs. Myogenin (main effect of group per time point). Note: effect of time for each group is reported in the previous graphs.

Figure 5: *The whole-muscle and systemic BNDF response to exercise-induced muscle damage.*
(**5a**) *BDNF* mRNA from whole-muscle expressed as fold change from Pre. *p<0.05 from Pre
(**5b**) Plasma BDNF concentration. *p<0.05 from Pre