Regulation of satellite cells by extrinsic factors during recovery from exercise in horses

Amanda M. Brandt

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Sally E. Johnson, Committee Chair
Samer W. El-Kadi
Madlyn Frisard
Robert P. Rhoads

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SCIENTIFIC ABSTRACT

The vast majority of horses engage in some form of exercise, whether it be for leisure or competition. Despite almost half of the approximately 7.2 million horses engaging in structured athletic work, very little is known about one of the most critical facets of recovery: satellite cells (SCs). Satellite cells lie adjacent to the myofiber of skeletal muscle, poised to enter the myogenic program and fuse to the nearby muscle after a damaging event. *Hepatocyte growth factor* (HGF) and *insulin-like growth facto*r-1 (IGF-1) transcript abundance increased after an exhaustive bout of endurance exercise in concert with myogenic regulator factors and preceding increased SC abundance in a previous study. This suggests that SCs may participate in repair of exercise-induced muscle damage. To assess the role of HGF in this process, equine SCs (eqSCs) were isolated from the gluteus medius of mature thoroughbred geldings for activation, proliferation and differentiation assays. Activation was not accelerated by 1, 5, 10, or 25 ng/mL HGF. Instead, 25 ng/mL HGF increased the proliferation rate of eqSC via protein kinase C δ and decreased differentiation. The influence of dietary L-citrulline, an amino acid that has the potential to influence SC activity and nutrient availability by its metabolism to L-arginine, was assessed during recovery from exercise in unfit adult horses. To model submaximal exercise, horses were exercised for 1 h at an average heart rate of 116 bpm, suggested to be typical of a heavy exercise session by the National Research Council. L-citrulline decreased *myogenin* mRNA abundance compared to controls while exercise increased *peroxisome proliferator-activated receptor gamma*

coactivator 1- α (*PGC1α*) mRNA abundance, a master regulator of energy metabolism, at 1 d post-exercise. Although SCs were not activated in response to a single bout of submaximal exercise, metabolic regulators increased in the early period of recovery. Through these studies eqSC dynamics during exercise are better defined.

GENERAL AUDIENCE ABSTRACT

The horse is well-known as an athletic creature and is often used in amateur and professional athletic events. Despite its popularity as a pastime in low and high-stakes competition, certain facets directly related to performance during exercise remain relatively unstudied. One crucial component of recovery from exercise is the intrinsic ability of skeletal muscle to repair exercise-induced muscle damage. This is accomplished largely through the incorporation of new nuclei, which originate from a position orbiting the muscle, hence the name satellite cells. This cell is essential to muscle regeneration from injury as often demonstrated in rodent models, but the role of satellite cells in recovery from exercise remains elusive in all species, but particularly so in horses. For instance, whether satellite cells only contribute nuclei after exercise to stimulate gains in muscle mass or whether they may also play a role in the process of adaptation to exercise is not clearly understood. The purpose of my work was to define the response of satellite cells to hepatocyte growth factor, a factor present in skeletal muscle during exercise that is already well-studied in rodent models. Additionally, to determine whether the addition of the non-essential amino acid, citrulline, would influence satellite cells and nutrient reserves after a session of submaximal exercise. I found that hepatocyte growth factor does not influence satellite cells isolated from horses in the same way it influences those from rodents, nor through the same mechanisms. Additionally, I found that satellite cells were not stimulated after a session of submaximal exercise, but a factor involved in regulation of genetic expression that is associated with satellite cells and skeletal muscle was downregulated with the addition of citrulline. Together, these results suggest that satellite cells may behave like other species in some

ways, such as some responses to hepatocyte growth factor and the lack of response to a submaximal bout of exercise, but that there is still much to be learned in order to begin to influence management and training decisions as regards skeletal muscle recovery.

DEDICATION

For all horses, hunter and cow ponies alike.

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Education is a long journey that often begins before we arrive to and hopefully continues after we leave these hallowed halls. Many people were beside me throughout, but none quite so long as my mother. Every evening she read to my brother and I, transporting us to destinations beyond our wildest imaginings. During these nightly pilgrimages to distant lands we tested our periphery, carefully prodding and nudging the edges further and further, expanding little by little. This gift emboldened me to test the limits of this physical world by pursuing a dream I hadn't believed was meant for me. Imagination fueled this dream; and a vision of growth and depth into new, unexplored spaces propelled me to the last great frontier, science.

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CHAPTER 1

Literature Review

Introduction

Skeletal muscle comprises 40-50% of body weight and is essential to functions such as locomotion and metabolism (Abramowitz et al., 2018; Kearns et al., 2002; Zurlo et al., 1990). Displaying a remarkable plasticity, skeletal muscle can regenerate from injury, respond to mechanical load, and alter intrinsic metabolic properties to meet energetic demands (Egner et al., 2016; Lepper et al., 2011; Li et al., 2006). Exercise training capitalizes on this flexibility, increasing strength and metabolism that may be beneficial for health or athletic events (Gollnick et al., 1972; Shefer et al., 2010). Though the resultant phenotype is well-characterized, the mechanisms governing adaptation to exercise continue to be elucidated, particularly in the equid.

Satellite cells

Discovery, origin, and abundance

Plasticity of skeletal muscle is due in part to the presence of muscle stem cells, termed satellite cells (SCs; Dumont et al., 2015; Flamini et al., 2018). Satellite cells were first identified and described in the tibialis anticus of Xenopus by Alexander Mauro in 1961. Electron micrographs depict a cell composed of nucleus with very little cytoplasm nestled between the sarcolemma of the myofiber and the basal lamina of the basement membrane. Though the origin of SCs was unknown to Mauro, he posited that SCs assist in regeneration of skeletal muscle following damage (Mauro, 1961). Trunk and limb musculature as well as SCs arise from the somite of the developing embryo (Horst et al., 2006; Lepper and Fan, 2010). Satellite cells marked by Pax7, a paired box transcription

factor required for SC specification, are found in skeletal muscle of mice at embryonic day 12.5 (Lepper and Fan, 2010; Seale et al., 2000). Notch signals prompt occupation of the sublaminal position at embryonic day 16.5, a position maintained throughout postnatal life (Bröhl et al., 2012; Lepper and Fan, 2010).

Satellite cells are most abundant in early postnatal life when hypertrophic growth is occurring. Because skeletal muscle nuclei are post-mitotic, fusion of SCs to the myofiber syncytium is essential for nuclear accretion, a pre-requisite for increased protein synthesis associated with early muscle growth (Moss and Leblond, 1971; Shefer et al., 2006; White et al., 2010). Genetic ablation of *Pax7*-expressing SCs decreased muscle mass, increased weakness, and led to death at postnatal week 2 in mice (Seale et al., 2000). As maturity is reached and aging continues SC numbers decline, an effect more profound in females (Day et al., 2010; Gibson and Schultz, 1983; Shefer et al., 2006). Notch activity, a transcriptional activator essential for SC proliferation and stemness, is decreased in aged mice. Impaired proliferation of SCs may lead to the gradual decline of the SC pool observed in aging populations (Conboy and Rando, 2002; Conboy et al., 2005; Day et al., 2010; Pasut et al., 2016). Serum from young animals restored the regenerative potential of aged SCs, indicating that extrinsic signals are attenuated in aged mice (Conboy et al., 2005; Shefer et al., 2006). Exercise may lessen the age-related loss of SCs in males and females (Shefer et al., 2010). Researchers continue to characterize the mechanisms by which extrinsic and intrinsic stimuli regulate SC dynamics, partially to enhance their efficacy and stem their decline.

Intrinsic regulation of satellite cell dynamics

Necessity of SCs does not abate with maturity, as conditional knockout of *Pax7* in SCs completely abolished muscle repair following injury (Lepper et al., 2011; von Maltzahn et al., 2013). Regeneration of myofibers following injury or exercise-induced damage is a highly coordinated, tightly regulated process. Ultimately, SCs are prompted to enter the cell cycle, proliferate, and exit the cell cycle to either differentiate or return to the stem-like pool (Dumont et al., 2015). Perturbations disrupt effective myofiber repair and often compromise the SC pool, thus impacting future regeneration.

During normal, resting conditions SCs are quiescent, small in size, and display little metabolic activity (Pala et al., 2018; Rodgers et al., 2014). Quiescent SCs express Pax7, which is required for SC specification, maintenance, proliferation, and regeneration of skeletal muscle (Lepper et al., 2011; Rocheteau et al., 2012; Seale et al., 2000; von Maltzahn et al., 2013). Quiescence is a reversible state that is essential for continuation of the SC population. Phosphorylation of eukaryotic initiation factor 2 (eiF2)-α, which prevents mRNA translation, and upregulation of sprouty1 (Spry1), an inhibitor of receptor tyrosine kinases, most notably fibroblast growth factor-2 (FGF-2) signaling through FGF receptor-1 (FGFR-1) which is dysregulated during aging, are essential for return to quiescence (Chakkalakal et al., 2012; Shea et al., 2010; Zismanov et al., 2016). Exit from quiescence in vitro is protracted compared to subsequent divisions, typically occurring between 42 and 60 hours after seeding in adult rat SC isolates (Allen et al., 1995; Sheehan and Allen, 1999). This effect may be more pronounced in SCs from aged animals or those that are more abundant in Pax7 protein, a characteristic of the more

stem-like of the heterogeneous SC pool (Johnson and Allen, 1993; Rocheteau et al., 2012).

The SC population is comprised of both stem-like SCs and progenitors committed to the myogenic program. To maintain the population, SCs must balance the need for reserve stem-like cells essential for future regeneration while providing the progeny necessary for the immediate round of regeneration (Kuang et al., 2007). Planar division is driven by Vangl2 induction of non-canonical Wnt7a-Frizzled7 signaling to expand the stem-like SC pool, while apical-basal division typically yields asymmetric daughter cells (Dumont et al., 2015b; Kuang et al., 2007; Le Grand et al., 2009). Cells from transgenic Pax7-nGFP mice were sorted by GFP using fluorescence-activated cell sorting. Cells that highly expressed GFP, representing approximately 10% of the population, displayed a protracted lag phase and asymmetric DNA segregation compared to those 10% of the population expressing less GFP (Rocheteau et al., 2012). Additionally, Pax7 overexpression resulted in cell cycle withdrawal in primary myoblasts (Olguin and Olwin, 2004). Some 90% of $Pax7^{+}$ SCs have at one point co-expressed myogenic factor 5 (Myf5), while 10% of SCs through lineage tracing were found to have never expressed *Myf5*. This population may divide in an apical-basal manner and, despite having never expressed *Myf5*, divide asymmetrically to yield one $Pax7^{+}Myf5^{+}$ progeny (Kuang et al., 2007). Dystrophin regulated polarity and maintained asymmetric divisions by p38γ/Carm1 dependent epigenetic modification of the *Pax7* gene to activate *Myf5* transcription (Dumont et al., 2015a; Chang et al., 2018). Numb, an antagonist of Notch-1, is more abundant in committed SCs in concert with Myf5 (Conboy and Rando, 2002).

Notch signaling is required for maintenance of undifferentiated, $Pax7^+$ stem-like cells (Kuang et al., 2007).

Proliferating cells express *Pax7* in combination with either basic helix-loop-helix myogenic regulatory factor *Myf5* or *myogenic differentiation 1* (*MyoD*), the latter of which was downregulated by *Pax7* overexpression in MM14 and mouse primary SCs (Kuang et al., 2007; Olguin and Olwin, 2004). Myf5 and MyoD can bind the same E-box binding sites, and are consecutively or co-expressed to activate myogenic genes (Conerly et al., 2016; Cooper et al., 1999). Loss of *MyoD* impaired differentiation in single fiber explants and SC mass cultures from mice (Yablonka-Reuvni et al., 1999). Regeneration following injury was also compromised, likely due to SC contribution to self-renewal instead of to myogenic progenitors (Megeney et al., 1996). Upregulation of *myogenin*, another member of the basic helix-loop-helix family of transcription factors, occurs in late-stage differentiation of myoblasts following or in combination with *MyoD*, *Myf5*, or *MRF4*, the fourth and final myogenic regulatory factor (Cornelison and Wold, 1997; Flamini et al., 2018). Conversely, *Pax7* and *myogenin* are expressed in a mutually exclusive manner (Olguin and Olwin, 2004). Loss of *myogenin in utero* resulted in embryonic lethality while loss postnatally reduced body size by 30% in mice (Knapp et al., 2005; Meadows et al., 2008). However, skeletal muscle development was not compromised suggesting that *myogenin* may exert effects beyond that of postnatal myogenesis (Knapp et al., 2005). $Myogenin^+$ SCs upregulated p21 protein, a cyclin dependent kinase inhibitor, to withdraw from the cell cycle before fusing to one another to form a new myotube or fuse into an existing myofiber syncytium (Andrés and Walsh, 1996; Olguin and Olwin, 2004).

The balance between self-renewal and differentiation is imperative for continuing the SC population. Both FOXO3-Notch and p53 signaling have been implicated as critical nodes between differentiation and self-renewal. Conditional knockout of *FOXO3*, which interacts with *Notch 1* and *3* promoters, increased differentiating cells at the expense of the self-renewing population possibly by reduced Notch signaling (Gopinath et al., 2014). Progression from proliferation to differentiation depends on the downregulation of *Notch* and upregulation of the Wnt/β-catenin signaling pathway (Brack et al., 2008; Rudolf et al., 2016). Differentiation and maintenance of stem-like SCs depends on the level of p53 protein abundance which upregulates retinoblastoma protein (pRb) (Flamini et al., 2018; Porrello et al., 2000). Retinoblastoma protein is a modulator of both cell cycle withdrawal and myocyte enhancer factor (MEF) 2 activity, a family of transcription factors associated with differentiation of myoblasts (Estrella et al., 2015; Novitch et al., 1999).

The effect of growth factors on satellite cell dynamics

Early work sought to identify factors responsible for initiating the regenerative response of SCs following muscle damage. Crushed muscle extract (CME) specifically stimulated proliferation of SCs, not fibroblasts, from rat single fiber explants, rat embryos, and chick embryos as measured by ^{3}H-thymidine incorporation. This same extract promoted differentiation of SCs on rat single fiber explants (Bischoff, 1986). Heparin affinity chromatography was utilized to isolate transferrin, FGF-2, the BB isoform platelet-derived growth factor (BB-PDGF), as well as an unidentified fourth mitogen from mouse CME (Chen and Quinn, 1992; Chen et al., 1994). Crushed muscle extract from rats not only stimulated proliferation of young and old rat SCs, but also

shortened the lag period as determined by proliferating cell nuclear antigen (PCNA) abundance (Johnson and Allen, 1993). In 1995, Allen and colleagues identified hepatocyte growth factor (HGF) as the factor likely responsible for activation of quiescent SCs by CME.

Hepatocyte growth factor is produced by a variety of cells and bound in the extracellular matrix of skeletal muscle (Tatsumi et al., 1998). Proteolytic processing of the 92 kDa single chain to a disulfide linked 32/34 kDa α-chain and 64 kDa β-chain enables activity (Hartmann et al., 1992). Cleavage of pro-HGF to form active HGF occurs by the 34 kDa serine protease, HGF activator (HGFA), itself a product of thrombin cleavage of a single-chain pro-HGFA to form a disulfide-linked heterodimer (Shimomura et al., 1993). To initiate intracellular signaling, the β-chain of HGF binds the extracellular β-chain of the disulfide-linked heterodimer tyrosine kinase known as hepatocyte growth factor receptor or c-met located on SCs (Bottaro et al., 1990; Naldini et al., 1991; Tatsumi et al., 1998). Upon ligand binding of c-met, the receptor homodimerizes, intracellular residues are phosphorylated, and signaling pathways are initiated (Organ and Tsao, 2011).

Hepatocyte growth factor shortened the lag phase of SCs in mass culture, and the effect was not replicated by FGF-2 (Allen et al., 1995; Johnson and Allen, 1993). This response occurred in a dose-dependent manner, but as HGF concentrations exceeded 500 ng/mL, SC activity decreased due to increased myostatin-stimulated p21 (Allen et al., 1995; McCroskery et al., 2003; Yamada et al., 2010). After injury HGF colocalized with c-met on SCs (Tatsumi et al., 1998). Additionally, thrombin and pro-HGFA increased following injury, and administration of serum from injured mice to uninjured mice

decreased time to first division in SCs by approximately 10 h (Rodgers et al., 2017). Purified, active HGFA enhanced recovery from injury and serum from injured mice activated the mTORC1 signaling pathway in vivo, perhaps by increasing active HGF (Rodgers et al., 2017). This cascade stimulated cell cycle genes, mitochondrial activity, and activation of pS6 downstream of mTORC1, resulting in an overall 'alert' phenotype termed G_{alert} (Rodgers et al., 2014; Rodgers et al., 2017). Conditional knockout of the p110α subunit of PI3K upstream of mTORC1 prevented expression of c-Jun and exit from quiescence, possibly connecting c-met activation, which can activate the PI3K/Akt pathway, to mTORC1 (Brandt et al., 2018; Halevy et al., 2004; Wang et al., 2018). Satellite cells in G_{alert} proceed more rapidly than cells in G_0 through the cell cycle, exhibiting a reduced time to first division and increased regenerative capacity (Rodgers et al., 2014). Despite activating G_{alert} ex vivo, exogenous administration of purified HGF to mice did not recapitulate the effects of serum from injured mice *in vivo*, contradictory to effects observed in rats (Miller et al., 2000; Rodgers et al., 2017; Tatsumi et al., 1998). Nor does exogenous HGF activate all c-met$^+$ cells on single fiber explants from mice (Wozniak et al., 2003). Single fiber culture may better maintain quiescence than mass culture, which increased *MyoD* and decreased *Pax7*, *Myf5*, *MRF4*, *Notch1-4*, *Hes1*, *Hey1*, *HeyL*, and *PPARA* expression as soon as 5 h after SC isolation (Cooper et al., 1999; Machado et al., 2017). Additionally, SC isolation increased trimethylation of histone 3 at lysine residue 4 and decreased acetylation of lysine residue 27 on histone 3 (Machado et al., 2017). The act of isolation apparently activates the transcriptional program and modifies histones, suggesting that studying activation or exit from quiescence is rather challenging in vitro and results should be interpreted carefully.

Media used to induce quiescence of murine and human SCs noticeably differs as human SC quiescence media lacks HGF or c-met suppressants and instead contains an FGFR inhibitor (Quarta et al., 2016; Quarta and Rando, 2017). At 18 h in culture, FGF-2 was bound to SCs isolated from young rats. This effect was replicated at 42 h in SCs from old rats, but FGF-2, IGF-1 and -2, transforming growth factor- β (TGF- β) 1 and 2, and PDGF were unable to increase cell number in the first 48 h after isolation (Johnson and Allen, 1995). Satellite cells on single fiber explants from young and old rats have more $PCNA^+$ and $MAPK^+$ cells when FGF2 is administered (Yablonka-Reuvni et al., 1999). To date, there is just one published report examining activation of mass cultured equine SCs. In this study, HGF did not increase DNA synthesis until 72 h of treatment, well beyond the time of activation (Brandt et al., 2018). Taken together, FGF2 and HGF (or its upstream activators) appear to initiate activity in at least a portion of cells. The efficacy of either growth factor may be dependent on species and perhaps on the intrinsic heterogeneity of SCs.

Numerous growth factors, including HGF and FGF-2, affect proliferation and/or differentiation of SCs. These factors may be present in skeletal muscle or circulation at rest or after injury (Hayashi et al., 2004; Rodgers et al., 2017; Pinol-Jurado et al., 2017; Chen et al., 1994;). Hepatocyte growth factor, PDGF-BB and FGF-2 are present in CME (Chen et al., 1994). Fibroblast growth factor isoforms 1, 2, 4, 6, and 9 increased the number of rat SCs (Allen and Boxhorn, 1989; Sheehan and Allen, 1999). This effect was additive when HGF was included in cultures for all but FGF-1. Hepatocyte growth factor, IGF-1, and FGF-1 increased *FGFR1* message in early proliferating rat SCs while PDGF-BB and IGF-2 decreased *FGFR1* message (Sheehan and Allen, 1999). Insulin-like

growth factor-1 and FGF-2 stimulated proliferation of SCs isolated from aged rats, young chicks, and 10 d old horses (Allen and Boxhorn, 1989; Allen et al., 1995; Gal-Levi et al., 1998; LaVigne et al., 2015). Satellite cells from yearling horses also proliferated in response to FGF, but not when treated with IGF-1 (Byrne et al., 2000). To increase proliferation of C2C12 myoblasts, FGF-2 induced transient phosphorylation of extracellular signal-regulated kinase (ERK)1/2 through protein kinase C (PKC) and sustained phosphorylation through growth factor receptor bound protein (Grb)2 (Nagata et al., 2010). Hepatocyte growth factor increased proliferation in eqSC and despite phosphorylation of both ERK1/2 and Akt the mitogenic effect depended on PKCδ (Brandt et al., 2018). Hepatocyte growth factor decreased p27 protein abundance, a cyclin dependent kinase inhibitor, in young chicks resulting in phosphorylated or inactive pRb. Proliferation was promoted by increased *E2F1* promoter activity which often occurs in tandem with inhibited differentiation (Leshem et al., 2000; Leshem and Halevy, 2002).

In addition to proliferation, IGF-1 also stimulates differentiation in aged rats and 10 d old horses but not yearling horses (Allen and Boxhorn, 1989; Byrne et al., 2000; LaVigne et al., 2015). Conversely, both FGF2 to SCs from young horses and HGF to SCs from young chicks and mature horses suppressed differentiation (Gal-Levi et al., 1998; LaVigne et al., 2015). Despite divergent effects elicited by growth factors, often similar signaling pathways are relied upon. For instance, both HGF and IGF-1 elicit PI3K/Akt and MAPK/ERK signaling but exert differential effects on differentiation. This may be mediated by the catalytic subunits of PI3K recruited, intensity of phosphorylation of either Akt or ERK1/2, and duration of phosphorylation (Halevy et al., 2004). Ultimately,

it is the concerted actions and integration of signaling pathways stimulated by growth factors at rest, after injury, or following exercise that influence SC population dynamics.

Adaptation to Exercise

Horses are athletic creatures that frequently engage in exercise for either recreational or professional purposes (American Horse Council, 2017). Performance during exercise is hinged in part upon the ability of skeletal muscle to recover from the previous session (Bergström et al., 1967; McGowan et al., 2002). Exercise perturbs homeostasis and recovery from exercise requires the coordinated efforts of complex systems to reestablish equilibrium. When exerted within the physiological system's ability to respond this event is a positive stress that confers advantageous adaptation to the individual when undertaken repeatedly (Selye, 1936; Hawley et al., 2014; Pereira et al., 2013). To repair damage and mount a more resistant response to future insults, transcription and translation are transiently upregulated (Perry et al., 2010; Stupka et al., 2001). Exercise training culminates in phenotypic changes via the accumulation of these repeated responses, which lessen the severity of exercise-induced damage in later phases of training (Damas et al., 2018; Kim et al., 2005; Perry et al., 2010). The initial response and resultant training phenotype is entirely dependent upon the modality, frequency, and intensity of exercise encountered (Egan and Zierath, 2013; Hill et al., 2010).

Signaling pathways and satellite cell contribution during adaptation to endurance exercise

Divergent adaption occurs with varying modalities of exercise, particularly that of endurance and resistance training. Endurance training is associated with increased aerobic performance, increased reliance on fatty acid oxidation, increased skeletal muscle

glycogen, skeletal muscle fiber-type shift from fast glycolytic to slow oxidative, and mitochondrial biogenesis (Gollnick et al., 1972; Holloszy and Coyle, 1984; Wilkinson et al., 2008). Mitochondrial fractional synthesis rate (FSR) increased after a single bout of endurance exercise and continued after 10 weeks of endurance training (Wilkinson et al., 2008). In the 6-18 h following a single bout of swimming, the triceps of rats exhibited increased PGC1-α, binding of nuclear respiratory factor (NRF)-1 to the *aminolevulinic acid (ALA) synthase* promoter, and binding of NRF-2 to the *cytochrome c subunit IV* promoter. Five days of swimming increased proteins involved in mitochondrial metabolism and glucose import including ALA synthase, citrate synthase, cytochrome oxidase subunit I, cytochrome c, and GLUT4 protein (Baar et al., 2002). The family of peroxisome proliferator-activated receptors (PPARs) in cooperation with PPARγ coactivator α (PGC1-α) have been implicated in the coordination of these phenotypes (Liang and Ward, 2006). Peroxisome proliferator activated receptors form heterodimers with retinoid X receptors to elicit gene transcription often associated with improved metabolism of carbohydrate and lipids (Tyagi et al., 2011; Wagner and Wagner, 2010). Muscle specific deletion of PPARβ/δ inhibits carnitine palmitoyl-transferase 1b, the enzyme responsible for fatty acid transport into mitochondria, pyruvate dehydrogenase kinase 4, which prevents glucose-derived pyruvate oxidation in the mitochondria, and decreases running time in mice after 4 weeks of endurance training (Fan et al., 2017). Despite the effect of PPARβ/δ on substrate utilization and performance, there was not an effect on mitochondria or fiber-type. This stands in contrast to work by Wang and colleagues (2004) who found that genetic activation of *PPARβ/δ* increased running time in addition to mitochondrial DNA, mitochondrial enzymes, myoglobin, and type 1 fibers

in mice. Overexpression of PPARβ/δ prevents ubiquitination of PGC1-α, thereby increasing its abundance, and serves as a transcription factor for *NRF-1* (Koh et al., 2017). Nuclear respiratory factor-1lies upstream of MEF2A and calcium calmodulin-dependent protein kinase kinase-β (CaMKK), which activates adenosine monophosphate-activated protein kinase (AMPK), a sensor of intracellular energy availability (Koh et al., 2017).

Fiber-type shift and expression of hexokinase II, an enzyme responsible for glucose phosphorylation, may be regulated by AMPK downstream of PPARβ/δ, during exercise training (Koh et al., 2017; Röckl et al., 2007). The α1 subunit of AMPK worked cooperatively with nitric oxide (NO), a signaling molecule produced during conversion of arginine to citrulline, to phosphorylate PGC1-α in C2C12 and L6 myoblast cultures (Jäger et al., 2007; Lira et al., 2010). Mitochondrial enzymes and type 1 fibers increase in mice genetically induced to express physiological levels of PGC1-α (Lin et al., 2002). These effects appear to be dependent upon PGC1-α interaction with MEF2, possibly in coordination with calcineurin, which is not required for NO-mediated PGC1-α activation (Lin et al., 2002). Calcineurin and CaM kinase rely on MEK signaling to elicit MEF2 and nuclear factor of activated T cells (NFAT) regulation of the *myosin heavy chain IIa* promoter in C2C12 (Allen and Leinwand, 2002). The influence of calcineurin in vivo, however, may be dependent upon muscle phenotype and is essential with NFAT to enact PPARβ/δ-dependent myonuclear accretion (Giordano et al., 2009; Talmadge et al., 2004).

Metabolic adaptations may be accompanied by increased SC activity and abundance independent of myofiber hypertrophy, the trait for which SCs are required during growth. Six weeks of non-hypertrophic moderate intensity continuous and high

intensity interval training stimulated SC activity and differentiation, in healthy and overweight men and women with expansion of the pool in hybrid fibers in sedentary, overweight women (Joanisse et al., 2013; Joanisse et al., 2015). Voluntary running increased SC number, myonuclear number, and citrate synthase activity without myofiber hypertrophy in adult male rats (Kurosaka et al., 2009). Interestingly, when SCs are ablated in mice, running performance, spindle fibers, and coordination are reduced (Jackson et al., 2015). In horses subject to a single bout of inclined treadmill exercise to exhaustion, SC content increased in type 1 and 2a fibers 1 week post-exercise and increased 2 weeks post-exercise in type 2a/x fibers in the absence of hypertrophy (Kawai et al., 2013). Satellite cell activity, expansion, and differentiation may serve to remodel myofibers when endurance exercise is non-hypertrophic. Fry and colleagues (2014) found SC content increased preferentially in type 1 fibers, but also observed increased cross-sectional area after aerobic training in humans. Intensity and volume of exercise may determine whether SCs respond to the aerobic stimulus and for what purpose, remodeling or hypertrophy (Kurosaka et al., 2009). This may be mediated at least in part by constituents of serum, as proliferation and differentiation of SCs isolated from the biceps femoris of unexercised weanling quarter horses increased in the presence of serum from endurance exercised weanlings (Greene et al., 1995). Ultimately, the role of SCs in adaptation to exercise requires further study.

Myogenic regulatory factors typically associated with SCs may also influence adaptation to aerobic exercise. *MyoD* mRNA and protein accumulated in fast fiber types while *myogenin* mRNA accumulated in slow fiber types of mice (Hughes et al., 1993; Hughes et al., 1997). Expression of each of these factors originated from the myonucleus

and is malleable, as thyroid hormone, cross-innervation, and loss of *MyoD* shifted fiber type and myogenic regulatory factor expression accordingly (Hughes et al., 1993; Hughes et al., 1997). Maintenance of slow type myofibers at rest or in response to exercise depended on NFATc1-dependent inhibition of MyoD-p300 interaction at myogenic promoters in *NFATc1* knockout mice (Ehlers et al., 2014). However, overexpression of *myogenin* by transfection in mice and C2C12 increased oxidative enzymes without appreciable changes in myosin heavy chain expression (Zhu et al., 2013). Myogenin protein expression increased in myonuclei after a single session of low or high intensity endurance exercise (Kadi et al., 2004). Perhaps somewhat surprisingly, *myogenin* knockout enhanced the running capacity of mice at both low and high intensities by increased efficiency of oxidative and glycolytic metabolism determined by blood lactate, blood glucose, respiratory exchange ratio, and oxygen consumption rates. With training, the percentage of type 1 fibers and succinate dehydrogenase activity also increased (Flynn et al., 2010). These studies underscore how the roles of myogenic regulatory factors beyond their role in SCs are relatively unexplored.

Signaling pathways and satellite cell contribution during adaptation to resistance exercise

While acute resistance exercise increased both myofibrillar and mitochondrial fractional synthesis rate (FSR), only myofibrillar FSR increased in the trained state (Wilkinson et al., 2008). Resistance training typically increases myofiber cross sectional area, myonuclear number, satellite cell number, and strength while shifting fiber type away from type 1 towards 2a (Kadi et al., 1999; Damas et al., 2018; Wilkinson et al., 2008). Despite the association of PGC1-α with the endurance phenotype, PGC1-α4, a

splice variant of the gene, may modulate skeletal muscle hypertrophy through regulation of *IGF1* and *myostatin* promoters (Ruas et al., 2012). Hypertrophy may be initiated by IGF1 stimulation of the Akt-mTOR-p70s6k signaling pathway, which regulates protein synthesis and degradation (Léger et al., 2006; Wilkinson et al., 2008). Inducible activation of Akt in mice increased muscle mass and force, but did not appear to utilize SCs for this process (Blauuw et al., 2009).

Indeed, whether SCs are absolutely required for hypertrophy has been subject to debate (McCarthy and Esser, 2007; O'Connor and Pavlath, 2007). SCs are postulated to donate nuclei necessary to increase cytoplasmic volume following hypertrophic stimuli, thus maintaining a myonuclei:cytoplasm ratio termed the myonuclear domain (Gunderson, 2016). Genetic ablation of *Pax7* expressing SCs attenuated compensatory hypertrophy in young mice (Murach et al., 2017). A lesser degree of hypertrophy appears to occur in mature mice lacking SCs, but statistical analysis between SC^+ and SC^- animals subject to compensatory hypertrophy were not reported (Murach et al., 2017). Another group found that when myofibers contributing to regeneration were excluded SCs were required for hypertrophy (Egner et al., 2016). Loss of *Tmem8c*, commonly known as myomaker, a protein essential for fusion of progenitors, prevents overload-induced hypertrophy. Nuclei from SC are not incorporated and myofiber protein synthesis is reduced, suggesting that nuclear addition is required to increase protein synthetic capacity of the myofiber (Goh and Millay, 2017). The response of SCs and myonuclear addition during resistance training may be dependent on subject genetic and training history. The concept of "muscle memory" has emerged which may explain discrepancies in SC and myonuclear addition during hypertrophic conditioning

(Gundersen, 2016; Petrella et al., 2008). Myonuclei are hypothesized to retain a so-called "memory" of previous phenotypes. For instance, muscle fibers that previously experienced hypertrophy but have since atrophied may contain more myonuclei per cytoplasm. Nuclei are more numerous in these previously hypertrophic fibers and may enhance the protein synthetic machinery without the addition of new nuclei because they had been previously exposed to this event. Additionally, SCs downregulate collagen production via microRNA-206 secretion, which acts to suppress ribosomal binding protein 1 (Rrbp1) and thus collagen deposition by fibroblasts (Fry et al., 2017). Together, it appears that there is likely some level of flexibility in hypertrophy, perhaps as determined by genetic background or prior history, and other purposes SCs are essential for during hypertrophy.

The role of hepatocyte growth factor and insulin-like growth factor-1 during acute exercise

Insulin like growth factor-1 mRNA increased up to 7 d after an exhaustive bout of exercise in untrained thoroughbred horses. Expression of *IGF-1* preceded that of *HGF*, and occurred concomitant with *Pax7*, *MyoD*, and *myogenin* (Kawai et al., 2013). *Mechano growth factor* (*MGF*), a splice variant of IGF-1 localized to skeletal muscle after mechanical damage, mRNA increased in a pattern similar to that of MyoD in the first 7 d after stretch stimulation in the tibialis anterior of rats (Hill and Goldsplink, 2003). Knee extensor lengthening contractions did not influence serum IGF-1 but increased *MGF* in correlation with *Myf5* and the *IGF-1* splice variants *IGF-1Ea* and *-1Eb* in correlation to *MRF4* (McKay et al., 2008). Stretch activated a small portion of $cMet^{+}$ SCs (Anderson, 2000; Tatsumi et al., 2001; Wozniak et al., 2003). Stretch also enhanced

nitric oxide synthase (NOS)-dependent production of nitric oxide (NO), a signaling molecule that released HGF from the ECM (Tatsumi et al., 2001; Tatsumi et al., 2002). The activity of these events appears to be dictated by the surrounding pH, as HGF may only be released from the ECM between pH 7.1 and 7.5, a range typical of skeletal muscle interstitial space at rest (pH 7.38) and during recovery from exercise (Street et al., 2001; Tatsumi et al., 2002). Satellite cell regulation following exercise may be directly related to degree of stretch sufficient to increase MGF or NO-stimulated HGF release.

Nutrient availability and supplementation to enhance exercise performance

Recovery from and performance during endurance exercise is dependent on the availability of energy substrates (Pizza et al., 1995). Glycogen is one of the primary fuel sources during exercise and its abundance is related to performance (Bergström et al., 1967; Pizza et al., 1995). Skeletal muscle and liver are the main repositories of glycogen where it is stored as either acid-soluble or insoluble (Barnes et al., 2009). Exercise of sufficient intensity or duration reduces glycogen in both liver and skeletal muscle (Baldwin et al., 1975; Lindholm et al., 1974). Efficient and rapid replenishment of muscle glycogen is of utmost importance when exercise is undertaken shortly after the previous bout.

While humans replenish glycogen within 24-48 h, horses require up to 72 h to replenish glycogen stores if sufficiently reduced (Egan and Zierath, 2013; Lacombe et al., 2004). Replenishment is dependent upon glucose availability, glucose absorption by the small intestine via sodium glucose transporter-1 (SGLT-1), subsequent uptake by GLUT1 and GLUT4 in muscle, and the activity of glycogen synthase 1(Adeva-Andany et al., 2016; Dyer et al., 2009; Egan and Zierath, 2013; Jentjens and Jeukendrup, 2003). Efforts

have mainly targeted glucose availability and uptake by nutritional manipulation. Soluble carbohydrates, which are rapidly digested and available in the small intestine of horses and humans, are often supplemented prior to or following exercise as a means of increasing circulating glucose (Alghannam et al., 2018; Pizza et al., 1995). Glucose derived from dietary sources is absorbed in the small intestine by SGLT-1, which may be limiting in horses unaccustomed to diets containing a large amount of soluble carbohydrates (Daly et al., 2009). Horses have a voluminous hindgut that is easily disturbed by excess soluble carbohydrates that escape small intestinal digestion with potentially fatal consequences. Therefore, diets high in soluble carbohydrates are a less viable option to increase glycogen replenishment in horses (Daly et al., 2009; Lacombe et al., 2004; Clarke et al., 1990). Addition of protein to carbohydrate supplementation may enhance glycogen resynthesis with the additional benefit of encouraging regeneration from exercise-induced muscle damage (Essén-Gustavsson et al., 2010; Ivy, 2005; Morifuji et al., 2010; Wang et al., 2015).

L-arginine, an amino acid, is an effective insulin segretagogue and possibly effective tool for increasing glucose for glycogen synthesis (Robertson et al., 2014). Dietary arginine increased insulin-stimulated glucose uptake at the skeletal muscle membrane by GLUT4, and enhanced activity of insulin-stimulated glycogen synthase 1. Carbohydrate oxidation decreased post-exercise in L-arginine supplemented animals (Yaspelkis and Ivy, 1999; Egan and Zierath, 2013). Dietary L-citrulline, a non-essential amino acid prevalent in watermelon, is actually more efficient at increasing systemic arginine than dietary L-arginine (Agarwal et al., 2017; Figueroa et al., 2017; Morita et al., 2014; Rimando et al., 2005). This is because L-citrulline bypasses sphlanchnic extraction

and is converted to L-arginine by the kidneys, vasculature, and peripheral tissues while arginine undergoes first-pass metabolism (Agarwal et al., 2017; Hartman et al., 1994). L-arginine may then be utilized for various metabolic pathways, including NO synthesis (Curis et al., 2005). As previously discussed, NO may be involved in liberation of HGF which regulates SC dynamics (Tatsumi et al., 2001; Tatsumi et al., 2002). Nitric oxide also increased glucose uptake during muscle contraction and stimulated vascular flow, an important mechanism for thermoregulation during exercise in the horse (Roberts et al., 1997; Mills et al., 1997; Mills et al., 1999; Morita et al., 2014). Fatigue is delayed during exercise in mice receiving citrulline supplementation. This effect is likely due to increased metabolic efficiency and recycling capacity, as citrulline reduced NH_3 and lactate accumulation while blood glucose remained elevated compared to control groups (Takeda et al., 2011; Villareal et al., 2018). Additionally, L-citrulline increased *PGC1-α* expression alongside *vascular endothelial growth factor* (*VEGF*) and *IGF-1*, which was attenuated with addition of L-NAME, a nitric oxide inhibitor (Villareal et al., 2018). These qualities make L-citrulline an attractive candidate for enhancing recovery from and performance during exercise in horses by nutritional manipulation.

Summary

Exercise utilizes metabolic reserves and stimulates SC activity, though the extent of each is dependent upon intensity and modality, and recovery depends on the actions of both of these components. In the equine, a single bout of exhaustive exercise increases transcriptional regulators of SCs and growth factors associated with SC activity, proliferation, and differentiation. Serum from exercised horses stimulates proliferation and differentiation of SCs, and SCs responded to a variety of growth factors in culture.

Prior to the studies performed herein, there were no studies on the effect of HGF, an important regulator of SC dynamics in other species that is increased in the post-exercise state in horses, on equine SCs. Additionally, there are very few studies examining the role of SCs during the post-exercise recovery period in the equine, let alone examining both metabolic and SC dynamics in concert during this time.

Objectives

The objectives were two-fold. First, to determine whether HGF mediates activation, proliferation, and differentiation of equine SCs. Second, to determine whether L-citrulline, which is critically placed to influence both facets of recovery, would enhance the metabolic and SC response during recovery from exercise.

Literature Cited

Abramowitz, MK, CB Hall, A Amodu, D Sharma, L Androga, and M Hawkins. 2018. Muscle mass, BMI, and mortality among adults in the United States: A population-based cohort study. PLoS ONE 13(4): e0194697. doi:10.1371/journal.pone.0194697.

Adeva-Andany, M.M., M. González-Lucán, C. Donapetry-García, C. Fernández-Fernández, E. Ameneiros-Rodríguez. 2016. Glycogen metabolism in humans. BBA Clin. 5: 85-100. doi: 10.1016/j.bbacli.2016.02.001.

Agarwal, U., Didelija, I. C., Yuan, Y., Wang, X., and J. C. Marini. 2017. Supplemental citrulline is more efficient than arginine in increasing systemic arginine availability in mice. J. Nutr. 147: 596-602.

Alghannaam, A.F., J.T. Gonzalez, and J.A. Betts. 2018. Restoration of muscle glycogen and functional capacity: role of post-exercise carbohydrate and protein co-ingestion. Nutrients 10(2): 253. doi: 10.3390/nu10020253.

Allen, R.E. and L.K. Boxhorn. 1989. Regulation of skeletal muscle satellite cell proliferation and differentiation by transforming growth factor-beta, insulin-like growth factor I, and fibroblast growth factor. J. Cell. Physiol. 138: 311-315.

Allen, R. E., S. M. Sheehan, R. G. Taylor, T. L. Kendall, and G. M. Rice. 1995. Hepatocyte growth factor activates quies- cent skeletal muscle satellite cells in vitro. J. Cell. Physiol. 165:307–312. doi:10.1002/jcp.1041650211.

Allen, D.L., and L.A. Leinwand. 2002. Intracellular calcium and myosin isoform transitions. Calcineurin and calcium-calmodulin kinase pathways regulate preferential activation of the IIa myosin heavy chain promoter. J. Biol. Chem. 277:45323-45330. doi: 10.1074/jbs.M208302200.

American Horse Council Foundation. 2017. Economic impact study of the U.S. horse industry. https://www.horsecouncil.org/resources/economics/ (Accessed 20 February 2019).

Anderson, J. E. 2000. A role for nitric oxide in muscle repair: nitric oxide-mediated activation of muscle satellite cells. Mol. Biol. Cell. 11(5): 1859-1874. doi: 10.1091/mbc.11.5.1859.

Andrés, V., and K. Walsh. 1996. Myogenin expression, cell cycle withdrawal, and phenotypic differentiation are temporally separable events that precede cell fusion upon myogenesis. J. Cell Biol. 132(4): 657-666.

Baar, K., A.R. Wende, T.E. Jones, M. Marison, L.A. Nolte, M. Chen, D.P. Kelly, and J.O. Holloszy. 2002. Adaptations of skeletal muscle to exercise: rapid increase in the transcriptional coactivator PGC-1. FASEB J. 16(14): 1879-1886.

Baldwin, K.M., R.H. Fitts, F.W. Booth, W.W. Winder, and J.O. Holloszy. 1975. Depletion of muscle and liver glycogen during exercise. Pflügers Archiv. 354(3): 203-212.

Barnes, P.D., A. Singh, and P.A. Fournier. 2009. Homogenization-dependent responses of acid-soluble and acid-insoluble glycogen to exercise and refeeding in human muscles. Metab. 58(12): 1832-1839. doi: 10.1016/j.metabol.2009.06.016.

Bergström, J., Hermansen, L., Hultman, E., and B. Saltin. 1967. Diet, muscle glycogen and physical performance. Acta Physiol. Scand. 71(2): 140-150. doi: 10.1111/j.1748-1716.1967.tb03720.x.

Bischoff, R. 1986. A satellite cell mitogen from crushed adult muscle. Dev. Biol. 115: 140-147.

Blauuw, B., M. Canato, L. Agatea, L. Toniolo, C. Mammucari, E. Masiero, R. Abraham, M. Sandri, S. Schiaffino, and C. Reggiani. 2009. Inducible activation of Akt increases skeletal muscle mass and force without satellite cell activation. FASEB J. 23(11): 3896-3905. doi: 10.1096/fj.09-131870.

Bottaro, D.P., J.S. Rubin, D.L. Faletto, A.M.L. Chan, T.E. Kmiecik, G.F. Vande Woude, and S.A. Aaronson. 1990. Identification of the hepatocyte growth factor receptor as the c-met proto-oncogene product. Science 251:802-804.

Brack, A.S., I.M. Conboy, M.J. Conboy, J. Shen, and T.A. Rando. 2008. A temporal switch from Notch to Wnt signaling in muscle stem cells is necessary for normal adult myogenesis. Cell Stem Cell. 2:50-59. doi: 10.1016/j.stem.2007.10.006.

Brandt, A. M., Kania, J. M., Gonzalez, M. L., and S. E. Johnson. 2018. Hepatocyte growth factor acts as a mitogen for equine satellite cells via protein kinase C δ-directed signaling. J. Anim. Sci. 96(9): 3645-3656. doi: 10.1093/jas/sky234.

Bröhl, D, E Vasyutina, MT Czajkowski, J Griger, C Rassek, H Rahn, B Purfürst, H Wende, and C Birchmeier. 2012. Colonization of the satellite cell niche by skeletal muscle progenitor cells depends on Notch signals. Developmental Cell 23: 469-481. doi: 10.1016/j.devcel.2012.07.014.

Byrne, K.M., J. Vierck, and M.V. Dodson. 2010. In vitro model of equine muscle regeneration. 32(5): 401-405. doi: 10.2746/042516400777591020.

Chakkalakal, J.V., K.M. Jones, M. A. Basson, and A.S. Brack. 2012. The aged niche disrupts muscle stem cell quiescence. Nature 490:355-362. doi: 10.1038/nature11438.

Chang, N.C., M. Sincennes, F.P. Chevalier, C.E. Brun, M. Lacaria, J. Segalés, P. Muñoz-Cánoves, H. Ming, and M.A. Rudnicki. 2018. The dystrophin glycoprotein complex

regulates the epigenetic activation of muscle stem cell commitment. Cell Stem Cell 22: 755-768. doi: 10.1016/j.stem.2018.03.022.

Chen, G., and L.S. Quinn. 1992. Partial characterization of skeletal myoblast mitogens in mouse crushed muscle extract. J. Cell. Physiol. 153(3): 563-574. doi: 10.1002/jcp.1041530318.

Chen, G., R. S. Birnbaum, Z. Yablonka-Reuveni, and L.S. Quinn. 1994. Separation of mouse crushed muscle extract into distinct mitogenic activities by heparin affinity chromatography. J. Cell. Physiol. 160: 563-572. doi: 10.1002/jcp.1041600320.

Clarke, L.L., M.C. Roberts, and R.A. Argenzio. 1990. Feeding and digestive problems in horses. Physiologic responses to a concentrated meal. Vet. Clin. North Am. Equine Pract. 6(2): 433-450.

Conboy, IM, and TA Rando. 2002. The regulation of notch signaling controls satellite cell activation and cell fate determination in postnatal myogenesis. Dev Cell 3: 397-409. doi: 10.1016/S1534-5807(02)00254-X.

Conboy, IM, MJ Conboy, AJ Wagers, ER Girma, IL Weissman, and TA Rando. 2005. Rejuvenation of aged progenitor cells by exposure to a young systemic environment. Nature 433:760-764. doi: 10.1038/nature03260.

Conerly, M.L., Z. Yao, J.W. Zhong, M. Groudine, and S.J. Tapscott. 2016. Distinct activities of Myf5 and MyoD indicate separate roles in skeletal muscle lineage specification and differentiation. Dev. Cell 36: 375-385. doi: 10.1016/j.devcel.2016.01.021.

Cooper, R.N., S. Tajbakhsh, V. Mouly, G. Cossu, M. Buckingham, and G.S. Butler-Browne. 1999. In vivo satellite cell activation via Myf5 and MyoD in regenerating mouse skeletal muscle. J Cell Sci. 112: 2895-2901.

Cornelison, D. D. W., and B.J. Wold. 1997. Single-cell analysis of regulatory gene expression in quiescent and activated mouse skeletal muscle satellite cells. Dev. Biol. 191: 270-283.

Curis, E., I. Nicolis, C. Moinard, S. Osowska, N. Zerrouk, S. Bénazeth, and L. Cynober. 2005. Almost all about citrulline in mammals. 29: 177-205. doi: 10.1007/s00726-005-0235-4.

Damas, F., C.A. Libardi, C. Ugrinowitsch, F.C. Vechin, M.E. Lixandrão, T. Snijders, J.P. Nederveen, A.V. Bacurau, P. Brum, V. Tricoli, H. Roschel, G. Parise, and S.M. Phillips. 2018. Early- and later-phases satellite cell responses and myonuclear content with resistance training in young men. PLoS ONE 13: e0191039. doi: 10.1371/journal.pone.0191039.

Day, K, G Shefer, A Shearer, and Z Yablonka-Reuveni. 2010. The depletion of skeletal muscle satellite cells with age is concomitant with reduced capacity of single progenitors to produce reserve progeny. Dev Biol 340: 330-343. doi: 10.1016/j.ydbio.2010.01.006.

Dumont, NA, YX Wang, J von Maltzahn, A Pasut, CF Bentzinger, CE Brun, and MA Rudnicki. 2015. Dystrophin expression in muscle stem cells regulates their polarity and asymmetric division. Nature Med. 21(12): 1455-1463. doi: 10.1038/nm.3990.

Dumont, NA, YX Wang, and MA Rudnicki. 2015. Intrinsic and extrinsic mechanisms regulating satellite cell function. Development 142: 1572-1581. doi: 10.1242/dev.114223.

Dyer, J., M. Al-Rammahi, L. Waterfall, K.S.H. Salmon, R.J. Geor, L. Bouré, F. Barrie Edwards, C.J. Proudman, S.P. Shirazi-Beechey. 2009. Adaptive response of equine intestinal Na^+/glucose co-transporter (SGLT1) to an increase in dietary soluble carbohydrate. Pflügers Arch. Eur. J. Physiol. 458: 419-430. doi: 10.1007/s00424-008-0620-4.

Egan, B., and J. R. Zierath. 2013. Exercise metabolism and the molecular regulation of skeletal muscle adapatation. Cell Metab. 17: 162-184.

Egner, IM, JC Bruusgaard, and K Gundersen. 2016. Satellite cell depletion prevents fiber hypertrophy in skeletal muscle. Development. 143: 2898-2906. doi: 10.1242/dev.134411.

Ehlers, M.L., B. Celona, and B.L. Black. 2014. NFATc1 controls skeletal muscle fiber type and is a negative regulator of MyoD activity. Cell Rep. 8: 1639-1648. doi: 10.1016/j.celrep.2014.08.035.

Essén-Gustavsson, B., Connysson, M., and A. Jansson. 2010. Effects of crude protein intake from forage-only diets on muscle amino acids and glycogen levels in horses in training. Equine Vet. J. 42(38): 341- 346. doi: 10.1111/j.2042-3306.2010.00283.x.

Estrella, N.L., C.A. Desjardins, S.E. Nocco, A.L. Clark, Y. Maksimenko, and F.J. Naya. 2015. MEF2 transcription factors regulate distinct gene programs in mammalian skeletal muscle differentiation. J. Biol. Chem. 290(2): 1256-1268. doi: 10.1074/jbc.M114.589838.

Fan, W., W. Waizenegger, C. Shi Lin, V. Sorrentino. M. He, C.E. Wall, H. Li, C. Liddle, R.T. Yu, A.R. Atkins, J. Auwerx, M. Downes, and R.M. Evans. 2017. PPARδ promotes running endurance by preserving glucose. Cell Metab. 25: 1186-1193. doi: 10.1016/j.cmet.2017.04.006.

Figueroa, A., Wong, A., Jaime, S. J., and J. U. Gonzales. 2017. Influence of L-citrulline and watermelon supplementation on vascular function and exercise performance. Curr. Opin. Clin. Nutr. Metab. Care. 20(1): 92-98.

Flamini, V., Ghadiali, R. S., Antczak, P., Rothwell, A., Turnbull, J. E., and A. Pisconti. 2018. The satellite cell niche regulates the balance between myoblast differentiation and self-renewl via p53. Stem Cell Rep. 10(3): 970-983. doi: 10.1016/j.stemcr.2018.01.007.

Flynn, J. M., Meadows, E., Fiorotto, M., and W. H. Klein. 2010. Myogenin regulates exercise capacity and skeletal muscle metabolism in the adult mouse. PLoS ONE. 5(10): e13535. doi: 10.1371/journal.pone.0013535.

Fry, C.S., B. Noehren, J. Mula, M.F. Ubele, P.M. Westgate, P.A. Kern, and C.A. Peterson. 2014. Fibre type-specific satellite cell response to aerobic training in sedentary adults. J. Physiol. 592(12): 2625-2635. doi: 10.1113/jphysiol.2014.271288.

Fry, C. S., Kirby, T. J., Kosmac, K., McCarthy, J. J., and C. A. Peterson. 2017. Myogenic progenitor cells control extracellular matrix production by fibroblasts during skeletal muscle hypertrophy. Cell Stem Cell. 20: 56-69.

Gal-Levi, R., Y. Leshem, S. Aoki, T. Nakamura, and O. Halevy. 1998. Hepatocyte growth factor plays a dual role in regulating skeletal muscle satellite cell proliferation and differentiation. Biochim. Biophys. Acta.1402: 39-51.

Gibson, MC, and E Schultz. 1983. Age-related differences in absolute numbers of skeletal muscle satellite cells. Muscle Nerve 6: 574-580. doi: 10.1002/mus.880060807.

Giordano, C. A.S. Rosseau, N. Wagner, C. Gaudel, J. Murdaca, C. Jehl-Piétri, B. Sibille, P.A. Grimaldi, and P. Lopez. 2009. Peroxisome proliferator-activated receptor β activation promotes myonuclear accretion in skeletal muscle of adult and aged mice. Pflügers Arch. Eur. J. Physiol. 458: 901-913. doi: 10.1007/s00424-009-0676-9.

Goh, Q., and D.P. Millay. 2017. Requirement of myomaker-mediated stem cell fusion for skeletal muscle hypertrophy. eLife 6: e20007. doi: 10.7554/eLife.20007.

Gopinath, S.D., A.E. Webb, A. Brunet, and T.A. Rando. 2014. FOXO3 promotes quiescence in adult muscle stem cells during the process of self-renewal. 2: 414-426. doi: 10.1016/j.stemcr.2014.02.022.

Gollnick, PD, RB Armstrong, CW Saubert IV, K Piehl, and B Saltin. 1972. Enzyme activity and fiber composition in skeletal muscle of untrained and trained men. J Applied Physiol. 33(3): 312-319. doi: 10.1152/jappl.1972.33.3.312.

Greene, E.A., S.G. Balch, R.H. Raub, and E. Gaughn. 1995. The effect of moderate exercise on equine muscle satellite cells in vitro. Equine Vet. J. 18: 218-222.

Gundersen, K. 2016. Muscle memory and a new cellular model for muscle atrophy and hypertrophy. J Exp Biol. doi: 10.1242/jeb.124495.

Halevy, O., and L.C. Cantley. 2004. Differential regulation of the phosphoinositide 3-kinase and MAP kinase pathways by hepatocyte growth factor vs. insulin-like growth factor-I in myogenic cells. 297: 224-234. doi: 10.1016/j.yexcr.2004.03.024.

Hartman, W. J., Torre, P. M., and R. L. Prior. 1994. Dietary citrulline but not ornithine counteracts arginine deficiency in rats by increasing splanchnic release of citrulline. J. Nutr. 124(10): 1950-1960.

Hartmann, G., L. Naldini, K.M. Weidner, M. Sachs, E. Vigna, P.M. Comoglio, and W. Birchmeier. 1992. A functional domain in the heavy chain of scatter factor/hepatocyte growth factor binds the c-Met receptor and induces cell dissociation but not mitogenesis. Proc. Nat. Acad. Sci. USA 89(23): 11574-11578.

Hayashi, S., H. Aso, K. Watanabe, H. Nara, M.T. Rose, S. Ohwada, and T. Yamaguchi. 2004. Sequence of IGF-1, IGF-2, and HGF expression in regenerating skeletal muscle. Histochem. Cell Biol. 122: 427-434. doi: 10.1007/s00418-004-0704-y.

Hawley, J.A., M. Hargreaves, M.J. Joyner, and J.R. Zierath. 2014. Integrative biology of exercise. Cell 159: 738-749. doi: 10.1016/j.cell.2014.10.029.

Hill, M., and G. Goldsplink. 2003. Expression and splicing of the insulin-like growth factor gene in rodent muscle is associated with muscle satellite (stem) cell activation following local tissue damage. J. Physiol. 549(2): 409-418. doi: 10.1113/jphysiol.2002.035832.

Hill, E.W., S.S. Eivers, B.A. McGivney, R.G. Fonseca, J. Gu, N.A. Smith, J.A. Browne, D.E. MacHugh, and L.M. Katz. 2010. Moderate and high intensity sprint exercise induce differential responses in *COX4I2* and *PDK4* gene expression in Thoroughbred horse skeletal muscle. Equine Vet. J. 42(38): 576-581. doi: 10.1111/j.2042-3306.2010.00206.x.

Holloszy, J.O., and E.F. Coyle. 1984. Adaptations of skeletal muscle to endurance exercise and their metabolic consequences. J. Appl. Physiol. 56(4): 831-838. doi: 10.1152/jappl.1984.56.4.831.

Horst, D, S Ustanina, C Sergi, G Mikuz, H Juergens, T Braun, and E Vorobyov. 2006. Comparative expression analysis of Pax3 and Pax7 during mouse myogenesis. Int J Dev Biol 50: 47-54. doi: 10.1387/ijdb.052111dh.

Hughes, S. M., Taylor, J. M., Tapscott, S. J., Gurley, C. M., Carter, W. J., and C. A. Peterson. 1993. Selective accumulation of MyoD and myogenin mRNAs in fast and slow adult skeletal muscle is controlled by innervation and hormones. Development. 118(4): 1137-1147.

Hughes, S.M., K. Koishi, M. Rudnicki, and A.M. Maggs. 1997. MyoD protein is differentially accumulated in fast and slow skeletal muscle fibres and required for normal type balance in rodents. Mech. Devel. 61: 151-163.

Ivy, J.L. 2004. Regulation of muscle glycogen repletion, muscle protein synthesis and repair following exercise. J. Sports Sci. Med. 3(3): 131-138.

Jackson, J.R., T.J. Kirby, C.S. Fry, R.L. Cooper, J.J. McCarthy, C.A. Peterson, and E.E. Dupont-Versteegden. 2015. Reduced voluntary running performance is associate with impaired coordination as a result of muscle satellite cell depletion in adult mice. Skel. Muscle. 5:41. doi: 10.1186/s13395-015-0065-3.

Jäger, S., C. Handschin, J. St-Pierre, and B.M. Spiegelman. 2007. AMP-activated protein kinase (AMPK) action in skeletal muscle via direct phosphorylation of PGC-1α. Proc. Natl. Acad. Sci. USA 104(29): 12017-12022. doi: 10.1073/pnas.0705070104.

Jentjens, R., and A. Jeukendrup. 2003. Determinants of post-exercise glycogen synthesis during short-term recovery. Sports Med. 33(2): 117-144. doi: 10.2165/00007256-200333020-00004.

Joanisse, S., J.B. Gillen, L.M. Bellamy, B.R. McKay, M.A. Tarnopolsky, M.J. Gibala, and G. Parise. 2013. FASEB J. 27(11): 4596-4605. doi: 10.1096/fj.13-229799.

Joanisse, S., B.R. McKay, J.P. Nederveen, T.D. Scribbans, B.J. Gurd, J.B. Gillen, M.J. Gibala, M.A. Tarnopolsky, and G. Parise. 2015. Satellite cell activity, without expansion, after nonhypertrophic stimuli. Am. J. Physiol. Regul. Integr. Comp. Physiol. 309: R1101-R1111. doi: 10.1152/ajpregu.00249.2015.

Johnson, SE, and RE Allen. 1993. Proliferating cell nuclear antigen (PCNA) is expressed in activated rat skeletal muscle satellite cells. J Cell Physiol 154: 39-43. doi: 10.1002/jcp.1041540106.

Johnson, S.E., and R.E. Allen. 1995. Activation of skeletal muscle satellite cells and the role of fibroblast growth factor receptors. Exp. Cell Res. 219: 449-453.

Kadi, F., A. Eriksson, S. Holmner, G.S. Butler-Browne, and L. Thornell. 1999. Cellular adaptation of the trapezius muscle in strength-trained athletes. Histochem. Cell Biol. 111: 189-195.

Kadi, F., Johansson, F., Johansson, R., Sjöström, M., and J. Henriksson. 2004. Effects of one bout of endurance exercise on the expression of myogenin in human quadriceps muscle. Histochem. Cell. Biol. 121: 329-334. doi: 10.1007/s00418-004-0630-z.

Kawai, M., H. Aida, A. Hiraga, and H. Miyata. 2013. Muscle satellite cells are activated after exercise to exhaustion in thoroughbred horses. Equine Vet. J. 45:512–517. doi:10.1111/evj.12010.

Kearns, CF, KH McKeever, and T. Abe. 2002. Overview of horse body composition and muscle architecture: implications for performance. Vet J 164:224-234. doi: 10.1053/tvjl.2001.0702.

Kim, J., Hinchcliff, K. W., Yamaguchi, M. , Beard, L. A., Markert, C. D., and S. T. Devor. 2005. Exercise training increases oxidative capacity and attenuates exercise-induced ultrastructural damage in skeletal muscle of aged horses. J. Appl. Physiol. 98(1): 334-342.

Knapp, J.R., J.K. Davie, A. Myer, E. Meadows, E.N. Olson, and W.H. Klein. 2006. Loss of myogenin in postnatal life leads to normal skeletal muscle but reduced body size. Dev. 133: 601-610. doi: 10.1242/dev.02249.

Koh, J., C.R. Hancock. S. Terada, K. Higashida, J.O. Holloszy, and D. Han. PPARβ is essential for maintaining normal levels of PGC1-α and mitochondria and for the increase in muscle mitochondria induced by exercise. Cell Metab. 25: 1176-1185. doi: 10.1016/j.cmet.2017.04.029.

Kuang, S, K Kuroda, F Le Grand, and MA Rudnicki. 2007. Asymmetric self-renewal and commitment of satellite stem cells in muscle. Cell 129: 999-1010. doi: 10.1016/j.cell.2007.03.044.

Kurosaka, M., H. Naito, Y. Ogura, A. Kojima, K. Goto, and S. Katamoto. 2009. Effects of voluntary wheel running on satellite cells in the rat plantaris muscle. J. Sports Sci. Med. 8: 51-57.

Lacombe, V. A., K. W. Hinchcliff, C. W. Kohn, S. T. Devor, and L. E. Taylor. 2004. Effects of feeding meals with various soluble carbohydrate content on muscle glycogen

synthesis after exercise in horses. Amer. J. Vet. Res. 65(7): 916-923. doi: 10.2460/ajvr.2004.65.916.

LaVigne, E.K., A.K. Jones, A. Sanchez-Londoño, A.S. Schauer, D.F. Patterson, J.A. Nadeau, and S.A. Reed. 2015. Muscle growth in young horses: effects of age, cytokines, and growth factors. J. Anim. Sci. 93: 5672-5680. doi: 10.2527/jas2015-9634.

Le Grand, F., A.E. Jones, V. Seale, A. Scimè, and M.A. Rudnicki. 2009. Wnt7a activates the planar cell polarity pathway to drive the symmetric expansion of satellite stem cells. Cell Stem Cell 4: 535-547. doi: 10.1016/j.stem.2009.03.013.

Léger, B., R. Cartoni, M. Praz, S. Lamon, O. Dériaz, A. Crettenand, C. Gobelet, P. Rohmer, M. Konzelmann, F. Luthi, and A.P. Russell. 2006. Akt signalling through GSK-3β, mTOR and Foxo1 is involved in human skeletal muscle hypertrophy and atrophy. J. Physiol. 576(3): 923-933. doi: 10.1113/jphysiol.2006.116715.

Lepper, C and C Fan. 2010. Inducible lineage tracing of Pax7-descendant cells reveals embryonic origin of adult satellite cells. Genesis 48(7): 424-436. doi: 10.1002/dvg.20630.

Lepper, C, TA Partridge, and C Fan. 2011. An absolute requirement for Pax7-positive satellite cells in acute injury-induced skeletal muscle regeneration. Development 138: 3639-3646. doi: 10.1242/dev.067595.

Leshem, Y., D. B. Spicer, R. Gal-Levi, and O. Halevy. 2000. Hepatocyte growth factor (HGF) inhibits skeletal muscle cell differentiation: a role for the Bhlh protein twist and the cdk inhibitor p27. J. Cell. Physiol. 184:101–109. doi:10.1002/(SICI)1097-4652(200007).

Leshem, Y., and O. Halevy. 2002. Phosphorylation of pRb is required for HGF-induced muscle cell proliferation and is $p27^{kip1}$-dependent. J. Cell. Physiol. 191: 173-182. doi: 10.1002/jcp.10089.

Li, P, T Akimoto, M Zhang, RS Williams, and Z Yan. 2006. Resident stem cells are not required for exercise-induced fiber-type switching and angiogenesis but are necessary for activity-dependent muscle growth. Am J Physiol Cell Physiol 290: C1461-C1468. doi: 10.1152/ajpcell.00532.2005.

Liang, H., and W.F. Ward. 2006. PGC-1α: a key regulator of energy metabolism. Adv. Physiol. Educ. 30: 145-151. doi: 10.1152/advan.00052.2006.

Lin, J., H. Wu, P.T. Tarr, C.Y. Zhang, Z. Wu, O. Boss, L.F. Michael, P. Puigserver, E. Isotani, E.N. Olson, B.B. Lowell, R. Bassel-Duby, and B.M. Spiegelman. 2002. Transcriptional co-activator PGC-1α drives the formation of slow-twitch muscle fibers. Nature 418: 797-801. doi: 10.1038/nature00904.

Lindholm, A., Bjerneld, H, and B. Saltin. 1974. Glycogen depletion pattern in muscle fibres of trotting horses. Acta Physiologica. 90(2): 475-484. doi: 10.1111/j.1748-1716.1974.tb05610.x.

Lira, V.A., D.L. Brown, A.K. Lira, A.N. Kavazis, Q.A. Soltow, E.H. Zeanah, and D. S. Criswell. Nitric oxide and AMPK cooperatively regulate PGC-1α in skeletal muscle cells. J. Physiol. 588(18): 3551-3566. doi: 10.1113/jphysiol.2010.194035.

Machado, L., J. Esteves de Lima, O. Fabre, C. Proux, R. Legendre, A. Szegedi, H. Varet, L. Roed Ingerslev, R. Barrès, F. Relaix, and P. Mourikis. 2017. In situ fixation redefines quiescence and early activation of skeletal muscle stem cells. Cell Rep. 21: 1982-1993. doi: 10.1016/j.celrep.2017.10.080.

Mauro, A. 1961. Satellite cell of skeletal muscle fibers. J Biophys Biochem Cytol. 9: 493-495. doi: 10.1083/jcb.9.2.493.

McCarthy, J.J., and K.A. Esser. 2007. Counterpoint: Satellite cell addition is not obligatory for skeletal muscle hypertrophy. J. Appl. Physiol. 103(3): 1100-1103. doi: 10.1152/japplphysiol.00101.2007a.

McCroskery, S., M. Thomas, L. Maxwell, M. Sharma, and R. Kambadur. 2003. Myostatin negatively regulates satellite cell activation and self-renewal. J. Cell Biol. 162(6): 1135-1147. doi: 10.1083/jcb.200207056.

McGowan, C.M., L.C. Golland, D.L. Evans, D.R. Hodgson, and R.J. Rose. 2002. Effects of prolonged training, overtraining and detraining on skeletal muscle metabolites and enzymes. Equine Vet. J. 34:257-263. doi: 10.1111/j.2042-3306.2002.tb05429.x.

McKay, B.R., C.E. O'Reilly, S.M. Phillips, M.A. Tarnopolsky, and G. Parise. 2008. Co-expression of IGF-1 family members with myogenic regulatory factors following acute damaging muscle-lengthening contractions in humans. J. Physiol. 586(22): 5549-5560. doi: 10.1113/jphysiol.2008.160176.

Meadows, E., J. Cho, J.M. Flynn, and W.H. Klein. 2008. Myogenin regulates a distinct genetic program in adult muscle stem cells. Dev. Biol. 322(2): 406-414. doi: 10.1016/j.ydbio.2008.07.024.

Megeney, L.A., B. Kablar, K. Garret, J.E. Anderson, and M.A. Rudnicki. 1996. MyoD is required for myogenic stem cell function in adult skeletal muscle. Genes Dev. 10(10): 1173-1183.

Miller, K.J., D. Thaloor, S. Matteson, and G.K. Pavlath. 2000. Hepatocyte growth factor affects satellite cell activation and differentiation in regenerating skeletal muscle. Am. J. Physiol. Cell Physiol. 278: C174-C181. doi: 10.1152/ajpcell.2000.278.1.C174.

Mills, P. C., Scott, C. M., and D. J. Marlin. 1997. Effects of nitric oxide inhibition on thermoregulation during exercise in the horse. Ann N Y Acad. Sci. 813: 591-599. doi: 10.1111/j.1749-6632.1997.tb51750.x.

Mills, P. C., Marlin, D. J., Scott, C. M., and N. C. Smith. 1999. Metabolic effects of nitric oxide synthase inhibition during exercise in the horse. Res. Vet. Sci. 66(2): 135-138. doi: 10.1053/rvsc.1998.0258.

Morifuji, M., Kanada, A., Koga, J., Kawanaka, K., and M. Higuchi. 2010. Post-exercise carbohydrate plus whey protein hydrolysates supplementation increases skeletal muscle glycogen level in rats. Amino Acids. 38: 1109-1115. doi: 10.1007/s00726-009-0321-0.

Morita, M., Hayashi, T., Ochiai, M., Maeda, M., Yamaguchi, T., Ina, K., and M. Kuzuya. 2014. Oral supplementation with a combination of l-citrulline and l-arginine rapidly increases plasma l-arginine concentration and enhances NO bioavailability. Biochem. Biophys. Res. Comm. 454(1): 53-57. doi: 10.1016/j.bbrc.2014.10.029.

Moss, FP, and CP Leblond. 1971. Satellite cells as the source of nuclei in muscles of growing rats. Anat. Rec. 170(4): 421-435. doi: 10.1002/ar.1091700405.

Murach, K. A., S. H. White, Y. Wen, A. Ho, E. E. Dupont- Versteegden, J. J. McCarthy, and C. A. Peterson. 2017. Differential requirement for satellite cells during over- load-induced muscle hypertrophy in growing versus mature mice. Skelet. Muscle. 7:14. doi:10.1186/s13395-017-0132-z.

Nagata, Y., Y. Honda, and R. Matsuda. 2010. FGF2 induces ERK phosphorylation through Grb2 and PKC during quiescent myogenic cell activation. Cell Struct. Funct. 35: 63-71. doi: 10.1247/csf.09024.

Naldini, L., K.M. Weidner, E. Vigna, G. Gaudino, A. Bardelli, C. Ponzetto, R.P. Narsimhan, G. Hartmann, R. Zarnegar, and G.K. Michalopoulos. 1991. Scatter factor and hepatocyte growth factor are indistinguishable ligands for the MET receptor. EMBO J. 10(10): 2867-2878.

Novitch, B.G., D.B. Spicer, P.S. Kim, W.L. Cheung, and A.B. Lassar. 1999. pRb is required for MEF2-dependent gene expression as well as cell-cycle arrest during skeletal muscle differentiation. Curr. Biol. 9: 449-459.

O'Connor, R.S., and G.K. Pavlath. 2007. Point: Satellite cell addition is obligatory for skeletal muscle hypertrophy. J. Appl. Physiol. 103(3): 1099-1100. doi: 10.115/japplphysiol.00101.2007.

Olguin, H. C., and B. B. Olwin. 2004. Pax-7 up-regulation inhibits myogenesis and cell cycle progression in satellite cells: a potential mechanism for self-renewal. Dev. Biol. 275: 375-388. doi: 10.1016/j.ydbio.2004.08.015.

Organ, S.L., and M.S. Tsao. 2011. An overview of the c-MET signaling pathway. Ther. Adv. Med. Oncol. 3(1): S7-S19. doi: 10.1177/1758834011422556.

Pala, F, D Di Girolamo, S Mella, S Yennek, L Chatre, M Ricchetti, and S Tajbakhsh. 2018. Distinct metabolic states govern skeletal muscle stem cell fates during prenatal and postnatal myogenesis. J Cell Sci 131: jcs212977. doi: 10.1242/jcs.212977.

Pasut, A, NC Chang, U Gurriaran-Rodriguez, S Faulkes, H Yin, M Lacaria, H Ming, and MA Rudnicki. 2016. Notch signaling rescues loss of satellite cells lacking Pax7 and

promotes brown adipogenic differentiation. Cell Reports 16: 333-343. doi: 10.1016/j.celrep.2016.06.001.

Pereira, D.S., B.Z. de Queiroz, A.S. Miranda, N.P. Rocha, D.C. Felício, E.C. Mateo, M. Favero, F.M. Coelho, F. Jesus-Moraleida, D.A. Gomes Pereira, A.L. Teixeira, and L.S. Máximo Pereira. 2013. Effects of physical exercise on plasma levels of brain-derived neurotrophic factor and depressive symptoms in elderly women-a randomized clinical trial. Arch. Phys. Med. Rehab. 94(8): 1443-1450. doi: 10.1016/j.apmr.2013.03.029.

Perry, C.G.R., J. Lally, G.P. Holloway, G.J.F. Heigenhauser, A. Bonen, and L.L. Spriet. 2010. Repeated transient mRNA bursts precede increases in transcriptional and mitochondrial proteins during training in human skeletal muscle. J. Physiol. 588(23): 4795-4810. doi: 10.1113/jphysiol.2010.199448.

Petrella, J.K., J. Kim, D.L. Mayhew, J.M. Cross, and M.M. Bamman. 2008. Potent myofiber hypertrophy during resistance training in humans is associated with satellite cell-mediate myonuclear addition: a cluster analysis. J. Appl. Physiol. 104: 1736-1742. doi: 10.1152/japplphysiol.01215.2007.

Piñol-Jurado, P., E. Gallardo, N. de Luna, X. Suárez-Calvet, C. Sánchez-Riera, E. Fernández-Simón, C. Gomis, I. Illa, and J. Díaz. 2017. Platelet-derived growth factor BB influences muscle regeneration in Duchenne Muscle Dystrophy. Am. J. Pathol. 187(8): 1814-1827. doi: 10.1016/j.ajpath.2017.04.011.

Pizza, F. X., Flynn, M. G., Duscha, B. D., Holden, J., and E. R. Kubitz. 1995. A carbohydrate loading regimen improves high intensity, short duration exercise performance. Internat. J. Sport Nutr. 5: 110-116. doi: 10.1123/ijsn.5.2.110.

Porrello, A., M.A. Cerone, S. Coen, A. Gurtner, G. Fontemaggi, L. Cimino, G. Piaggio, A. Sacchi, and S. Soddu. 2000. p53 regulates myogenesis by triggering the differentiation activity of pRb. J. Cell Biol. 151(6): 1295-1303.

Quarta, M., J.O. Brett, R. DiMarco, A. De Morree, S.C. Boutet, R. Chacon, M.C. Gibbons, V.A. Garcia, J. Su, J.B. Shrager, S. Heilshorn, and T.A. Rando. 2016. An artificial niche preserves the quiescence of muscle stem cells and enhances their therapeutic efficacy. Nat. Biotech. 34(7): 752-759. doi: 10.1038/nbt.3576.

Quarta, M., and T.A. Rando. 2017. Methods and compositions to maintain stem cell quiescence. US Patent Appl. Pub. Pub. No. US 2017/0335287 A1.

Rimando, A. M., and P. M. Perkins-Veazie. Determination of citrulline in watermelon rind. 2005. J. Chromatog. A. 1078: 196-200.

Roberts, C. K., Barnard, R. J., Scheck, S. H., and T. W. Balon. 1997. Exercise-stimulate glucose transport in skeletal muscle is nitric oxide dependent. Am. J. Physiol. 273(1): E220-225. doi: 10.1152/ajpendo.1997.273.1.E220.

Robertson, R. P., Raymond, R. H., Lee, D. S., Calle, R. A., Ghosh, A., Shankar, S. S., Vassileva, M. T., Weir, G. C., and D. A. Fryburg. 2014. Arginine is preferred to glucagon for stimulation testing of β-cell function. Am. J. Physiol. Endrinol. Metab. 307(8): E720-727. doi: 10.1152/ajpendo.00149.2014.

Rocheteau, P, B Gayraud-Morel, I Siegl-Cachedenier, Ma Blasco, and S Tajbakhsh. 2012. A subpopulation of adult skeletal muscle stem cells retains all template DNA strands after cell division. Cell 148:112-125. doi: 10.1016/j.cell.2011.11.049.

Röckl, K.S., M.F. Hirshman, J. Brandauer, N. Fuji, L.A. Witters, L.J. Goodyear. 2007. Skeletal muscle adaptation to exercise training: AMP-activated protein kinase mediates muscle fiber type shift. Diabetes 56(8): 2062-2069. doi: 10.2337/db07-0255.

Rodgers, JT, KY King, JO Brett, MJ Cromie, GW Charville, KK Maguire, C Brunson, N Mastey, L Liu, C Tsai, MA Goodell, and TA Rando. 2014. mTORC1 controls the adaptive transition of quiescent stem cells from G_0 to G_{Alert}. Nature 510: 393-396. doi: 10.1038/nature13255.

Rodgers, J.T., M.D. Schroder, C. Ma, and T.A. Rando. 2017. HGFA is an injury-regulated systemic factor that induces the transition of stem cells into G_{Alert}. Cell Rep. 19: 479-486. doi: 10.1016/j.celrep.2017.03.066.

Ruas, J.L., J.P. White, R.R. Rao, S. Kleiner, K.T. Branna, B.C. Harrison, N.P. Greene, J.Wu, J.L. Estall, B.A. Irving, I.R. Lanza, K.A. Rasbach, M. Okutsu, K. Sreekumaran Nair, Z. Yan, LA. Leinward, and B.M. Spiegelman. 2012. A PGC1-α isoform induced by resistance training regulates skeletal muscle hypertrophy. Cell 151: 1319-1331. doi: 10.1016/j.cell.2012.10.050.

Rudolf, A. E. Schirwis, L. Giordani, A. Parisi, C. Lepper, M.M. Taketo, and F. Le Grand. 2016. β-catenin activation in muscle progenitor cells regulates tissue repair. Cell Rep. 15: 1277-1290. doi: 10.1016/j.celrep.2016.04.022.

Seale, P, LA Sabourin, A Girgis-Gabardo, A Mansouri, P Gruss, and MA Rudnicki. 2000. Pax7 is required for the specification of myogenic satellite cells. Cell 102 (6): 777-786. doi: 10.1016/S0092-8674(00)00066-0.

Selye, H. 1936. A syndrome produced by diverse nocuous agents. Nature 138: 32.

Shea, KL, W Xiang, VS LaPorta, JD Licht, C Keller, MA Basson, AS Brack. 2010. Sprouty1 regulates reversible quiescence of a self-renewing adult muscle stem cell pool during regeneration. Cell Stem Cell 6: 117-129. doi: 10.1016/j.stem.2009.12.015.

Sheehan, SM, and RE Allen. 1999. Skeletal muscle satellite cell proliferation in response to members of the fibroblast growth factor family and hepatocyte growth factor. J Cell Physiol 181:499-506. doi: 10.1002/(SICI)1097-4652(199912)181:3<499::AID-JCP14>3.0.CO;2-1.

Sheehan, S.M., R. Tatsumi, C.J. Temm-Grove, and R.E. Allen. 2000. HGF is an autocrine growth factor for skeletal muscle satellite cells in vitro. Muscle Nerve 23: 239-245.

Shefer, G, DP Van de Mark, JB Richardson, and Z Yablonka-Reuveni. 2006. Satellite-cell pool size does matter: defining the myogenic potency of aging skeletal muscle. Dev Biol 294(1): 50-66. doi: 10.1016/j.ydbio.2006.02.022.

Shefer, G, G Rauner, Z Yablonka-Reuveni, and D. Benayahu. 2010. Reduced satellite cell numbers and myogenic capacity in aging can be alleviated by endurance exercise. PLoS ONE 5(10): e13307. doi: 10.1371/journal.pone.0013307.

Shimomura, T., J. Kondo, M. Ochia, D. Naka, K. Miyazawa, Y. Morimoto, and N. Kitamura. 1993. Activation of the zymogen of hepatocyte growth factor activator by thrombin. J. Biol. Chem. 268(30): 22927-22932

Street, D., J. Bangsbo, and C. Juel. 2001. Interstitial pH in human skeletal muscle during and after dynamic graded exercise. J. Physiol. 537(3): 993-998.

Stupka, N., M.A. Tarnopolsky, N.J. Yardley, and S.M. Phillips. 2001. Cellular adaptation to repeated eccentric exercise-induced muscle damage. J. Appl. Physiol. 91(4): 1669-1678. doi: 10.1152/jappl.2001.91.4.1669.

Takeda, K., Machida, M., Kohara, A., Omi, N., and T. Takemasa. 2011. Effects of citrulline supplementation on fatigue and exercise performance in mice. J. Nutr. Sci. Vitaminol. 57: 246-250. doi: 10.3177/jnsv.57.246.

Talmadge, R.J., J.S. Otis, M.R. Rittler, N.D. Garcia, S.R. Spencer, S.J. Lees, and F.J. Naya. 2004. Calcineurin activation influences muscle phenotype in a muscle-specific fashion. BMC Cell Biol. 5: 28. doi: 10.1186/1471-2121-5-28.

Tatsumi, R., J.E. Anderson, C.J. Nevoret, O. Halevy, and R.E. Allen. 1998. HGF/SF is present in normal adult skeletal muscle and is capable of activating satellite cells. Dev. Biol. 194: 114-128.

Tatsumi, R., S.M. Sheehan, H. Iwasaki, A. Hattori, and R.E. Allen. 2001. Mechanical stretch induces activation of skeletal muscle satellite cells in vitro. Exp. Cell Res. 267: 107-114. doi: 10.1006/excr.2001.5252.

Tatsumi, R., A. Hattori, Y. Ikeuchi, J. E. Anderson, and R. E. Allen. 2002. Release of hepatocyte growth factor from mechanically stretched skeletal muscle satellite cells and role of Ph and nitric oxide. Mol. Biol. Cell. 13:2909–2918. doi:10.1091/mbc.E02-01-0062.

Tyagi, S., P. Gupta, A. Singh Saini, C. Kaushal, and S. Sharma. 2011. The peroxisome proliferator-activated receptor: A family of nuclear receptors role in various diseases. J. Adv. Pharm. Technol. Res. 2(4): 236-240. doi: 10.4103/2231-4040.9089.

Villareal, M. O., Matsukawa, T., and H. Isoda. 2018. L-Citrulline Supplementation-Increased Skeletal Muscle PGC-1α Expression Is Associated with Exercise Performance and Increased Skeletal Muscle Weight. Mol. Nutr. Food Res. 62: e1701043. doi: 10.1002/mnfr.201701043.

von Maltzahn, J, AE Jones, RJ Parks, and MA Rudnicki. 2013. Pax7 is critical for the normal function of satellite cells in adult skeletal muscle. Proc Nat Acad Sci 110(41): 16474-16479. doi: 10.1073/pnas.1307680110.

Wagner, K.D., and N. Wagner. 2010. Peroxisome proliferator-activated receptor beta/delta (PPARbeta/delta) acts as regulator of metabolism linked to multiple cellular functions. Pharmacol. Ther. 125(3): 423-435. doi: 10.1016/j.parmthera.2009.12.001.

Wang, Y., C. Zhang, R.T. Yu, H.K. Cho, M.C. Nelson, C.R. Bayuga-Ocampo, J. Ham, H. Kang, and R.M. Evans. 2004. Regulation of muscle fiber type and running endurance by PPARδ. PLoS Biol. 2(10): e294. doi: 10.1371/journal.pbio.0020294

Wang, B., Ding, Z., Wang, W., Hwang, J., Liao, Y., and J. L. Ivy. 2015. The effect of an amino acid beverage on glucose response and glycogen replenishment after strenuous exercise. Eur. J. Appl. Physiol. 115: 1283-1294. doi: 10.1007/s00421-015-3098-8.

Wang, G., H. Zhu, C. Situ, L. Han, Y. Yu, T.H. Cheung, K. Liu, and Z. Wu. 2018. p110α of PI3K is necessary and sufficient for quiescence exit in adult muscle satellite cells. EMBO J. 37: e98239. doi: 10.15252/embj.201798239.

White, RB, Biérinx, VF Gnocchi, and PS Zammit. 2010. Dynamics of muscle fibre growth during postnatal mouse development. Dev Biol 10:21. doi:10.1186/1471-213X-10-21.

Wilkinson, S.B., S.M. Phillips, P.J. Atherton, R. Patel, K.E. Yarasheski, M.A. Tarnopolsky, and M.J. Rennie. 2008. Differential effects of resistance and endurance exercise in the fed state on signaling molecule phosphorylation and protein synthesis in human muscle. J. Physiol. 586(15): 3701-3717. doi: 10.1113/jphysiol.2008.153916.

Wozniak, A.C., O. Pilipowicz, Z. Yablonka-Reuveni, S. Greenway, S. Craven, E. Scott, and J.E. Anderson. 2003. C-met expression and mechanical activation of satellite cells on cultured muscle fibers. J. Histochem. Cytochem. 51(11): 1437-1445. doi: 10.1177/002215540305101104.

Yablonka-Reuveni, M.A. Rudnicki, A.J. Rivera, M. Primig, J.E. Anderson, and P. Natanson. 1999. The transition from proliferation to differentiation is delayed in satellite cells from mice lacking MyoD. Dev. Biol. 210: 440-455. doi: 10.1006/dbio.1999.9284.

Yamada, M., R. Tatsumi, K. Yamanouchi, T. Hosoyama, S. Shiratsuchi, A. Sato, W. Mizunoya, Y. Ikeuchi, M. Furuse, and R. E. Allen. 2009. High concentrations of HGF inhibit skeletal muscle satellite cell proliferation in vitro by inducing expression of myostatin: a possible mechanism for reestablishing satellite cell quiescence in vivo. Am. J. Physiol. Cell Physiol. 298: C465-C476.doi: 10.1152/ajpcell.00449.2009.

Yaspelkis, B. B., and J. L. Ivy. 1999. The effect of a carbohydrate-arginine supplement on postexercise carbohydrate metabolism. Internat. J. Sport Nutr. 9(3)241-250. doi: 10.1123/ijsn.9.3.241.

Zhu, L., Y. Ren, J. Chen, and Y. Wang. 2013. Effects of myogenin on muscle fiber types and key metabolic enzymes in gene transfer mice and C2C12 myoblasts. Gene 532: 246-252. doi: 10.1016/j.gene.2013.09.028.

Zismanov, V, V Chichkov, V Colangelo, S Jamet, S Wang, A Syme, AE Koromilas, and C Crist. 2016. Phosphorylation of eIF2α is a translational control mechanism regulating muscle stem cell quiescence and self-renewal. Cell Stem Cell 18: 79-90. doi: 10.1016/j.stem.2015.09.020.

Zurlo, F, K Larson, C. Bogardus, and E. Ravussin. 1990. Skeletal muscle metabolism is a major determinant of resting energy expenditure. J Clinical Investigation 86: 1423-1427. doi: 10.1172/JCI11485.

CHAPTER 2

Hepatocyte growth factor acts as a mitogen for equine satellite cells via protein kinase δ-directed signaling

Co-authors: Joanna M. Kania, Madison L. Gonzalez, and Sally E. Johnson

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ABSTRACT

Hepatocyte growth factor (HGF) signals mediate mouse skeletal muscle stem cell, or satellite cell (SC), reentry into the cell cycle and myoblast proliferation. Because the athletic horse experiences exercise-induced muscle damage, the objective of the experiment was to determine the effect of HGF on equine SC (eqSC) bioactivity. Fresh isolates of adult eqSC were incubated with increasing concentrations of HGF and the initial time to DNA synthesis was measured. Media supplementation with HGF did not shorten ($P > 0.05$) the duration of G_0/G_1 transition suggesting the growth factor does not affect activation. Treatment with 25 ng/mL HGF increased ($P < 0.05$) eqSC proliferation that was coincident with phosphorylation of extracellular signal-regulated kinase (ERK)1/2 and AKT serine/threonine kinase 1 (AKT1). Chemical inhibition of the upstream effectors of ERK1/2 or AKT1 elicited no effect ($P > 0.05$) on HGF-mediated 5-eth- ynyl-2′-deoxyuridine (EdU) incorporation. By contrast, treatment of eqSC with 2 μm Gö6983, a pan-protein kinase C (PKC) inhibitor, blocked ($P < 0.05$) HGF-initiated mitotic activity. Gene expression analysis revealed that eqSC express PKCα, PKCδ, and

PKCε isoforms. Knockdown of PKCδ with a small interfering RNA (siRNA) prevented ($P > 0.05$) HGF-mediated EdU incorporation. The siPKCδ was specific to the kinase and did not affect ($P > 0.05$) expression of either PKCα or PKCε. Treatment of confluent eqSC with 25 ng/mL HGF suppressed ($P < 0.05$) nuclear myogenin expression during the early stages of differentiation. These results demonstrate that HGF may not affect activation but can act as a mitogen and modest suppressor of differentiation.

INTRODUCTION

Satellite cells (SC), a population of muscle stem and progenitor cells, are located adjacent to the skeletal muscle fiber under the basal lamina (Mauro, 1961). The SC typically resides in a quiescent state but becomes mitotically active in response to muscle damage. Genetic ablation of SC within the adult mouse completely abolishes skeletal muscle regeneration demonstrating its essential role in damage repair (Sambasivan et al., 2011; von Maltzahn et al., 2013). A case for their involvement in muscle hypertrophy also exists. Removal of $\geq 90\%$ of SC in adult mice prevented fiber hypertrophy in response to synergist ablation (SA) of soleus and gastrocnemius muscles (Egner et al., 2016). Using a different approach, disruption of SC fusogenic capabilities prevented fiber hypertrophy during SA further supporting a role for the cell type during growth (Goh and Millay, 2017). These findings are in stark contrast to others demonstrating that SC are not required for overload-induced hypertrophy (McCarthy et al., 2011), atrophic recovery (Jackson et al., 2012), or fiber remodeling and plasticity (Lee et al., 2016; Murach et al., 2017a, 2017b). Differences may be attributed to genetic ablation models, animal age, and subtleties in hypertrophy measures.

Contribution of the SC to growth and regeneration requires exit from G_0 followed

by commitment to the myoblast lineage and progression through myogenesis; a subpopulation undergoes self-renewal to maintain the muscle precursor pool. Multiple growth factors, cytokines, and other signaling molecules that reside within the fiber niche work in concert to regulate myogenesis. For example, Notch signals are required for SC self-renewal and quiescence (Bjornson et al., 2012; Bi et al., 2016). Genetic ablation of *Hes*/*Hey*, Notch target genes, causes SC to undergo precocious differentiation (Fukada et al., 2011). Migration and motility of SC to sites of microdamage are controlled by spatial localization of ephrins and their ligands on myofibers and interstitial cells (Stark et al., 2011; Gu et al., 2016). Expansion of the SC pool to support fiber damage repair relies upon the mitogenic actions of fibroblast growth factor (FGF) 2 and FGF receptor signaling (Yablonka-Reuveni et al., 2015; Galimov et al., 2016; Pawlikowski et al., 2017). Fusion of SC is positively regulated by IGF-1 and multiple genetic models demonstrate the hypertrophic effects of the growth factor on skeletal muscle (Kandalla et al., 2011; Schiaffino and Mammucari, 2011). The presence and diverse actions of these niche-localized factors underscore the complexity of signals present during normal growth as well as injury repair.

Hepatocyte growth factor (HGF) is an autocrine growth factor produced by the SC that participates in multiple stages of myogenesis (Anderson, 2016). Early work established that HGF activates G_0 exit of quiescent SC and inhibition of HGF signaling delays cell cycle entry in vitro (Allen et al., 1995; Miller et al., 2000; Sheehan et al., 2000). The bio- availability of HGF to the SC is controlled sequentially by nitric oxide production, which in turn causes the enzymatic release of membrane-tethered HGF allowing the ligand to dock with the HGF receptor (MET; Tatsumi et al., 2002; Wozniak

et al., 2003; Wozniak and Anderson, 2007, 2009; Tatsumi et al., 2009). In addition to its role as an activator, HGF promotes SC proliferation through recruitment of GRB2-associated-binding protein (Gab1) and tyrosine-protein phosphatase nonreceptor type-11 (SHP2) to the MET kinase domain which may allow for sustained ERK1/2 phosphorylation (Leshem et al., 2002; Halevy and Cantley, 2004; Li et al., 2009). The local concentration of HGF and intracellular ERK1/2 signal intensity are critical to SC biology as elevation in either leads to cell cycle exit (Reed et al., 2007; Yamada et al., 2010). The role of HGF during myoblast differentiation remains unresolved. Satellite cell-specific ablation of MET impairs migration, motility, and myoblast fusion during muscle regeneration (Webster and Fan, 2013). Human primary myoblasts cultured in differentiation permissive conditions increase fusion and myotube formation in response to HGF treatment (Walker et al., 2015). Treatment of mice with a synthetic, bivalent HGF protein initiates MET signal transduction through AKT serine/threonine kinase 1 (AKT1) to promote muscle fiber hypertrophy (Cassano et al., 2008). By contrast, others report that HGF inhibits SC differentiation in vitro and injection of the growth factor blunts fiber formation during damage repair (Leshem et al., 2000; Miller et al., 2000).

The athletic horse, similar to humans, experiences skeletal muscle damage upon completion of strenuous sporting activities. Several growth factors are expressed in muscle during the immediate post- race recovery period including HGF (Kawai et al., 2013). Due to the importance of HGF as a regulator SC function, the objectives of the experiment were to determine its effects on equine SC (eqSC) bioactivity and to identify the signaling systems that mediate HGF effects.

MATERIALS AND METHODS

Animal Care

Animal work was reviewed and approved by the Institutional Animal Care and Use Committee at Virginia Polytechnic Institute and State University (16–074).

Satellite Cell Isolation and Culture

Adult light breed geldings (n = 6; 3–8 yr of age) were utilized for all studies. Animals were sedated with xylazine (1 mg/kg; Bimeda-MTC Animal Health, Cambridge, ON) and administered a local anesthetic (Lidocaine; Aspen Veterinary Resources, Liberty, MO) subcutaneously atop the middle gluteal muscle. The muscle was chosen for sampling due to the large amount of data available and its participation in strenuous exercise. Biopsies (~200 mg/sample) were retrieved with a vacuum-assisted biopsy device (Vacora Biopsy System, 10 G; C. R. Bard, Tempe, AZ) and maintained individually in wash buffer [WB; 5% fetal bovine serum (FBS), 5% penicillin-streptomycin, 0.2% gentamicin in PBS] on ice prior to transport and processing in the lab. Muscle samples were washed three times with WB, dissected free of fat and connective tissue, and minced using sterile dis- section scissors. Minced tissue was incubated at 37 °C for 40 min in 1 mg/Ml protease (Type XIV, Sigma Aldrich, St. Louis, MO) in PBS. The tissue slurry was passed through a 70 μm cell strainer and retained fiber fragments were collected in WB, vortexed 2 min, and passed through a 40 μm cell strainer. The filtrate containing eqSC was collected by centrifugation at 800 × g for 5 min. The pellet was resuspended in Dulbecco's Modified Eagle Medium (DMEM) containing 20% FBS, 1% penicillin-streptomycin, 0.2% gentamicin, and 4 ng/mL recombinant human FGF-2 (growth media; GM). Equine SC were seeded onto entactin–

collagen–laminin (ECL; EMD Millipore) coated plates and cultured at 37 °C in a humidified, 5% CO_2-controlled atmosphere. All culture media and supplements were purchased from Gibco-ThermoFisher, Waltham, MA. Myogenicity (>90%) was confirmed by Pax7, myogenin, and myosin heavy chain (MyHC) reactivity prior to commencement of experiments. Cells were manually dissociated from the substratum with a sterile cell scraper for passage and expansion prior to proliferation and differentiation assays. Biological specimens were maintained and treated individually throughout all assays. All assays included a minimum of four biological replicates in duplicate.

Lag Period Measurement

Immediately following eqSC isolation, cells (n = 5) were seeded equally into ECL-coated 48-well plates and allowed to attach to the matrix over- night. At 22 h post-seeding, the cells were washed repeatedly with WB and re-fed GM or placed into DMEM + 1% FBS + 1% penicillin-streptomycin + 0.2% gentamicin (basal medium; BM) supplemented with 1, 5, 10, or 25 ng/mL recombinant human HGF (HGF; R&D Systems, Minneapolis, MN), doses of HGF demonstrated to affect cell cycle or regeneration, or 0.1% BSA in PBS control (Miller et al., 2000). At 24-h intervals subsequent, eqSC were pulsed for 2 h with 10 Mm 5-ethynyl-2′-deoxyuridine (EdU), a thymidine analog. Cells were fixed with 4% paraformaldehyde (PFA) in PBS for 15 min at room temperature at the end of the EdU pulse period. EdU was detected with click chemistry (Click-It EdU AlexaFluor488, Invitrogen, Carlsbad, CA) and total nuclei were visualized with Hoechst 33342 (10 μg/mL). A mitotic index was calculated as EdU (+)/total Hoechst (+) × 100.

Proliferation and Differentiation Assays

Serially passaged eqSC at 30% confluency were treated with 1, 5, 10, or 25 ng/mL HGF or 0.1% BSA PBS control in BM for 48-h proliferation assays, with EdU included during the final 2 h prior to PFA fixation. For assays involving the inhibition of signaling intermediates, chemical inhibitors or an equal amount of dimethyl sulfoxide were added for 1 h prior to and for the duration of the stimulus. Chemical inhibitors and concentrations were 25 mM PD98059 (Cell Signaling Technology, Danvers, MA), 200 nM wortmannin (Cell Signaling Technology), and 2 mM Gö6983 (Tocris Bioscience, Minneapolis, MN).

Equine SC at 80% confluency were treated with 25 ng/mL HGF or 0.1% BSA in PBS in low-glucose DMEM containing 1% FBS, 1% penicillin-streptomycin, and 0.2% gentamicin (differentiation media) for 24 or 48 h for analysis of early and late differentiation, respectively. Cells were fixed with PFA for 15 min at room temperature or acidified formalin alcohol on ice for 4 mins then washed repeatedly with PBS. Nonspecific antigen sites were blocked with 3% BSA in PBS containing 0.1% Triton ×100 for 20 min followed by overnight incubation with anti-myogenin hybridoma supernatant (F5D, Developmental Studies Hybridoma Bank, Iowa City, IA) or anti-MyHC hybridoma supernatant (MF20, Developmental Studies Hybridoma Bank) diluted 1:5 in blocking buffer. Cells were washed with PBS prior to 1-h incubation with goat anti- mouse IgG AlexaFluor568 (1:200; ThermoFisher), 10 μg/mL Hoechst 33342. Representative photomicrographs were captured at equal shutter speeds to ensure consistent intensity at 200-fold magnification with a Nikon Eclipse TS100 epifluorescent microscope (Nikon Imaging Corp., Melville, NY) connected to a CoolSNAP HQ2

camera (Photometrics, Tuscon, AZ) and digitized with NIS Elements AR Ver4.13.00 software (Nikon). As an indicator of early differentiation, an index was calculated as myogenin (+)/Hoechst (+) × 100. Fusion index was calculated as (number of Hoechst 33342 positive cells residing within the myotube)/ (total number of Hoechst 33342 positive cells) × 100. Representative images are shown in black and white for ease of reading.

Small Interfering RNA Knockdown of PKC Isoforms

Semi-confluent eqSC were transiently transfected with 5 nM of scrambled or target-specific small interfering RNA (siRNA) (Lipofectamine RNAiMAX; Invitrogen), according to manufacturer's protocols. Oligonucleotides were custom synthesized by Invitrogen (Table 1). After 24 h, the media was replaced with BM supplemented with 25 ng/mL HGF or an equivalent amount of 0.1% BSA in PBS. Cells were pulsed with 10 mM EdU 2 h prior to fixation at 24 h in treatment. Detection of EdU was performed using Click-it EdU detection kit (Click-It EdU AlexaFluor 488; Invitrogen) and nuclei were detected with Hoechst 33342 (10 μg/mL). EdU (+) and Hoechst (+) cells were visualized and enumerated for the calculation of mitotic index, as described above.

Quantitative Reverse-Transcription PCR

Total RNA was extracted using Trizol reagent coupled with column purification (PureLink RNA Mini Kit, Invitrogen). Genomic DNA was removed by DNAse I (Invitrogen) digestion. RNA quantification and purity were assessed by spectrophotometry (Nanodrop, ThermoFisher). One microgram of total RNA was reverse transcribed (High Capacity Cdna kit, ThermoFisher) in a final volume of 20 mL. Fifty nanograms of cDNA was amplified with DNA polymerase (GoTaq Green master mix,

Promega, Madison, WI) and gene-specific primers for glyceraldehyde phosphate dehydrogenase (F, 5-CCACCCCTAACGTGTCAGTC, R, 5-AATCGCAGGAGACAACCTGG, efficiency = 0.89), pan-protein kinase C (PKC) α (F, 5-GACTCCCTGGTATGTGCTCG, R, 5-GCTC CTCACAAGACCGGAAA, efficiency = 0.84), PKCδ (F, 5-GTTCCCAACAATGAACCGCC, R, 5-CAGAAGGTGGGCTGCCTAAA, efficiency = 0.85), and PKCε (F, 5-TGTCAACATG CCCCACAAGT, R, 5-TGTCATTGCACAACAG AGG, efficiency = 0.85). Polymerase chain reaction conditions were 95 °C for 2 min followed by 40 cycles of 95 °C for 30 s, 60 °C for 30 s, and 72 °C for 30 s. A final extension of 72 °C for 5 min was performed. Amplicons were electrophoretically separated through 1.2% TAE agarose gels impregnated with SYBR-Safe dye (Invitrogen). Amplicon size was confirmed by comparison to a molecular weight ladder (*ex*ACTG*ene* DNA Ladder, Fisher Scientific, Waltham, MA). Quantitative PCR was performed with 5 ng cDNA and gene-specific primers using SYBR chemistry (Power SYBR Green PCR, ThermoFisher) in an Eppendorf Realplex thermocycler (Eppendorf, Hamburg, Germany). The optimum thermal cycling parameters included 95 °C for 10 min, 40 cycles of 95 °C for 15 s and 60 °C for 1 min. A melt curve was generated by 95 °C for 15 s, 60 °C for 15 s followed by 1.75 °C/ min for 20 min. Glyceraldehyde phosphate dehydrogenase was used as a housekeeping gene for normalization and fold change for all the samples relative to its own scrambled control was calculated using the $2^{-\Delta\Delta Ct}$ method.

Western Blotting

Equine SC (n = 5) were lysed in ice-cold radioimmunoprecipitation assay buffer containing pro- tease and phosphatase inhibitors (Halt Inhibitor Cocktail, ThermoFisher).

Protein concentration was measured by bicinchoninic acid assay (ThermoFisher). Twenty micrograms of protein were denatured with lithium dodecyl sulfate sample buffer (ThermoFisher) at 95 °C for 5 min prior to electrophoretic separation through 10% Tris-glycine gels (Novex Wedge Well, ThermoFisher). Proteins were transferred to nitrocellulose (iBlot 2, ThermoFisher). Membranes were blocked in 0.1% Tween-20 Tris buffered saline (TBST) containing 5% nonfat dry milk for 1 h. Membranes were washed 3 × 5 min with TBST prior to incubation with primary antibodies diluted in TBST containing 5% BSA overnight at 4 °C. Antibodies (Cell Signaling Technology) and dilutions were anti-ERK1/2, 1:1000, anti-phosphoERK1/2, 1:2000, anti-AKT1, 1:1000, anti-phosphoAKT1, 1:2000, and anti- beta tubulin, 1:1000. Membranes were washed with TBST and incubated with goat anti-rabbit IgG peroxidase (1:1000, ThermoFisher) for 1 h at room temperature. Following TBST washes, the antigen-antibody complexes were visualized by chemiluminescence (ECL, ThermoFisher) using a Chemi-Doc MP system equipped with Image Lab software (BioRad, Hercules, CA).

Statistics

The GLM procedure of SAS (SAS Enterprise Guide, Cary, NC) was used to generate a one- or two-way ANOVA. The model included horse, concentration of growth factor, chemical additive, the interaction between growth factor and chemical additive, and hours in culture where appropriate. Technical replicates were averaged for each biological replicate prior to calculation of least square means and SEM. Post-test comparisons were performed using Tukey's adjustment to analyze pre-planned comparisons between groups. Significance was set at $P < 0.05$.

RESULTS

Hepatocyte growth factor is regarded as an activator of rodent SC that facilitates their exit from G_0 and decreases the time to first division in vitro (Anderson, 2016). To determine if the growth factor elicits similar effects on eqSC, isolates from adult horses were treated with increasing concentrations of HGF for 72 h and the numbers of mitotic cells were enumerated daily. Results demonstrate the presence of EdU (+) cells after 48 h of treatment indicating the cells have exited G_0 and transitioned into S-phase (Fig. 1). No differences ($P > 0.05$) in the percentage of cells incorporating the thymidine analog were evident in the presence of 0, 1, 5, 10, or 25 ng/mL HGF after 48 h. Treatment with 25 ng/mL increased ($P < 0.05$) proliferation at 72 h by comparison to controls receiving vehicle-only. No differences ($P > 0.05$) in proliferation rate were evident at 72 h for 0, 1, 5, or 10 ng/mL HGF.

To further evaluate the effects of HGF on proliferation, serial-passaged eqSC were treated for 48 h with BM supplemented with 0, 1, 5, 10, and 25 ng/mL HGF and a mitotic index calculated. Results demonstrate that only 25 ng/mL HGF stimulated ($P < 0.05$) cell proliferation (Fig. 2A). Previous studies indicate that ERK1/2 and AKT1 are activated in response to HGF (Halevy and Cantley, 2004). The status of these two signaling axes was investigated by Western blot using lysates prepared from cells treated with the various concentrations of HGF. After 20 min of stimulation, eqSC exhibited phosphorylation of both AKT1 and ERK1/2 (Fig. 2B). No apparent differences in chemiluminescent band intensity for the phosphoproteins was evident by visual appraisal suggesting that 1 ng/mL was as effective as 25 ng/mL at initiating AKT1 and ERK1/2 modification. Interestingly, HGF stimulates preferentially the phosphorylation of ERK2

by comparison to ERK1.

Using a dose of 25 ng/mL HGF, the necessity of ERK1/2 activation on eqSC proliferation was evaluated. Treatment with HGF increased ($P < 0.05$) proliferation, as measured by EdU incorporation, by comparison to control (CON; Fig. 3A). Incubation of eqSC with HGF in the presence of a MEK1/2 inhibitor (PD98059) did not suppress ($P > 0.05$) the proliferative effect. The concentration of MEK1/2 inhibitor was sufficient to block ERK1/2 phosphorylation (Fig. 3B). Thus, HGF initiation of ERK1/2 phosphorylation does not underlie its ability to stimulate eqSC proliferation. In a similar manner, eqSC were treated with HGF in the presence or absence of wortmannin. HGF increased proliferation ($P < 0.05$) that remained unaffected ($P > 0.05$) by inclusion of the phosphoinositide 3-kinase (PI3K) inhibitor (Fig. 3C). Western blot confirmed that 200 Nm wortmannin was sufficient to prevent phosphorylation and subsequent activation of AKT1 (Fig. 3D).

The finding that neither AKT1 nor ERK1/2 activity was required for HGF-initiated eqSC proliferation was unexpected. Because PKC isoforms can increase mitotic rates in other cells (Poli et al., 2014), we chose to examine this system as a modulator of HGF effects. Semi-confluent eqSC were treated for 48 h in basal media supplemented with 25 ng/mL HGF with or without 2 mM Gö6983, an inhibitor that preferentially targets both classical and novel PKC isoforms. HGF stimulated ($P < 0.05$) eqSC proliferation by comparison to CON treated with vehicle-only (Fig. 4A). Co-incubation of cells with HGF and Gö6983 diminished ($P < 0.05$) EdU incorporation to levels observed in CON. Thus, a PKC isoform likely is involved in HGF-driven eqSC division. To narrow the list of potential candidates, total RNA was isolated from semi-confluent

eqSC, reverse transcribed and amplified with gene-specific primers for PKCα, PKCβ, PKCδ, PKCε, and PKCζ. End-point PCR reveals that eqSC express PKCα, PKCδ, and PKCε; no amplicons for PKCβ and PKCζ were detected (Fig. 4B).

The contribution of the PKC isoforms to HGF-mediated proliferation was examined using siRNA methodology. In brief, siRNA specific for PKCα, PKCδ, and PKCε (siPKCα, siPKCδ, siPKCε) were designed and transfected into semi-confluent eqSC. Scrambled oligonucleotides for each of the PKC isoforms were included as controls (siCON) for nonspecific effects. Twenty-four hours after siRNA loading, eqSC were treated with HGF for 24 h and a mitotic index calculated. Results demonstrate that treatment of eqSC with siCON or siPKCα proliferated ($P < 0.05$) in response to 25 ng/mL HGF (Fig. 5A). A similar result was found for siPKCε (Fig. 5B). By contrast, eqSC ectopically expressing siPKCδ failed to respond ($P > 0.05$) to HGF treatment, retaining a proliferation rate similar to vehicle-treated siCON (Fig. 5C). Despite multiple attempts, antibodies against the PKC isoforms failed to detect the respective proteins by western blot. Thus, Mrna knockdown was confirmed by real-time PCR for the siPKCα, siPKCδ, and siPKCε oligonucleotides (Fig. 5D). Transfection of the isoform-specific siRNA resulted in an approximate 70, 50, and 60% reduction ($P < 0.05$) in PKCα, PKCδ, and PKCε, respectively, in the absence of detect- able off-targeting ($P > 0.05$; Fig. 5E–G). Reduced PKCδ mRNA prevented eqSC response to 25 ng/mL HGF, in contrast to loss of either PKCα or PKCε. Thus, the results provide a role for PKCδ as a transducer of HGF signals that support eqSC proliferation.

Increased differentiation was reported following treatment of human SC with HGF (Walker et al., 2015). The role of HGF during eqSC myotube formation was

examined in vitro. Cultures at 80% confluency were treated with 25 ng/mL HGF for 24 h or 48 h followed by fixation. Immunocytochemical detection of myogenin revealed fewer cells express the transcription factor at 24 h (Fig. 6A). Enumeration of total and myogenin (+) nuclei indicates HGF reduces ($P < 0.05$) the percentage of terminally differentiated myoblasts by approximately 30% (Fig. 6B). By 48 h in culture both CON and HGF cultures contain large multinucleated myotubes (Fig. 6C) with no difference ($P > 0.05$) in the calculated fusion index (Fig. 6D). Thus, HGF serves to delay but not prevent myogenesis.

DISCUSSION

The skeletal muscle of athletes performing strenuous exercise undergoes a period of post- event recovery that includes activation of SC to repair fiber microdamage (Paulsen et al., 2012; Kawai et al., 2013; Bryan et al., 2017). Increased expression of several cytokines and growth factors is observed prior to SC activation suggesting they may play a role in modulating SC bioactivity. In the horse, HGF and Pax7 mRNA expression increase nearly 2-fold at day 3 of the exercise-recovery period (Kawai et al., 2013). Because HGF plays a significant role during SC exit from G in rodents, we investigated the ability of the growth factor to perform a similar function in eqSC. Interestingly, our results demonstrate that supplementation of eqSC with HGF does not alter the time to S-phase following initial seeding. Thus, HGF may not be the requisite activator of eqSC. Species-specific differences are noted between human and mouse SC activation (Quarta et al., 2016). To prolong quiescence, mouse SC are cultured with both MET and FGF receptor inhibitors while only the FGF receptor inhibitor is supplemented to the human SC. FGF2 is present in the initial eqSC plating media prior to its

replacement with low serum treatment medium thus, the mitogen could serve as an activator during that 24 h window. It should be noted that neither the metabolic activation status of the cells nor their residence in G_1 was measured. It is possible that the eqSC are primed (G_{alert}) and ready to progress into the cell cycle but arrested at a restriction point due to insufficient growth factors. The inability of HGF to reduce the initial lag period also may be attributed to subtle differences in ligand:receptor interactions. The human HGF used in these studies is 91% identical at the amino acid level to equine HGF. The strong amino acid homology coupled with the ability of human HGF to stimulate phosphorylation of intracellular signaling kinases supports effective ligand docking with the equine MET receptor. Importantly, SC synthesize and secrete HGF and autocrine HGF may cause local activation of the cell and prevent a response to exogenous growth factor due to receptor down-regulation (Sheehan et al., 2000). This aspect should be explored in future experiments. From the current experiments, we interpret the results to conclude that HGF-alone is unable to accelerate G_1 progression as indicated by no change in the initial lag period.

Satellite cell migration and proliferation are controlled by specific intracellular signaling pathways initiated following HGF binding to MET, the cognate receptor. Ligand docking and MET kinase domain *trans*phosphorylation lead to recruitment of the scaffold protein, Gab1. Genetic ablation of *Gab1* results in compromised muscle formation due to the inability of migratory muscle progenitor cells to populate the analages (Sachs et al., 2000). The adaptor protein serves as a binding sur- face for multiple intracellular signaling intermediates including Grb2, SHP2, and signal transducer and activator of transcription 3 (STAT3; Barrow- McGee and Kermorgant,

2014). The mediator of Gab1 migratory effects involves SHP2, a tyrosine phosphatase. Site-directed mutagenesis of the SHP2-docking site in *Gab1* results in both fewer muscle progenitor cells as well as their migration into the dorsal limb (Schaeper et al., 2007). Previous work by our group demonstrated that SHP2 is required for HGF-directed proliferation of mouse myoblasts likely through a MET/Gab1/ SHP2 signalosome (Li et al., 2009). Further, conditional ablation of *SHP2* in mouse SC disrupts activation and proliferation following injury (Griger et al., 2017). Extension of the cascade may include PKC isoforms. SHP2 is phosphorylated in vitro by multiple PKC isoforms including PKCδ (Strack et al., 2002). The phosphatase directs PKC to the insulin receptor complex thereby allowing PKC to phosphorylate and inactivate signal transduction (Müssig et al., 2005). Serving as a docking interface between PKCδ and MET, SHP2 may mediate the downstream signals that underlie HGF-initiated SC proliferation.

Satellite cells are required for the formation of new fibers following myotrauma and play an integral role during muscle hypertrophy through their ability to increase myonuclear content (von Maltzahn et al., 2013; Egner et al., 2016; Goh and Millay, 2017). Both functions are dependent upon SC fusion and subsequent expression of the myogenic differentiation program. In general, growth factors that act as SC mitogens (FGF2, PDGF) also suppress differentiation through mechanisms that are independent of proliferation (Pawlikowski et al., 2017). Our results indicate HGF elicits a reduction in early differentiation as determined by a lower percentage of myogenin (+) myoblasts. No differences were found in myoblast fusion or a subjective measure of apparent size of the myotubes. The ability of eqSC to form myotubes even in the presence of mitogenic concentrations of HGF may be partially attributed to ligand-induced phosphorylation of

AKT1, a positive effector of biochemical and morphological myocyte differentiation (Schiaffino et al., 2013). A synthetic MET agonist with structural similarity to native HGF causes the phosphorylation and activation of AKT1 in C2C12 myoblasts that results in increased fusion and myotube size (Cassano et al., 2008; Perini et al., 2015). Injection of the MET agonist into skeletal muscle or transgenic expression of the protein in skeletal muscle increases fiber cross-sectional area and improves exercise performance (Ronzoni et al., 2017). The decreased number of myogenin positive nuclei and absence of an effect on myoblast fusion point to a delay in the differentiation program in response to HGF. The inability of HGF to completely abrogate eqSC differentiation may be attributed to the strong predisposition of primary SC to spontaneously differentiate even the presence of FBS and mitogens (Charville et al., 2015).

In summary, our results extend upon existing literature to provide novel roles for HGF during SC myogenesis. Unlike rodent SC, the initial lag period between G exit and S-phase is not shortened in eqSC treated with HGF. The growth factor is capable of initiating intracellular signaling cascades in eqSC that include phosphorylation of ERK1/2 and AKT1. Signals through the nonclassical PKCδ, however, are required for HGF-mediated SC proliferation. These results provide a foundation for future efforts modulating PKCδ as a means of promoting eqSC bioactivity.

LITERATURE CITED

Allen, R. E., S. M. Sheehan, R. G. Taylor, T. L. Kendall, and G. M. Rice. 1995. Hepatocyte growth factor activates quies- cent skeletal muscle satellite cells in vitro. J. Cell. Physiol. 165:307–312. doi:10.1002/jcp.1041650211

Anderson, J. E. 2016. Hepatocyte growth factor and sat- 63igna cell activation. Adv. Exp.

Med. Biol. 900:1–25. doi:10.1007/978-3-319-27511-6_1

Barrow-McGee, R., and S. Kermorgant. 2014. Met endo- somal 64ignaling: in the right place, at the right time. Int. J. Biochem. Cell Biol. 49:69–74. doi:10.1016/j. biocel.2014.01.009

Bi, P., F. Yue, Y. Sato, S. Wirbisky, W. Liu, T. Shan, Y. Wen, D. Zhou, J. Freeman, and S. Kuang. 2016. Stage-specific effects of Notch activation during skeletal myogenesis. Elife. 5:3593. doi:10.7554/eLife.17355

Bjornson, C. R., T. H. Cheung, L. Liu, P. V. Tripathi, K. M. Steeper, and T. A. Rando. 2012. Notch signaling is nec- essary to maintain quiescence in adult muscle stem cells. Stem Cells. 30:232–242. doi:10.1002/stem.773

Bryan, K., B. A. McGivney, G. Farries, P. A. McGettigan, C. L. McGivney, K. F. Gough, D. E. MacHugh, L. M. Katz, and E. W. Hill. 2017. Equine skeletal muscle adaptations to exercise and training: evidence of differential regula- tion of autophagosomal and mitochondrial components. BMC Genomics. 18:595. doi:10.1186/s12864-017-4007-9

Cassano, M., S. Biressi, A. Finan, L. Benedetti, C. Omes, R. Boratto, F. Martin, M. Allegretti, V. Broccoli, G. Cusella De Angelis, et al. 2008. Magic-factor 1, a partial agonist of Met, induces muscle hypertrophy by protecting myo- genic progenitors from apoptosis.S. Wölfl, editor. PloS ONE. 3:e3223. doi:10.1371/journal.pone.0003223

Charville, G. W., T. H. Cheung, B. Yoo, P. J. Santos, G. K. Lee, J. B. Shrager, and T. A. Rando. 2015. Ex vivo expansion and in vivo self-renewal of human muscle stem cells. Stem Cell Reports. 5:621–632. doi:10.1016/j.stemcr.2015.08.004

Egner, I. M., J. C. Bruusgaard, and K. Gundersen. 2016. Satellite cell depletion prevents fiber hypertrophy in skel- etal muscle. Development. 143:2898–2906. doi:10.1242/ dev.134411

Fukada, S., M. Yamaguchi, H. Kokubo, R. Ogawa, A. Uezumi, T. Yoneda, M. M. Matev, N. Motohashi, T. Ito, A. Zolkiewska, et al. 2011. Hesr1 and hesr3 are essential to generate undifferentiated quiescent satellite cells and to maintain satellite cell numbers. Development. 138:4609– 4619. doi:10.1242/dev.067165

Galimov, A., T. L. Merry, E. Luca, E. J. Rushing, A. Mizbani, K. Turcekova, A. Hartung, C. M. Croce, M. Ristow, and J. Krützfeldt. 2016. MicroRNA-29a in adult muscle stem cells controls skeletal muscle regeneration during injury and exercise downstream of fibroblast growth factor-2. Stem Cells. 34:768–780. doi:10.1002/stem.2281

Goh, Q., and D. P. Millay. 2017. Requirement of myomak- er-mediated stem cell fusion for skeletal muscle hypertro- phy. Elife. 6:e20007. doi:10.7554/eLife.20007

Griger, J., R. Schneider, I. Lahmann, V. Schöwel, C. Keller, S. Spuler, M. Nazare, and C. Birchmeier. 2017. Loss of Ptpn11 (Shp2) drives satellite cells into quiescence. Elife. 6:633. doi:10.7554/eLife.21552

Gu, J. M., D. J. Wang, J. M. Peterson, J. Shintaku, S. Liyanarachchi, V. Coppola, A. E. Frakes, B. K. Kaspar, D. D. Cornelison, and D. C. Guttridge. 2016. An NF-κb–ephrinA5-dependent communication between NG2(+) interstitial cells and myoblasts promotes muscle growth in neonates. Dev. Cell. 36:215–224. doi:10.1016/j. devcel.2015.12.018

Halevy, O., and L. C. Cantley. 2004. Differential regulation of the phosphoinositide 3-

kinase and MAP kinase path- ways by hepatocyte growth factor vs. Insulin-like growth factor-I in myogenic cells. Exp. Cell Res. 297:224–234. doi:10.1016/j.yexcr.2004.03.024

Jackson, J. R., J. Mula, T. J. Kirby, C. S. Fry, J. D. Lee, M. F. Ubele, K. S. Campbell, J. J. McCarthy, C. A. Peterson, and E. E. Dupont-Versteegden. 2012. Satellite cell depletion does not inhibit adult skeletal muscle regrowth following unloading-induced atrophy. Am. J. Physiol. Cell Physiol. 303:C854–C861. doi:10.1152/ajpcell.00207.2012

Kandalla, P. K., G. Goldspink, G. Butler-Browne, and V. Mouly. 2011. Mechano growth factor E peptide (MGF- E), derived from an isoform of IGF-1, activates human muscle progenitor cells and induces an increase in their fusion potential at different ages. Mech. Ageing Dev. 132:154–162. doi:10.1016/j.mad.2011.02.007

Kawai, M., H. Aida, A. Hiraga, and H. Miyata. 2013. Muscle satellite cells are activated after exercise to 66ignali- tion in thoroughbred horses. Equine Vet. J. 45:512–517. doi:10.1111/evj.12010

Lee, J. D., C. S. Fry, J. Mula, T. J. Kirby, J. R. Jackson, F. Liu, L. Yang, E. E. Dupont-Versteegden, J. J. McCarthy, and C. A. Peterson. 2016. Aged muscle demonstrates fib- er-type adaptations in response to mechanical overload, in the absence of myofiber hypertrophy, independent of satellite cell abundance. J. Gerontol. A. Biol. Sci. Med. Sci. 71:461–467. doi:10.1093/66ignal/glv033

Leshem, Y., I. Gitelman, C. Ponzetto, and O. Halevy. 2002. Preferential binding of grb2 or phosphatidylinositol 3-kinase to the met receptor has opposite effects on HGF-induced myoblast proliferation. Exp. Cell Res. 274:288– 298.

doi:10.1006/excr.2002.5473

Leshem, Y., D. B. Spicer, R. Gal-Levi, and O. Halevy. 2000. Hepatocyte growth factor (HGF) inhibits skeletal mus- cle cell differentiation: a role for the Bhlh protein twist and the cdk inhibitor p27. J. Cell. Physiol. 184:101–109. doi:10.1002/(SICI)1097-4652(200007)

Li, J., S. A. Reed, and S. E. Johnson. 2009. Hepatocyte growth factor (HGF) signals through SHP2 to regulate primary mouse myoblast proliferation. Exp. Cell Res. 315:2284– 2292. doi:10.1016/j.yexcr.2009.04.011

von Maltzahn, J., A. E. Jones, R. J. Parks, and M. A. Rudnicki. 2013. Pax7 is critical for the normal function of satellite cells in adult skeletal muscle. Proc. Natl. Acad. Sci. U. S. A. 110:16474–16479. doi:10.1073/pnas.1307680110

Mauro, A. 1961. Satellite cell of skeletal muscle fibers. J. Biophys. Biochem. Cytol. 9:493–495. doi:10.1083/jcb.9.2.493

McCarthy, J. J., J. Mula, M. Miyazaki, R. Erfani, K. Garrison, A. B. Farooqui, R. Srikuea, B. A. Lawson, B. Grimes, C. Keller, et al. 2011. Effective fiber hypertrophy in satellite cell-depleted skeletal muscle. Development. 138:3657–3666. doi:10.1242/dev.068858

Miller, K. J., D. Thaloor, S. Matteson, and G. K. Pavlath. 2000. Hepatocyte growth factor affects satellite cell activation and differentiation in regenerating skeletal muscle. Am. J. Physiol. Cell Physiol. 278:C174–C181. doi:10.1152/ajpcell.2000.278.1.C174

Murach, K. A., A. L. Confides, A. Ho, J. R. Jackson, L. S. Ghazala, C. A. Peterson, and E. E. Dupont-Versteegden. 2017a. Depletion of pax7+ satellite cells does not

affect diaphragm adaptations to running in young or aged mice. J. Physiol. 595:6299–6311. doi:10.1113/JP274611

Murach, K. A., S. H. White, Y. Wen, A. Ho, E. E. Dupont- Versteegden, J. J. McCarthy, and C. A. Peterson. 2017b. Differential requirement for satellite cells during over-load-induced muscle hypertrophy in growing versus mature mice. Skelet. Muscle. 7:14. doi:10.1186/s13395-017-0132-z

Müssig, K., H. Staiger, H. Fiedler, K. Moeschel, A. Beck, M. Kellerer, and H. U. Häring. 2005. Shp2 is required for pro- tein kinase C-dependent phosphorylation of serine 307 in insulin receptor substrate-1. J. Biol. Chem. 280:32693– 32699. doi:10.1074/jbc.M506549200

Paulsen, G., U. R. Mikkelsen, T. Raastad, and J. M. Peake. 2012. Leucocytes, cytokines and satellite cells: what role do they play in muscle damage and regeneration following eccentric exercise? Exerc. Immunol. Rev. 18:42–97.

Pawlikowski, B., T. O. Vogler, K. Gadek, and B. B. Olwin. 2017. Regulation of skeletal muscle stem cells by fibro- blast growth factors. Dev. Dyn. 246:359–367. doi:10.1002/ dvdy.24495

Perini, I., I. Elia, A. Lo Nigro, F. Ronzoni, E. Berardi, H. Grosemans, S. Fukada, and M. Sampaolesi. 2015. Myogenic induction of adult and pluripotent stem cells using recombinant proteins. Biochem. Biophys. Res. Commun. 464:755–761. doi:10.1016/j.bbrc.2015.07.022

Poli, A., S. Mongiorgi, L. Cocco, and M. Y. Follo. 2014. Protein kinase C involvement in cell cycle modulation. Biochem. Soc. Trans. 42:1471–1476. doi:10.1042/BST20140128

Quarta, M., J. O. Brett, R. DiMarco, A. De Morree, S. C. Boutet, R. Chacon, M. C. Gibbons, V. A. Garcia, J. Su, J. B. Shrager, et al. 2016. An artificial niche preserves the quiescence of muscle stem cells and enhances their thera- peutic efficacy. Nat. Biotechnol. 34:752–759. doi:10.1038/ nbt.3576

Reed, S. A., S. E. Ouellette, X. Liu, R. E. Allen, and S. E. Johnson. 2007. E2F5 and LEK1 translocation to the nucleus is an early event demarcating myoblast quiescence. J. Cell. Biochem. 101:1394–1408. doi:10.1002/jcb.21256

Ronzoni, F., G. Ceccarelli, I. Perini, L. Benedetti, D. Galli, F. Mulas, M. Balli, G. Magenes, R. Bellazzi, G. C. De Angelis, et al. 2017. Met-activating genetically improved chimeric factor-1 promotes angiogenesis and hypertrophy in adult myogenesis. Curr. Pharm. Biotechnol. 18:309– 317. doi:10.2174/1389201018666170201124602

Sachs, M., H. Brohmann, D. Zechner, T. Müller, J. Hülsken, I. Walther, U. Schaeper, C. Birchmeier, and W. Birchmeier. 2000. Essential role of Gab1 for signaling by the c-Met receptor in vivo. J. Cell Biol. 150:1375–1384. doi:10.1083/ jcb.150.6.1375

Sambasivan, R., R. Yao, A. Kissenpfennig, L. Van Wittenberghe, A. Paldi, B. Gayraud-Morel, H. Guenou, B. Malissen, S. Tajbakhsh, and A. Galy. 2011. Pax7-expressing satellite cells are indispensable for adult skel- etal muscle regeneration. Development. 138:3647–3656. doi:10.1242/dev.067587

Schaeper, U., R. Vogel, J. Chmielowiec, J. Huelsken, M. Rosario, and W. Birchmeier. 2007. Distinct requirements for Gab1 in met and EGF receptor signaling in vivo. Proc. Natl. Acad. Sci. U. S. A. 104:15376–15381. doi:10.1073/ pnas.0702555104

Schiaffino, S., K. A. Dyar, S. Ciciliot, B. Blaauw, and M. Sandri. 2013. Mechanisms

regulating skeletal muscle growth and atrophy. Febs J. 280:4294–4314. doi:10.1111/febs.12253

Schiaffino, S., and C. Mammucari. 2011. Regulation of skeletal muscle growth by the IGF1-Akt/PKB path- way: insights from genetic models. Skelet. Muscle 1:4. doi:10.1186/2044-5040-1-4

Sheehan, S. M., R. Tatsumi, C. J. Temm-Grove, and R. E. Allen. 2000. HGF is an autocrine growth factor for skeletal muscle satellite cells in vitro. Muscle Nerve. 23:239–245. doi:10.1002.(SCI)1097-4598

Stark, D. A., R. M. Karvas, A. L. Siegel, and D. D. Cornelison. 2011. Eph/ephrin interactions modulate muscle satellite cell motility and patterning. Development. 138:5279– 5289. doi:10.1242/dev.068411

Strack, V., J. Krützfeldt, M. Kellerer, A. Ullrich, R. Lammers, and H. U. Häring. 2002. The protein-tyrosine-phosphatase SHP2 is phosphorylated on serine residues 576 and 591 by protein kinase C isoforms alpha, beta 1, beta 2, and eta. Biochemistry. 41:603–608. doi:10.1021/bi011327v CCC

Tatsumi, R., A. Hattori, Y. Ikeuchi, J. E. Anderson, and R. E. Allen. 2002. Release of hepatocyte growth factor from mechanically stretched skeletal muscle satellite cells and role of Ph and nitric oxide. Mol. Biol. Cell. 13:2909–2918. doi:10.1091/mbc.E02-01-0062

Tatsumi, R., A. L. Wuollet, K. Tabata, S. Nishimura, S. Tabata, W. Mizunoya, Y. Ikeuchi, and R. E. Allen. 2009. A role for calcium-calmodulin in regulating nitric oxide produc- tion during skeletal muscle satellite cell activation. Am. J. Physiol. Cell Physiol. 296:C922–C929. doi:10.1152/ ajpcell.00471.2008

Walker, N., T. Kahamba, N. Woudberg, K. Goetsch, and C. Niesler. 2015. Dose-dependent modulation of myo- genesis by HGF: implications for c-Met expression and downstream 71ignaling pathways. Growth Factors 33:229– 241. doi:10.3109/08977194.2015.1058260

Webster, M. T., and C-M. Fan. 2013. c-MET regulates myo- blast motility and myocyte fusion during adult skele- tal muscle regeneration.V. Mouly, editor. PloS ONE. 8:e81757. doi:10.1371/journal.pone.0081757

Wozniak, A. C., and J. E. Anderson. 2007. Nitric oxide-de- pendence of satellite stem cell activation and quiescence on normal skeletal muscle fibers. Dev. Dyn. 236:240–250. doi:10.1002/dvdy.21012

Wozniak, A. C., and J. E. Anderson. 2009. The dynamics of the nitric oxide release-transient from stretched muscle cells. Int. J. Biochem. Cell Biol. 41:625–631. doi:10.1016/j. biocel.2008.07.005

Wozniak, A. C., O. Pilipowicz, Z. Yablonka-Reuveni, S. Greenway, S. Craven, E. Scott, and J. E. Anderson. 2003. C-met expression and mechanical activation of satellite cells on cultured muscle fibers. J. Histochem. Cytochem. 51:1437–1445. doi:10.1177/002215540305101104

Yablonka-Reuveni, Z., M. E. Danoviz, M. Phelps, and P. Stuelsatz. 2015. Myogenic-specific ablation of Fgfr1 impairs FGF2-mediated proliferation of satellite cells at the myofiber niche but does not abolish the capacity for muscle regeneration. Front. Aging Neurosci. 7:85. doi:10.3389/fnagi.2015.00085

Yamada, M., R. Tatsumi, K. Yamanouchi, T. Hosoyama, S. Shiratsuchi, A. Sato, W. Mizunoya, Y. Ikeuchi, M. Furuse, and R. E. Allen. 2010. High concentrations of

HGF inhibit skeletal muscle satellite cell proliferation in vitro by inducing expression of myostatin: a possible mechanism for reestab- lishing satellite cell quiescence in vivo. Am. J. Physiol. Cell Physiol. 298:C465–C476. doi:10.1152/ajpcell.00449.2009

Table 2.1. Small interfering RNA sequences

	Sense (5′-3′)	Antisense (5′-3′)
PKCα	CGACGACUCUCUGUAGAAA	UUUCUACAGAGAUCGUCG
PKCα—scramble	CGACGACUGAGUGUAGAAA	UUUCUACACUCAGUCGUCG
PKCδ	GCAUGAACGUGCACCAUAA	UUAUGGUGCACGUUCAUGC
PKCδ—scramble	GCAUGAACCACCACCUAA	UUAUGGUGGUGGUUCAUGC
PKCε	ACCUCGAAUUAAAACCAAA	UUUGGUUUUAAUUCGAGGU
PKCε—scramble	ACCUCGAAAAUAAACCAAA	UUUGGUUUAUUUUCGAGG

Figure 2.1.

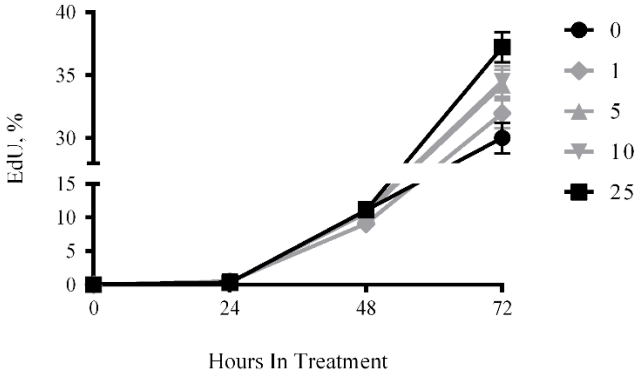

Figure 2.1. Hepatocyte growth factor supplementation does not alter time to S-phase. Fresh isolates of eqSC (n = 5) were allowed to attach overnight followed by treatment with 0, 1, 5, 10, and 25 ng/mL HGF for 72 h. Cells were pulsed with EdU for 2 h prior to fixation at 24-h intervals. EdU (+) and total nuclei were enumerated. Percent EdU = EdU(+)/Hoechst 33342 (+) × 100. Means and SEMs shown. Black symbols (0 and 25 ng/mL) are different ($P < 0.05$) from one another at 72 h only. eqSC = equine satellite cell.

Figure 2.2.

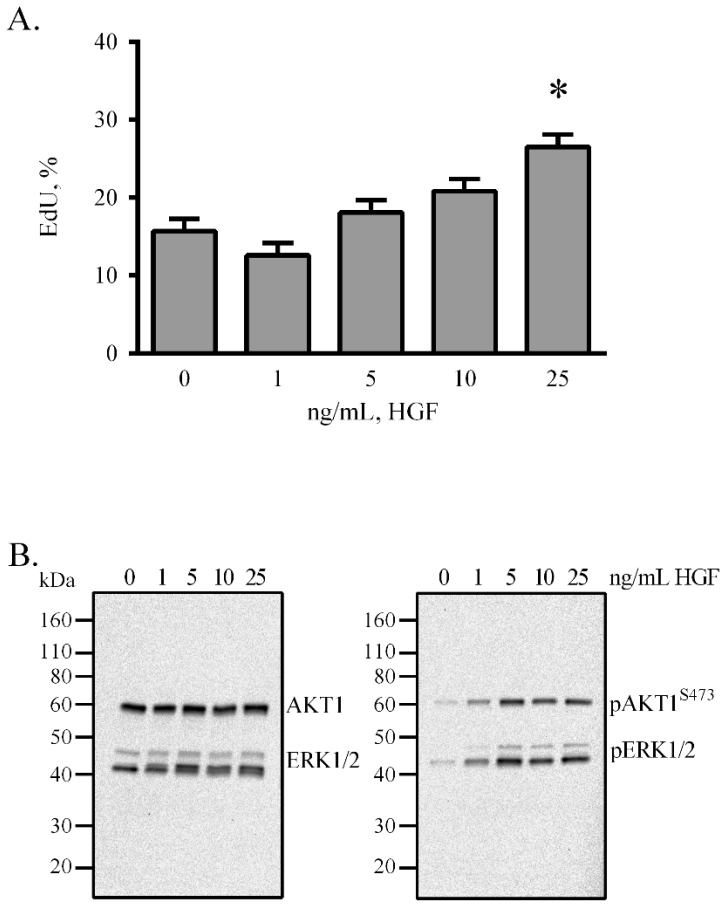

Figure 2.2. Hepatocyte growth factor stimulates proliferation and phosphorylation of AKT1 and ERK1/2.

Semi-confluent eqSC (n = 5) were treated with HGF for 48 h with a 2-h pulse of EdU prior to fixation (A). Cells were treated with the indicated concentration of HGF for 20 min, lysed and analyzed by Western blot for total and phosphorylated forms of AKT1 and ERK1/2 (B). EdU (+) and total nuclei were enumerated. Western blot exposure time was 2 mins. Percent EdU = EdU (+)/Hoechst 33342 (+) × 100. Means and SEMs shown. *Significance between control and treatment at $P < 0.05$. AKT1 = AKT serine/threonine

kinase 1; eqSC = equine satellite cell.

Figure 2.3. The mitotic actions of HGF are not mediated by phosphorylation of either ERK1/2 or AKT1.

Semi-confluent eqSC (n = 4) were treated with 25 ng/mL HGF in the presence or absence of 25 μM PD98059 (A), a MEK1/2 inhibitor, or 200 nM wortmannin (B), a PI3K inhibitor. After 48 h, cells were fixed and EdU(+) and total nuclei were enumerated. Parallel plates were lysed for Western blot analysis of phosphoERK1/2 (C) and phosphoAKT1 (D). Percent EdU = EdU (+)/Hoechst 33342 (+) × 100. Means and SEMs shown. Means with different letters are significant at $P < 0.05$. AKT1 = AKT serine/threonine kinase 1; eqSC = equine satellite cell; PI3K = phosphoinositide 3-kinase.

Figure 2.4.

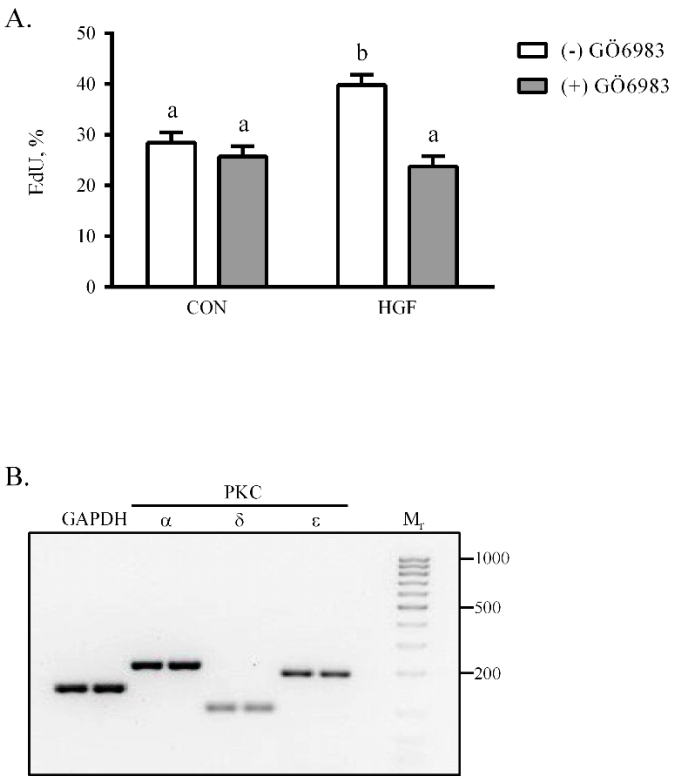

Figure 2.4. Protein kinase C mediates the proliferative effects of HGF.

Semi-confluent eqSC (*n* = 4) were treated with HGF in the pres- ence or absence of 2 μM Gö6983 for 48 h (A). Cells were pulsed with EdU 2 h prior to fixation. Total and EdU (+) nuclei were enumerated. Isoforms of PKC expressed by eqSC were identified by RT-PCR using gene-specific primers. Amplicons were separated electrophoretically through SYBR-Safe impregnated agarose gels. (B). RNA isolates from two animals are shown per gene of interest. Molecular weight lad- der ranges from 1 kb to 25 bp. Amplicon sizes are GAPDH, 150 bp, PKCα, 201 bp, PKCδ, 102 bp and PKCε, 191 bp. Percent EdU = EdU (+)/Hoechst (+) × 100. Means and SEMs shown. Means with dif- ferent letters are significant at $P < 0.05$. eqSC = equine satellite cell; GAPDH = glyceraldehyde phosphate dehydrogenase; RT-PCR = real- time PCR.

Figure 2.5.

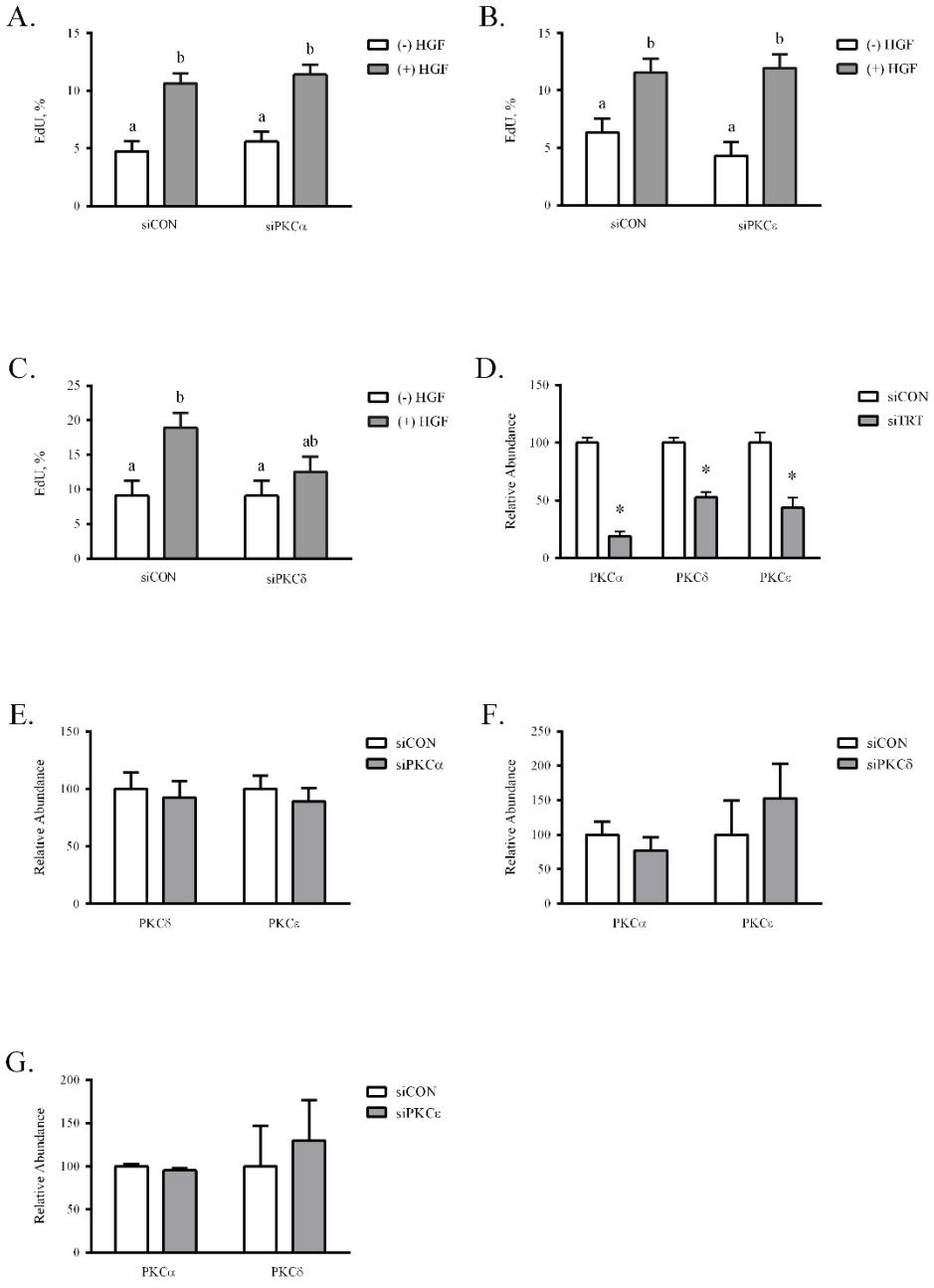

Figure 2.5. Hepatocyte growth factor signaling through PKCδ stimulates eqSC proliferation.

Semi-confluent eqSC (n = 5) were transfected with scrambled (siCON) or siRNA specific for PKC isoforms. After 24 h, the cells were treated with HGF for 24 h with a 2 h EdU

pulse prior to fixation. Total and EdU (+) nuclei were enumerated. Equine SC treated with siPKCα (A) or siPKCε (B) did not prevent HGF stimulated proliferation. Cells expressing siPKCδ did not respond to HGF (C). Means with different letters are significant at $P < 0.05$. Total RNA from siCON- and siP- KC-treated cells was analyzed by qPCR for specific knockdown (D) and off-target (E–G) reduction of PKCα, PKCδ, and PKCε mRNA. Relative expression was calculated by $2^{-\Delta\Delta Ct}$ method. *Significance at $P < 0.05$ within treatment. eqSC = equine satellite cell; qPCR = quantitative PCR; siRNA = small interfering RNA.

Figure 2.6.

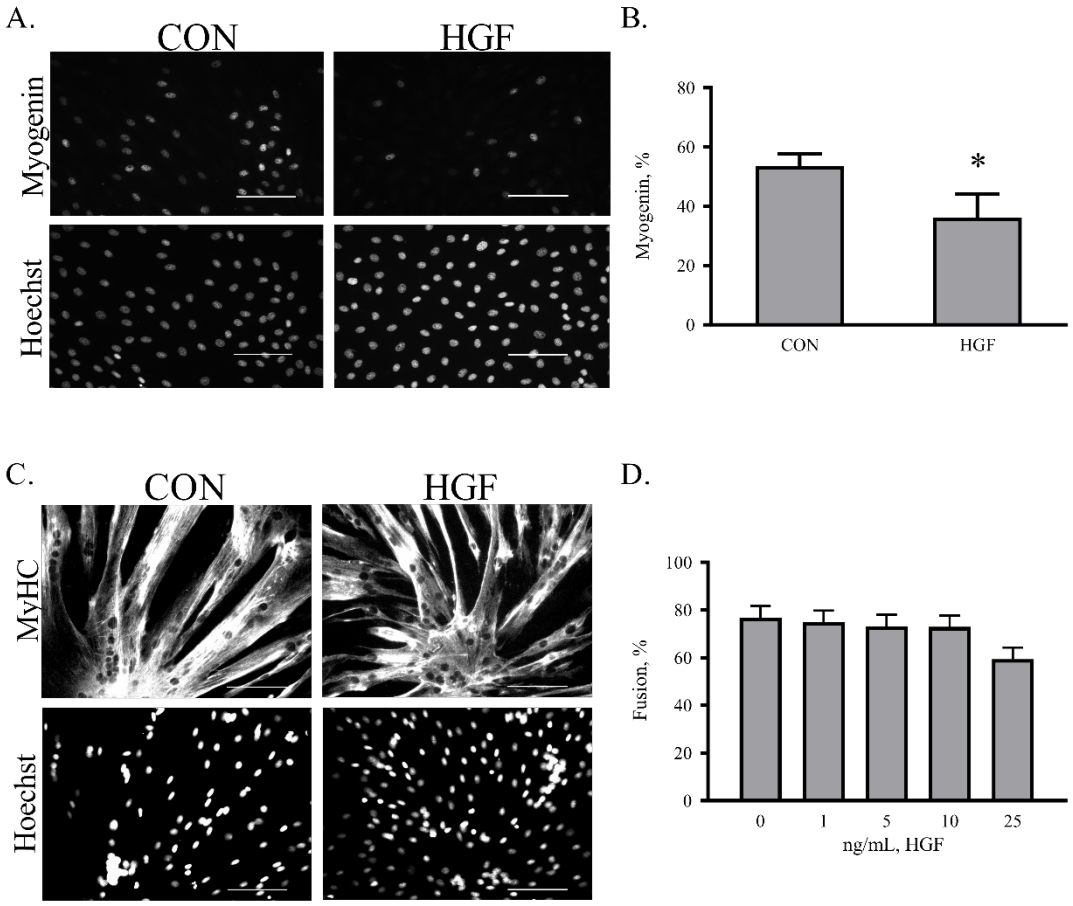

Figure 2.6. Hepatocyte growth factor inhibits eqSC differentiation.

Eighty percent confluent eqSC (n = 4) were treated with HGF for 24 or 48 h followed by fixation and immunocytochemical detection of myogenin or MyHC. Representative black and white photomicrographs of myogenin expression at equal shutter speeds for CON cells receiving vehicle and eqSC treated with 25 ng/mL HGF are shown (A). Percent myogenin (+) was calculated as total myogenin/total Hoechst 33342 × 100 (B). Representative black and white images of MyHC immunostaining at 48 h (C). Percent fusion was calculated as total number nuclei within a MyHC myotube/total number nuclei × 100 (D). Scale bar = 100 μm. *Significance at $P < 0.05$. eqSC = equine satellite cell.

CHAPTER 3

L-citrulline decreases myogenin in skeletal muscle of horses subject to an acute bout of submaximal exercise

Amanda M. Brandt, Madison L. Gonzalez, Jennifer L. Schlenker, Jamea N. Kidrick, Sally E. Johnson[#]

Department of Animal and Poultry Sciences, Virginia Polytechnic Institute and State University, Blacksburg VA 24061

[#]Corresponding author:

Virginia Polytechnic Institute and State University

Department of Animal and Poultry Sciences

3070 Litton Reaves (0306)

Blacksburg, VA 24061

(540) 231-0776

FAX: (540) 231-3010

sealy@vt.edu

Running Title: Citrulline and post-exercise muscle recovery

ABSTRACT

L-citrulline is a non-essential amino acid that improves human exercise performance. Nitric oxide and arginine, metabolic products of citrulline metabolism, affect satellite cell activity and glycogen stores, respectively. The objective of the study was to determine whether 26 d of L-citrulline supplementation (CIT; 0.0917 g/kg BW/d) would improve recovery from acute, submaximal exercise by increasing glycogen content and activating satellite cells. Citrulline supplementation increased ($P < 0.05$) plasma citrulline, arginine, and ornithine post-prandial relative to urea supplemented (CON; 0.0275 g/kg BW/d) horses. Exercise increased ($P < 0.05$) plasma lactate and packed cell volume from baseline, but dietary treatment did not affect ($P > 0.05$) these or heart rate variables. Neither diet nor exercise affected skeletal muscle glycogen content ($P > 0.05$) or *myosin heavy chain* mRNA content. However, *peroxisome proliferator-activated receptor gamma coactivator 1-α* (*PGC1-α*), a regulator of energy metabolism, expression increased ($P < 0.05$) in both dietary groups 1 d post-exercise relative to pre-exercise. Neither exercise nor dietary treatment affected ($P > 0.05$) *IGF-1* or *Pax7* expression. However, *myogenin* mRNA abundance decreased ($P < 0.05$) overall with CIT and decreased ($P < 0.05$) in CON 1 d post-exercise relative to CON pre-exercise. Submaximal exercise stimulates regulation of energy metabolism without activating SCs.

INTRODUCTION

Exercise is largely undertaken in the equine for recreational purposes; however, an almost equal number of horses are utilized for amateur or professional athletic events (American Horse Council, 2017). Acute or chronic bouts of exercise perturb homeostasis, placing the body in a state of stress. Oftentimes this is a positive state of eustress which, with repeated exposure, elicits signaling cascades leading to adaptation (Selye, 1936; Kim et al., 2005; Sanchis-Gomar et al., 2012; Egan and Zierath, 2013). Chronic exercise enhances performance, metabolism, and ameliorates symptoms of disease (Egan and Zierath, 2013; Fry et al., 2014; Aparcio et al., 2016). Ergogenic aids or exercise mimetics with the ability to reduce recovery time, improve performance, or enhance health would benefit the vast majority of horses and humans alike (Fan and Evans, 2017).

Citrulline is a non-essential amino acid found exogenously in watermelon but is also produced in the liver as a byproduct of urea cycle metabolism (Rimando et al., 2005; Figueroa et al., 2017). Unlike dietary arginine, dietary citrulline bypasses splanchnic extraction, enters circulation, and is converted to arginine and nitric oxide in the kidneys, vasculature, and peripheral tissues (Hartman et al., 1994; Agarwal et al., 2017; Daniel et al., 2017; Figueroa et al., 2017). While arginine or its metabolites serve as intermediates and stimulators of metabolic processes, nitric oxide is a signaling intermediate with vasoactive properties including vasodilation, which may aid in thermoregulation and metabolism during and after exercise, and possibly angiogenesis (Roberts et al., 1997; Mills et al., 1997; Mills et al., 1999; Morita et al., 2014; Fillipini et al., 2009; Figueroa et al., 2017). Through its metabolism to arginine and nitric oxide, citrulline has the potential to improve performance while decreasing time in recovery.

Response and adaptation to exercise may be mediated in part by muscle stem cells, or satellite cells (SCs), located between the basal lamina and sarcolemma of the myofiber (Mauro, 1961; Egner et al., 2016; Joanisse et al., 2018). Satellite cells are typically quiescent and express the transcription factor, *Paired box 7* (*Pax7)* (Seale et al., 2000). Hypertrophic stimuli appear to activate and increase SCs, though their necessity and the exact reason for SC contribution is unclear (Kadi et al., 2004b; Blaauw et al., 2009; McCarthy et al., 2011; Egner et al., 2016; Gunderson, 2016; Murach et al., 2017; Fry et al., 2017). General use and exercise training of a non-hypertrophic nature also appear to rely on SCs (Joanisse et al., 2013; Pawlikowski et al., 2015). Though SC contribution to endurance training is poorly understood, the contribution following acute submaximal exercise even less so (Yang et al., 2005; Dehghani et al., 2018).

Mechanical or chemical factors, such as insulin-like growth factor 1 (IGF-1) and hepatocyte growth factor (HGF), that may stimulate cell cycle entry are upregulated after exercise (Tatsumi et al., 2002; Hill and Goldsplink, 2003; Kawai et al., 2013). Proliferation may ensue wherein SCs are fated to either repopulate the stem cell niche or differentiate and fuse to the nearby myofiber (Olguin and Olwin, 2004; Rocheteau et al., 2012; Flamini et al., 2018). The stimuli and circumstances in which SCs contribute to the post-exercise response are not well defined in horses. However, Brandt et al. (2018) found that HGF, a paracrine/autocrine growth factor upregulated after exercise to exhaustion in horses, is a mitogen for equine SCs (Kawai et al., 2013). Nitric oxide increases SC activity in mice, possibly in conjunction with increased release of HGF from its extracellular tether, as seen in rats (Anderson, 2000; Tatsumi et al., 2002).

Skeletal muscle glycogen is a significant source of energy during exercise of greater intensity or moderate to long duration and its abundance at the start of exercise is related to performance (Bergstrom et al., 1967; Lindholm et al., 1974). Exercise may significantly reduce glycogen stores and full replenishment of these stores may take up to 72 h in horses (Lacombe et al., 2004). Dietary manipulation to increase skeletal muscle glycogen may delay fatigue as well as decrease time to replenishment (Pizza et al., 1995; Lacombe et al., 2004; Wang et al., 2015). Carbohydrate supplementation alters initial glycogen concentrations and accelerates rate of replenishment, though some diets pose an increased risk for gastrointestinal disturbance in horses (Bergstrom et al., 1967; Lacombe et al., 2004). Several groups have demonstrated that dietary proteins or amino acids in combination with carbohydrates may be more effective at replenishing glycogen (Essen-Gustavsson et al., 2010; Morifuji et al., 2010; Wang et al., 2015). Supplementation of arginine, a citrulline metabolite, decreases the rate of carbohydrate oxidation and may increase glycogen replenishment post-exercise in humans (Yaspelkis and Ivy, 1999).

The objective of the experiment was to determine whether L-citrulline supplementation would improve metabolic and SC responses to an acute bout of exercise in unfit adult horses. We hypothesized that L-citrulline would increase vascular properties in skeletal muscle, improve storage of muscle glycogen, and increase SC activity post-exercise.

MATERIALS AND METHODS

All protocols were reviewed and approved by the Institute for Animal Care and Use Committee at Virginia Polytechnic Institute and State University.

Animal Husbandry

Mature Thoroughbred geldings (n = 12) ranging from 3 to 12 y of age (mean = 7.417 y ± 0.5702) were used in a randomized 2-way crossover study. For the duration of the 80 d study, animals were housed in adjacent dry lot paddocks in groups of 3 with ad libitum water and mineral blocks. Body weight (BW) averaged 506.65 ± 8.05 kg and body condition score (BCS) was 5.69 ± 0.55 (Henneke et al., 1983).

Experimental Design

Horses were randomly assigned to receive either feed grade urea (>98%; Sigma Aldrich, St. Louis, MO; n=6) or L-citrulline (99.5%; Unichem Enterprises, Ontario, CA; n = 6) for a total of 26 d (7 d acclimation + 19 d experiment) during the first period of the crossover. This was followed by a 28 d dietary washout period prior to crossover and commencement of the second period.

Dietary Treatment

Body weight was rounded to the nearest 45 kg and horses assigned a diet of 0.5% BW pelleted concentrate with 1.5% BW fescue hay daily. Concentrate was fed individually in the morning and increased by 0.23 kg per d during 7 d acclimation. Crystallized urea (n = 12; 0.0275 g/kg BW) or powdered L-citrulline (n = 12; 0.0917 g/kg BW) were solubilized in water and top-dressed on the concentrate. Hay was divided into 2 daily feedings and provided in a group hay feeder. Ad libitum hay and 0.5% concentrate were provided during the 28 d washout.

Feed Analysis

Samples were collected weekly and stored at -20C. Samples were pooled and a subsample analyzed for proximate analysis and amino acids by Eurofins Food Integrity and Innovation (Table 3.1-3.2; Madison, WI).

Exercise Protocol

Horses were exercised in a free run 6-horse exerciser (EquiGym LLC, Paris, Kentucky). Three horses from each treatment group were worked at the same time with all horses performing the exercise protocol (Table 3.3) on the same day. The submaximal standard exercise test (SET) consisted of four consecutive replicates of 3 min ± 2 m/s, 7 min ± 5 m/s, and 5 min ± 7 m/s, corresponding to walk, trot and canter, respectively. Direction was alternated between reps. All horses were fitted with heart rate monitors (V800, Polar Electro Inc., Bethpage, NY) with data acquisition every 1 s from commencement of exercise until 5 min post-exercise. Variables collected for analysis included average heart rate per replicate, heart rate at 5 minutes of recovery, and maximum, minimum, and average heart rate achieved during the SET.

Venous Blood Collection and Analysis

Venous blood was collected on day 6 of the treatment period prior to the morning meal and 3.5 h post-prandial. Blood was drawn from the jugular vein using a 21 g x 1 ½ inch blood collection needle fitted to sodium heparin and K2EDTA blood collection tubes for the measurement of amino acids and plasma, respectively (Becton, Dickinson and Company, Franklin Lakes, NJ). Collection tubes were placed on ice prior to plasma isolation by centrifugation at 1300 x g for 15 min. Plasma aliquots were stored at -20 C until further analysis. Plasma amino acid and metabolite composition and concentration was measured by the University of Missouri Experiment Station Chemical Laboratory (Table 3.4). Blood samples were collected before and within 5 minutes of completion of the exercise test, as described above. Plasma lactate concentrations were measured

colorimetrically (Lactate Assay Kit; Sigma-Aldrich, St. Louis, MO) according to manufacturer's instructions.

Skeletal Muscle Biopsies

Horses were sedated with xylazine (0.66 mg/kg BW, XylaMed, VetOne, Boise, ID) and the area atop the gluteus medius surgically prepped. Lidocane hydrochloride (Lidocaine 2%, VetOne, Boise, ID) was administered to the biopsy site and a surgical grade razor used to pierce hide and fascia. A 10 gauge biopsy probe fitted in a vacuum assisted biopsy system (Vacora Assisted Biopsy System, Bard Biopsy, Tempe, AZ) was used to aseptically retrieve 2 biopsies (~150 mg ea.)from the incision site. Biopsies were collected on d 13, 15, 17, and 19 from alternating limbs at least 3 cm from the previous site and one snap frozen in liquid nitrogen while the other was placed in PBS on ice.

RNA Extraction and Isolation

Approximately 30 mg of frozen tissue was added to 2 mL Trizol Reagent (Invitrogen, Carlsbad, CA). Tissue was homogenized using a handheld rotor-stator polytron (Kinematica, Bohemia, NY) in bursts at maximum speed for 60 s. After a 5 min incubation at 25 C, chloroform was added as per manufacturer's recommendation. Samples were centrifuged for 15 min at 12,000 x g. The upper phase was added to 0.1 volumes sodium acetate (0.3 M) and 2.5-3 volumes 100% ice cold ethanol then precipitated at -80C overnight. Precipitates were pelleted by centrifugation for 30 min at 20,000 x g and 4 C and washed twice with ice cold 70% ethanol. RNA quantity and quality was assessed spectrophotometrically (NanoDrop, ND-1000, ThermoScientific) and a DNA removal step (DNase I, ThermoFisher Scientific) performed prior to RT-qPCR.

RT-qPCR

Fifty nanograms of DNA-free RNA was reverse transcribed to cDNA (High capacity cDNA Reverse Transcription Kit, Fisher Scientific). Five nanograms was then added to a mix of SYBRGreen (Power SYBRGreen PCR Master Mix, ThermoFisher Scientific), nuclease-free water, and primers (Table 3.5). Samples were denatured at 95 C for 10 min prior to 40 cycles of 15 s at 95C and 1 min at 62C. Data were normalized (ΔCt) to the geometric mean of two housekeeping genes previously found to be stable across dietary and exercise treatments, beta 2 microglobulin (Genorise Scientific, Glen Mills, PA) and glyceraldehyde phosphate dehydrogenase. Urea pre-exercise levels were chosen as the control sample for the calculation of $\Delta\Delta$Ct). Fold change was calculated by the $2^{(-\Delta\Delta Ct)}$ method.

Glycogen Analysis

Snap frozen tissue from skeletal muscle biopsies were ground by pellet pestle (Kimble Chase, Rockwood, TN). Ground samples (100 mg) were added to 1.25 M HCl (1 mL), vortexed, and lysed for 2.5 min. Lysates were incubated at 90C for 2 h before vortexing and centrifugation at 20817 x g for 5 min. Supernatants were added to 1.25 M KOH (500 µL) and maintained on ice. Two mL of a buffer containing triethanolamine, 200 mM EDTA (pH 7.4), 1 M $MgCl_2$, and NADP were added to test tubes. Diluted samples (1:2 with Nanopure water) were added to the buffer, vortexed, and 200 µL added to a 96 well place and optical density (OD_1) measured by BioTek PowerWave XS Plate Reader (Winooski, VT). Hexokinase (35 µL of 1:10 solution), glucose-6-phosphate dehydrogenase (35 µL of 1:10 solution), and Na-ATP (70 µL of 11 mg/mL) were added, vortexed, and incubated for 15 minutes. Two hundred microliters of each sample was

added to a separate 96 well plate and the optical density (OD_2) measured. Absorbance was calculated by (OD_1-OD_2). Concentration was then determined using the linear equation generated from the standard curve. Data was expressed as mmol glycosyl unit/kg tissue (wet weight).

Immunohistochemistry

Biopsies were fixed with 4% paraformaldehyde for 40 minutes on ice, washed with PBS, and immersed in 30% sucrose overnight at 4C. Tissues were then embedded in Neg-50 medium (ThermoScientific, Waltham, MA) and frozen on dry ice. Serial cryosections (10 μm) were collected on glass slides (SuperFrost Plus, ThermoFisher) and air dried, blocked, and permeabilized with 3% bovine serum albumin (BSA) containing 0.5% Triton X-100 for 20 min at room temperature. Sections were washed with PBS and incubated in blocking solution with anti-CD31 (1:20, ABCAM) or without anti-CD31 for 1 h at room temperature. Sections were incubated with goat anti-rabbit IgG Alexafluor 568 (8 μg/mL, Thermofisher), wheat germ agglutinin conjugated to Alexafluor-488 (2.5 μg/mL, Thermofisher), and Hoechst 33342 (1 μg/mL, Thermofisher) in blocking solution covered for 40 min at room temperature. Representative microscopic images at 200-fold magnification (Nikon Eclipse Ti, Melville, NY) were captured using a digital camera system ($CoolSNAP_{HQ}^2$, Photometrics, Tucson, AZ) with shutter speed and image acquisition controlled with NIS Elements software (Nikon). All CD31 immunopositive (+) images were normalized to background fluorescence detected in samples incubated without anti-CD31. Myofibers per field of view were enumerated and the area of CD31(+) expressed relative to myofiber number using the formula [(area $CD31^+$- area background fluorescence)/# myofibers]. Eight microscopic fields in three non-consecutive cryosections for each horse were evaluated and used in the calculation.

Statistical Analysis

All data were analyzed using the mixed procedure of SAS Enterprise Guide (Cary, NC). An analysis of variance was performed with previously planned post-hoc comparisons adjusted with Tukey's method. Horse was included in the random variable statement while treatment, time, and period were included in the model as appropriate. Significance was established at $P < 0.05$ and a tendency toward significance defined as $P > 0.05$ and < 0.10.

RESULTS

Neither CIT nor CON affected HR during performance of the SET (Table 3.6). Average HR during the SET was 116 bpm for both CON and CIT which decline to 72 and 75 bpm for CON and CIT, respectively, within 5 min post-exercise. The SET was sufficient to cause submaximal exercise responses as indicated by an increase ($P < 0.05$) in packed cell volume and plasma lactate (Fig 3.1).

A primary objective of the experiment was to monitor satellite cell activity during the post-exercise recovery period. Expression of *Pax7*, a satellite cell marker gene, did not differ from pre-SET amounts at d1, 3 or 5 for either the CON or CIT groups suggesting the SET was insufficient to initiate satellite cell mediated repair (Fig 3.2a). Expression of *IGF-1* and *myogenin*, early markers of muscle repair in horses, also remained unaffected by exercise (Fig 3.2b,c). However, CIT caused a reduction ($P < 0.05$) in total *myogenin* mRNA expression by comparison to CON.

Myogenin expression is greater in slow muscle fibers of mice by comparison to fast twitch fibers and knockdown of *myogenin* causes a reduction in slow myosin expression *in vitro* (Hughes et al., 1993; Hughes et al., 1999; Tatsumi et al., 2017). To

examine the effect of CIT on fiber type, expression of *Myh1*, *2* and *7* was measured. Results demonstrate that CIT does not affect expression of any of the myosin isoforms (Fig 3.3a-c). In a similar manner, the amount of *PGC1a* mRNA, a transcription factor abundant in slow oxidative muscle fibers, remained unaffected by CIT supplementation (Fig 3.3d). The absence of a fiber type switch was further supported by no difference in the amount of glycogen in CON or CIT gluteus medius muscle (Fig 3.3e). Slow twitch fibers also contain a denser capillary network than their fast counterparts. Capillary density was measured by immunostaining for CD31, a surface marker for endothelial cells. As shown in Fig 3.4a, regions of immunopositive CD31 are localized to the outer surface of the myofibers. Calculation of the CD31 area per fiber demonstrates no differences between CON and CIT (Fig 3.4b). Neither the angiogenic factor, VEGFA, nor its receptor, FLT1, were expressed differently as a function of diet (Fig 3.4c,d).

DISCUSSION

Dietary L-citrulline bypasses splanchnic extraction and rapidly increases in the blood of several mammals, including horses as in the current study. Conversion of citrulline to arginine and nitric oxide occurs in the kidney and other peripheral tissues. Citrulline also inhibits arginase, the enzyme responsible for arginine metabolism in enterocytes, thereby increasing blood arginine more effectively than would dietary arginine alone (Figueroa et al., 2017). Citrulline and nitric oxide are associated with increased vascular function, and as such would be expected to increase surface area, density of circulatory components, or the angiogenic growth factor VEGFA (Morita et al., 2014; Figueroa et al., 2017; Villareal et al., 2018). Contrary to other studies, in the current study there were no observed differences in vascular parameters though plasma citrulline did increase post-prandial alongside ornithine, arginine, and urea. It is possible that citrulline may be most beneficial post-exercise, which was not analyzed for vascular components in the present study (Figueroa et al., 2017; Villareal et al., 2018).

Other groups have found citrulline altered metabolism during exercise by sparing glycogen and reducing lactate accumulation (Takeda et al., 2011). We observed no differences in post-exercise lactate or glycogen concentration between urea and citrulline supplemented groups. Perhaps this lack of difference is due to insufficient intensity, and a disparity may have been observed if anaerobic threshold was met. Accordingly, we did not observe a shift in the metabolic phenotype of the muscle towards that of a more oxidative profile, as *PGC1α* increased at 24 h post-exercise irrespective of dietary treatment and there were no differences in myosin heavy chain mRNA. Though mRNA is not always directly related to protein expression, Eizema et al. (2005) demonstrated that

myosin heavy chain is regulated transcriptionally, preceding alterations at the level of protein in unfit horses. Some groups have relied upon electrophoretic separation of myosin heavy chain isoforms, but the predicted molecular weight of these isoforms is within 1 kDa of each other and as such electrophoretic separation within our lab was unsuccessful (Rivero et al., 1996).

Previous work by Kawai et al. (2013) showed exercise to exhaustion increased *IGF-1* mRNA in tandem with *myogenin* within 1 d of exercise, followed by *Pax7* at d 3. Villareal et al. (2018) found citrulline increased *IGF1* expression as well. Interestingly, while there were no effects on *Pax7* or *IGF-1*, citrulline suppressed *myogenin* expression. Myogenin expression is not limited to SCs, as myonuclei express myogenin following an acute bout of resistance exercise (Kadi et al., 2004a). Recently, *myogenin* has also been implicated in regulating response to exercise as well as metabolism. Some groups have found increased myogenin expression associated with both slow-twitch fibers and a more oxidative phenotype, while others have found *myogenin* knockout to enhance endurance capacity (Flynn et al., 2010; Hughes et al., 1993). Though the role of myogenin is equivocal, it is possible that the effect of myogenin is exerted at a metabolic level rather than that of influencing SC fate.

Though the exercise performed reached the mean heart rate achieved during a heavy to very heavy exercise session by NRC (2007) definition, glycogen was not reduced nor were SCs active in the 5 d following exercise. The lack of difference at 24 h does not preclude the possibility that glycogen was reduced in the early hours following exercise, but the anaerobic threshold was not met suggesting that any reductions would have been modest. Taken together, an acute session of heavy endurance exercise

stimulates the metabolic program as evidenced by stimulation of *PGC1α* and possibly altered *myogenin* in CON animals at 1 d post, but is insufficient to activate SCs. Chronic endurance exercise increases SC number, and as such it is possible that repeated bouts similar to that of this experiment may ultimately lead to SC adaption consequent to metabolic adaptation.

LITERATURE CITED

Agarwal, U., Didelija, I. C., Yuan, Y., Wang, X., and J. C. Marini. 2017. Supplemental citrulline is more efficient than arginine in increasing systemic arginine availability in mice. J. Nutr. 147: 596-602.

American Horse Council Foundation. 2017. Economic Impact Study of the United States Horse Industry. https://www.horsecouncil.org/resources/economics/. Accessed December 14, 2018.

Anderson, J. E. 2000. A role for nitric oxide in muscle repair: nitric oxide-mediated activation of muscle satellite cells. Mol. Biol. Cell. 11(5): 1859-1874. doi: 10.1091/mbc.11.5.1859.

Aparicio, V. A., I. Coll-Risco, D. Camiletti-Moirón, E. Nebot, R. Martínez, M. López-Jurado, P. Aranda. 2016. Interval aerobic training combined with strength-endurance exercise improves metabolic markers beyond caloric restriction in Zucker rats. Nutr. Metab. Cardiovasc. Dis. 26(8): 713-721.

Bergström, J., Hermansen, L., Hultman, E., and B. Saltin. 1967. Diet, muscle glycogen and physical performance. Acta Physiol. Scand. 71(2): 140-150. doi: 10.1111/j.1748-1716.1967.tb03720.x

Blaauw, B., M. Canato, L. Agatea, L. Toniolo, C. Mammucari, E. Masiero, R. Abraham,

M. Sandri, S. Schiaffino, and C. Reggiani. 2009. Inducible activation of Akt increases skeletal muscle mass and force without satellite cell activation. FASEB J. 23: 3896-3905.

Brandt, A. M., Kania, J. M., Gonzalez, M. L., and S. E. Johnson. 2018. Hepatocyte growth factor acts as a mitogen for equine satellite cells via protein kinase C δ-directed signaling. J. Anim. Sci. 96(9): 3645-3656. doi: 10.1093/jas/sky234.

Daniel, J. A., Stockwell-Goering, M. G., Crane, A. R., Hernandez, C. M., Qualley, D. F., and B. K. Whitlock. 2017. Oral administration of L-citrulline increases plasma concentrations of L-citrulline and argninine in horses. J. Anim. Sci. 95(4): 166. Abstr. doi:10.2527/asasann.2017.335.

Dehghani, M., Kargarfard, M., Rabiee, F., Nasr-Esfahani, M. H., and K. Ghaedi. 2018. A comparative study on the effects of acute and chronic downhill running vs uphill running exercise on the RNA levels of the skeletal muscles PGC1-α, FNDC5 and the adipose UCP1 in BALB/c mice. Gene. 679: 369-376. doi:10.1016/j.gene.2018.09.024

Egan, B., and J. R. Zierath. 2013. Exercise metabolism and the molecular regulation of skeletal muscle adapatation. Cell Metab. 17: 162-184.

Egner, I. M., J. C. Bruusgaard, and K. Gundersen. 2016. Satellite cell depletion prevents fiber hypertrophy in skeletal muscle. Dev. 143: 2898-2906. doi: 10.1242/dev.134411.

Eizema, K., van den Burg, M. M., de Jonge, H. W., Dingboom, H., Weijs, W. A., and M. E. Everts. 2005. Myosin Heavy Chain Isoforms in Equine Gluteus Medius

Muscle: Comparison of mRNA and Protein Expression Profiles. J. Histochem. Cytochem. 53(11): 1383-1390. doi: 10.1369/jhc.4A6609.2005.

Essén-Gustavsson, B., Connysson, M., and A. Jansson. 2010. Effects of crude protein intake from forage-only diets on muscle amino acids and glycogen levels in horses in training. Equine Vet. J. 42(38): 341- 346. doi: 10.1111/j.2042-3306.2010.00283.x.

Fan, W., and R. M. Evans. 2017. Exercise mimetics: impact on health and performance. Cell Metab. 25: 242-247.

Figueroa, A., Wong, A., Jaime, S. J., and J. U. Gonzales. 2017. Influence of L-citrulline and watermelon supplementation on vascular function and exercise performance. Curr. Opin. Clin. Nutr. Metab. Care. 20(1): 92-98.

Fillipini, L. I., Moreira, A. J., Marroni, N. P., and R. M. Xavier. 2009. Nitric oxide and repair of skeletal muscle injury. Nitric Oxide. 21: 157-163. doi:10.1016/j.niox.2009.08.002.

Flamini, V., Ghadiali, R. S., Antczak, P., Rothwell, A., Turnbull, J. E., and A. Pisconti. 2018. The satellite cell niche regulates the balance between myoblast differentiation and self-renewl via p53. Stem Cell Rep. 10(3): 970-983. doi: 10.1016/j.stemcr.2018.01.007.

Flynn, J. M., Meadows, E., Fiorotto, M., and W. H. Klein. 2010. Myogenin regulates exercise capacity and skeletal muscle metabolism in the adult mouse. PLoS ONE. 5(10): e13535. doi: 10.1371/journal.pone.0013535.

Fry, C. S., Kirby, T. J., Kosmac, K., McCarthy, J. J., and C. A. Peterson. 2017. Myogenic progenitor cells control extracellular matrix production by fibroblasts during skeletal muscle hypertrophy. Cell Stem Cell. 20: 56-69.

Gundersen, K. 2016. Muscle memory and a new cellular model for muscle atrophy and hypertrophy. J Exp Biol. doi: 10.1242/jeb.124495.

Hartman, W. J., Torre, P. M., and R. L. Prior. 1994. Dietary citrulline but not ornithine counteracts arginine deficiency in rats by increasing splanchnic release of citrulline. J. Nutr. 124(10): 1950-1960.

Henneke, D.R., G.D. Potter, J.L. Kreider, and B.F. Yeates. 1983. Relationship between condition score, physical measurements and body fat percentage in mares. Equine Vet. J. 15(4): 371-372. doi: 10.1111/j.2042-3306.1983.tb01826.x

Hill, M., and G. Goldsplink. 2003. Expression and splicing of the insulin-like growth factor gene in rodent muscle is associated with muscle satellite (stem) cell activation following local tissue damage. J. Physiol. 549(2): 409-418. doi: 10.1113/jphysiol.2002.035832.

Hughes, S. M., Taylor, J. M., Tapscott, S. J., Gurley, C. M., Carter, W. J., and C. A. Peterson. 1993. Selective accumulation of MyoD and myogenin mRNAs in fast and slow adult skeletal muscle is controlled by innervation and hormones. Development. 118(4): 1137-1147.

Joanisse, S., J. B Gillen, L. M. Bellamy, B. R. McKay, M. S. Tarnopolsky, M. J. Gibala, and G. Parise. 2013. Evidence for the contribution of muscle stem cells to nonhypertrophic skeletal muscle remodeling in humans. FASEB J. 27(11): 45964605.

Joanisse, S., Snijders, T., Nederveen, J. P., and G. Parise. 2018. The impact of aerobic exercise on the muscle stem cell response. Exerc. Sport Sci. Rev. 46(3): 180-187. doi: 10.1249/JES.0000000000000153.

Kadi, F., Johansson, F., Johansson, R., Sjöström, M., and J. Henriksson. 2004a. Effects of one bout of endurance exercise on the expression of myogenin in human quadriceps muscle. Histochem. Cell. Biol. 121: 329-334. doi: 10.1007/s00418-004-0630-z

Kadi, F., P. Schjerling, L.L. Andersen, N. Charifi, J.L. Madsen, L.R. Christensen, and J.L. Andersen. 2004b. The effects of heavy resistance training and detraining on satellite cells in human skeletal muscles. J. Physiol. 558(3): 1005-1012.

Kawai, M., H. Aida, A. Hiraga, and H. Miyata. 2013. Muscle satellite cells are activated after exercise to exhaustion in Thoroughbred horses. Equine Vet. J. 45: 512-517. doi: 10.1111/evj.12010.

Kim, J., Hinchcliff, K. W., Yamaguchi, M., Beard, L. A., Markert, C. D., and S. T. Devor. 2005. Exercise training increases oxidative capacity and attenuates exercise-induced ultrastructural damage in skeletal muscle of aged horses. J. Appl. Physiol. 98(1): 334-342.

Lacombe, V. A., K. W. Hinchcliff, C. W. Kohn, S. T. Devor, and L. E. Taylor. 2004. Effects of feeding meals with various soluble carbohydrate content on muscle glycogen synthesis after exercise in horses. Amer. J. Vet. Res. 65(7): 916-923. doi: 10.2460/ajvr.2004.65.916.

Lindholm, A., Bjerneld, H, and B. Saltin. 1974. Glycogen depletion pattern in muscle fibres of trotting horses. Acta Physiologica. 90(2): 475-484. doi: 10.1111/j.1748-

1716.1974.tb05610.x.

Mauro, A. 1961. Satellite cell of skeletal muscle fibers. J. Biophys. Biochem. Cytol. 9(2): 493-495. doi: 10.1083/jcb.9.2.493.

McCarthy, J.J., J. Mula, M. Miyazaki, R. Erfani, K. Garrison, A.B. Farooqui, R. Srikuea, B.A. Lawson, B. Grimes, C. Keller, G.V. Zant, K.S. Campbell, K.A. Esser, E.E. Dupont-Versteegden, and C.A. Peterson. 2011. Effective fiber hypertrophy in satellite cell-depleted skeletal muscle. Development. 138: 3657-3666.

Mills, P. C., Scott, C. M., and D. J. Marlin. 1997. Effects of nitric oxide inhibition on thermoregulation during exercise in the horse. Ann N Y Acad. Sci. 813: 591-599. doi: 10.1111/j.1749-6632.1997.tb51750.x.

Mills, P. C., Marlin, D. J., Scott, C. M., and N. C. Smith. 1999. Metabolic effects of nitric oxide synthase inhibition during exercise in the horse. Res. Vet. Sci. 66(2): 135-138. doi: 10.1053/rvsc.1998.0258.

Morifuji, M., Kanada, A., Koga, J., Kawanaka, K., and M. Higuchi. 2010. Post-exercise carbohydrate plus whey protein hydrolysates supplementation increases skeletal muscle glycogen level in rats. Amino Acids. 38: 1109-1115. doi: 10.1007/s00726-009-0321-0.

Morita, M., Hayashi, T., Ochiai, M., Maeda, M., Yamaguchi, T., Ina, K., and M. Kuzuya. 2014. Oral supplementation with a combination of l-citrulline and l-arginine rapidly increases plasma l-arginine concentration and enhances NO bioavailability. Biochem. Biophys. Res. Comm. 454(1): 53-57. doi: 10.1016/j.bbrc.2014.10.029.

Murach, K.A., S.H. White, Y. Wen, A. Ho, E.E. Dupont-Versteegden, J.J. McCarthy, and

C.A. Peterson. 2017. Differential requirement for satellite cells during overload-induced muscle hypertrophy in growing versus mature mice. Skeletal Muscle. 7(14). doi: 10.1186/s13395-017-0132-z.

NRC, 2007. Nutrient requirements of horses. 6th rev. ed. Natl. Acad. Press. Washington, DC.

Olguin, H. C., and B. B. Olwin. 2004. Pax-7 up-regulation inhibits myogenesis and cell cycle progression in satellite cells: a potential mechanism for self-renewal. Dev. Biol. 275: 375-388. doi: 10.1016/j.ydbio.2004.08.015.

Pawlikowski, B., Pulliam, C., Betta, N. D., Kardon, G., and B. B. Olwin. 2015. Pervasive satellite cell contribution to uninjured adult muscle fibers. Skeletal Muscle. 5(42). doi:10.1186/s13395-015-0067-1.

Pizza, F. X., Flynn, M. G., Duscha, B. D., Holden, J., and E. R. Kubitz. 1995. A carbohydrate loading regimen improves high intensity, short duration exercise performance. Internat. J. Sport Nutr. 5: 110-116. doi: 10.1123/ijsn.5.2.110.

Rimando, A. M., and P. M. Perkins-Veazie. Determination of citrulline in watermelon rind. 2005. J. Chromatog. A. 1078: 196-200.

Rivero, J. L., Talmadge, R. J., and V. R. Edgerton. 1996. Myosin heavy chain isoforms in adult equine skeletal muscle: an immunohistochemical and electrophoretic study. Anat. Rec. 246(2): 185-194. doi: 10.1002/(SICI)1097-0185(199610)246:2<185::AID-AR5>3.0.CO;2-0

Roberts, C. K., Barnard, R. J., Scheck, S. H., and T. W. Balon. 1997. Exercise-stimulate glucose transport in skeletal muscle is nitric oxide dependent. Am. J. Physiol. 273(1): E220-225. doi: 10.1152/ajpendo.1997.273.1.E220.

Robertson, R. P., Raymond, R. H., Lee, D. S., Calle, R. A., Ghosh, A., Shankar, S. S., Vassileva, M. T., Weir, G. C., and D. A. Fryburg. 2014. Arginine is preferred to glucagon for stimulation testing of β-cell function. Am. J. Physiol. Endrinol. Metab. 307(8): E720-727. doi: 10.1152/ajpendo.00149.2014.

Rocheteau, P., Gayraud-Morel, B., Siegl-Cachedenier, I., Blasco, M. A., and S. Tajbakhsh. 2012. A subpopulation of adult skeletal muscle stem cells retains all template DNA strands after cell division. Cell. 148: 112-125. doi: 10.1016/j.cell.2011.11.049.

Sanchis-Gomar, F., Garcia-Gimenez, J. L., Perez-Quilis, C., Gomez-Cabrera, M. C., Pallardo, F. V., and G. Lippi. 2012. Physical exercise as an epigenetic modulator: eustress, the "positive stress" as an effector of gene expression. J. Strength Condition. Res. 26(12): 3469-3472.

Seale, P., Sabourin, L. A., Girgis-Gabardo, A., Mansouri, A., Gruss, P., and M. A. Rudnicki. 2000. Pax7 is required for the specification of myogenic satellite cells. Cell. 102(6): 777-786. doi.org/10.1016/S0092-8674(00)00066-0.

Selye, H. 1936. A syndrome produced by diverse nocuous agents. Nature 138: 32.

Takeda, K., Machida, M., Kohara, A., Omi, N., and T. Takemasa. 2011. Effects of citrulline supplementation on fatigue and exercise performance in mice. J. Nutr. Sci. Vitaminol. 57: 246-250. doi: 10.3177/jnsv.57.246.

Tatsumi, R., A. Hattori, Y. Ikeuchi, J. E. Anderson, and R. E. Allen. 2002. Release of hepatocyte growth factor from mechanically stretched skeletal muscle satellite cells and role of pH and nitric oxide. Mol. Biol. Cell. 13(8): 2909-2918. doi: 10.1091/mbc.E02-01-0062.

Villareal, M. O., Matsukawa, T., and H. Isoda. 2018. L-Citrulline Supplementation-Increased Skeletal Muscle PGC-1α Expression Is Associated with Exercise Performance and Increased Skeletal Muscle Weight. Mol. Nutr. Food Res. 62: e1701043. doi: 10.1002/mnfr.201701043.

Wang, B., Ding, Z., Wang, W., Hwang, J., Liao, Y., and J. L. Ivy. 2015. The effect of an amino acid beverage on glucose response and glycogen replenishment after strenuous exercise. Eur. J. Appl. Physiol. 115: 1283-1294. doi: 10.1007/s00421-015-3098-8.

Yang, Y., Creer, A., Jemiolo, B., and S. Trappe. 2005. Time course of myogenic and metabolic gene expression in response to acute exercise in human skeletal muscle. J. Appl. Physiol. 98: 1745-1752. doi:10.1152/japplphysiol.01185.2004.

Yaspelkis, B. B., and J. L. Ivy. 1999. The effect of a carbohydrate-arginine supplement on postexercise carbohydrate metabolism. Internat. J. Sport Nutr. 9(3)241-250. doi: 10.1123/ijsn.9.3.241.

Table 3.1. Chemical Analysis of Feedstuffs (As-Fed)

Component (As-Fed)	Concentrate	Hay
Moisture, %	11.4	12.5
Crude Protein, %	13.1	10.5
Acid Detergent Fiber, %	18.7	28.3
Neutral Detergent Fiber, %	34.8	57.8
Starch, %	9.35	0.42
Fat, %	9.5	2
Ash, %	7.85	6.55
Calcium, ppm	11000	3350
Copper, ppm	52.5	5
Iron, ppm	675	47.5
Phosphorous, ppm	6610	4240
Magnesium, ppm	4760	3180
Manganese, ppm	180	45.7
Potassium, ppm	11800	18800
Sodium, ppm	3960	116
Zinc, ppm	139	14.4
Calculated DE, Mcal/kg DM	3.30	2.09

Table 3.2. Amino Acid Composition of Feedstuffs (As-Fed)

Amino Acid (Free, mg/g)	Concentrate	Hay
Alanine	0.228	0.751
Arginine	0.316	0.233
Asparagine	0.821	0.124
Aspartic Acid	0.278	0.186
Citrulline	<0.100	<0.100
Cystine	<0.100	<0.100
Glutamic Acid	0.324	0.128
Glutamine	<0.100	0.105
Glycine	<0.100	<0.100
Histidine	<0.100	<0.100
Isoleucine	<0.100	0.143
Leucine	<0.100	0.222
Lysine	0.414	0.206
Methionine	0.394	<0.100
Phenylalanine	<0.100	0.123
Proline	0.308	1.02
Serine	0.100	0.145
Threonine	<0.100	0.128
Tryptophan	0.288	<0.100
Tyrosine	<0.100	0.126
Valine	0.131	0.314

Table 3.3. Exercise Protocol

	Gait	Time (min)	Speed (m/s)
4X	Walk	3	1.983
	Trot	7	4.935
	Canter	5	7.264

Table 3.4. Plasma concentration of urea cycle components at 0 and 3.5 hr post-feeding

	Urea[1]		Citrulline[1]	
	Pre-prandial	Post-prandial	Pre-prandial	Post-prandial
Urea,μg/mL	324 ± 13^{a}	358 ± 13^{bc}	346 ± 13^{b}	362 ± 13^{c}
Citrulline, μg/mL	14 ± 6.6^{a}	15 ± 6.6^{a}	15 ± 6.6^{a}	120 ± 6.6^{b}
Arginine, μg/mL	13 ± 1.4^{a}	20 ± 1.4^{b}	17 ± 1.4^{ab}	33 ± 1.4^{c}
Ornithine, μg/mL	7.2 ± 0.7^{a}	9.0 ± 0.7^{ab}	10 ± 0.7^{b}	14 ± 0.7^{c}
Glutamine, μg/mL	43 ± 3.9^{a}	64 ± 3.9^{b}	48 ± 3.9^{a}	60 ± 3.9^{b}

[a,b,c] Denotes differences ($P < 0.05$) within row

Table 3.5. Real time PCR primer sequences

	Forward primer, 5′-3′	Reverse primer, 5′-3′	Size, bp	E ,%
Vegfa	TGCGGATCAAACCTCACCAA	CCCACAGGGATTTTCTTGCC	112	108
Flt-1	TAGACTGTCACGCTAACGGC	GTGCTGCTTCCTGGTCCTAA	105	85
Myog	TCACGGCTGACCCTACAGATG	GGTGATGCTGTCCACAATGG	64	87
Pax7	CATCGGCGGCAGCAA	TCCTCGATCTTTTTCTCCACATC	60	92
Igf-1	TGTCCTCCTCACATCTCTTCTACCT	CGTGGCAGAGCTGGTGAA	62	100
Ppargc1-α	CGCGTCTTGGGGGTTATCAT	TCGGAAAGGTCAAGTTCGG	104	107
Myh1	TACCGAGGCAAAAAGCGCCA	TCCCGGCACCAGATTCTCCA	127	88
Myh2	GTGACGGTGAAAACCGATGC	GAGGCCCGAGTAGGTGTAGA	186	87
Myh7	AGCGAGCTAAGCGGAAGCTG	CTGCCCAGAGCCTGCTCATC	160	83
Gapdh	CAAGGCTGTGGGCAAGGT	GGAAGGCCATGCCAGTGA	59	107

E = efficiency; Vegfa = vascular endothelial growth factor a; Flt-1 = vascular endothelial growth factor a receptor; Myog = myogenin; Ppargc1a = peroxisome proliferator-activated receptor gamma coactivator 1-alpha; Myh1 = myosin heavy chain 1; Myh2 = myosin heavy chain 2; Myh7 = myosin heavy chain 7; Gapdh = glyceraldehyde phosphate dehydrogenase. Efficiency was calculated using $-1+10^{(-1/\text{slope})}$.

Table 3.6. Heart rate variables during submaximal exercise

	Urea	Citrulline
Minimum	57.52 ± 4.07	60.47 ± 3.75
Average	115.86 ± 2.47	116.40 ± 2.38
Maximum	179.52 ± 6.35	176.51 ± 6.02
Recovery	71.57 ± 2.85	75.12 ± 2.57
Replicate		
1	122.78[a]	122.29[a]
2	113.68[b]	116.66[a]
3	114.87[ab]	114.84[a]
4	113.96[b]	116.02[a]

[a,b] Denotes differences ($P < 0.05$) within column between replicates

Figure 3.1.

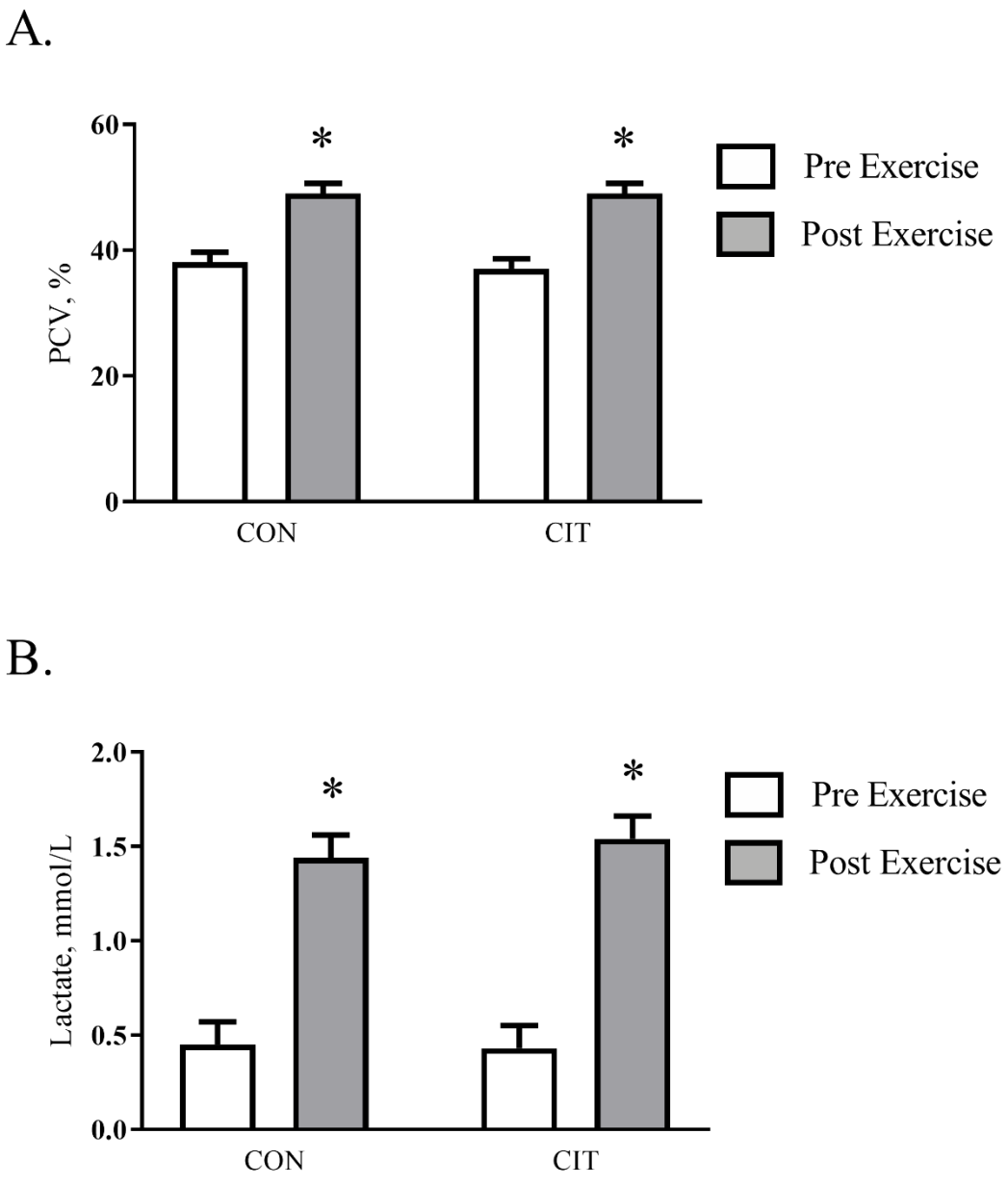

Figure 3.1. Exercise increases packed cell volume (PCV) and lactate.

Horses (n = 12) were fed urea (CON) or citrulline (CIT) for 21 d prior to undergoing a strenuous exercise protocol. Blood was collected and plasma retained immediately pre-

and post-exercise. Exercise increased (A) PCV and (B) plasma lactate. *$P < 0.05$. Means and SEMS are presented.

Figure 3.2.

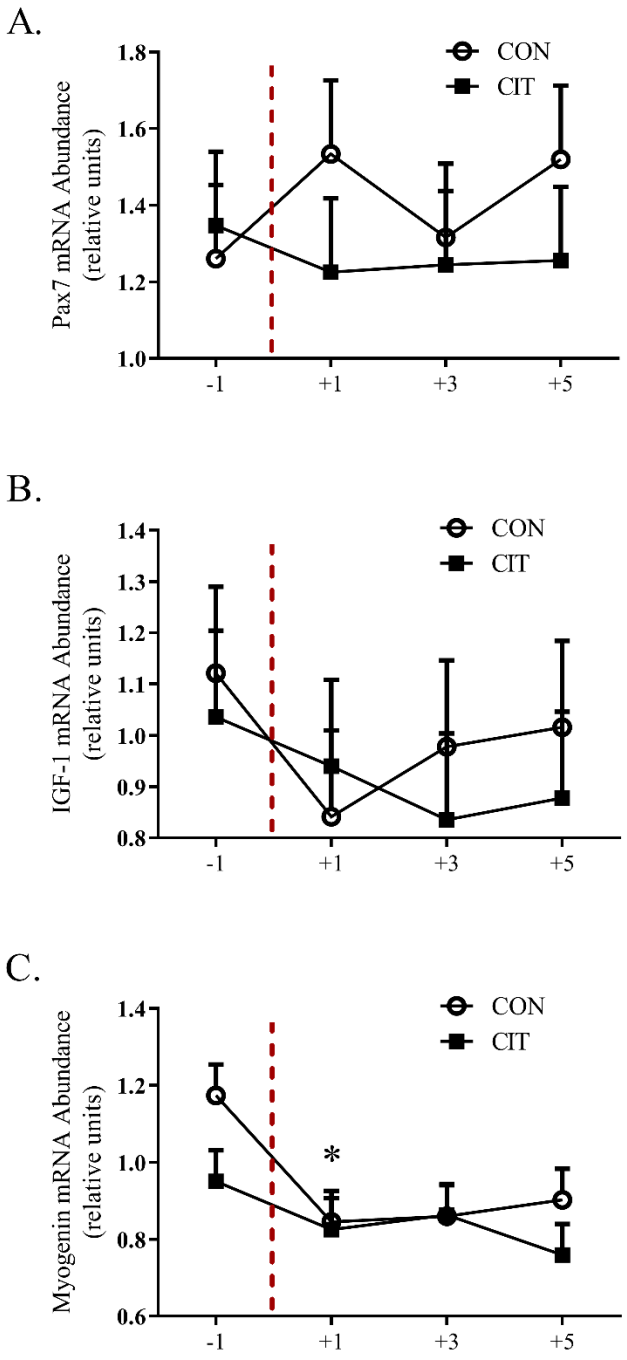

Figure 3.2. Citrulline decreases myogenin expression but does not alter other factors associated with SC activity.

Horses (n = 12) were fed urea (CON) or citrulline (CIT) for a total of 26 d. On d 21 horses underwent a strenuous endurance exercise protocol. Skeletal muscle biopsies were collected from the gluteus medius on d 20, 22, 24, and 26. Ribonucleic acid was extracted from biopsies, DNA removed, reverse transcribed to cDNA, and quantitative PCR performed. Neither diet nor exercise affected (A) *Pax7* or (B) *IGF-1* expression. (C) Citrulline decreased *myogenin* overall while exercise specifically reduced *myogenin* in CON at +1. The $2^{(-\Delta\Delta ct)}$ method was used to calculate relative expression. *$P < 0.05$ CON -1 v +1. Means and SEM presented. Dashed line represents exercise.

Figure 3.3.

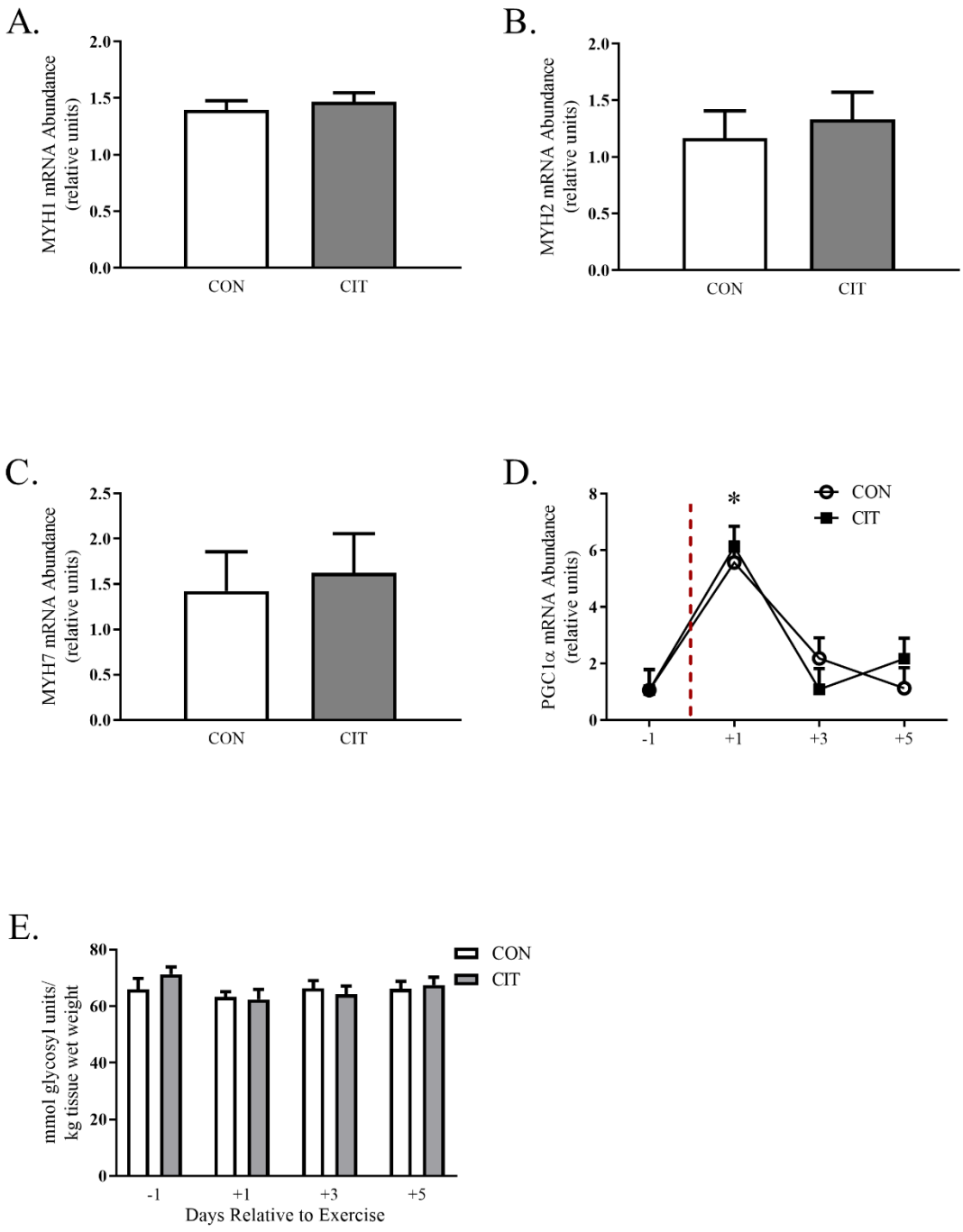

Figure 3.3. Citrulline does not alter metabolic properties of skeletal muscle. Horses (n = 12) were fed urea (CON) or citrulline (CIT) for a total of 26 d. On d 21 horses underwent a strenuous endurance exercise protocol. Skeletal muscle biopsies were

collected from the gluteus medius on d 20, 22, 24, and 26. Ribonucleic acid was extracted from biopsies, DNA removed, reverse transcribed to cDNA, and quantitative PCR performed. Citrulline did not affect (A) *MYH1*, (B) *MYH2*, or (C) *MYH7* pre-exercise. (D) Exercise did increase *PGC1-α* at 1 d post-exercise relative to pre-exercise. (E) Glycogen was not affected by diet or time. The $2^{(-\Delta\Delta ct)}$ method was used to calculate relative expression. **P* < 0.05 -1 v +1. Means and SEM presented. Dashed line represents exercise. MYH1 = myosin heavy chain 2x; MYH2 = myosin heavy chain 2a; MYH7 = myosin heavy chain 1; PGC1-α = peroxisome proliferator-activated receptor gamma coactivator 1-alpha.

Figure 3.4.

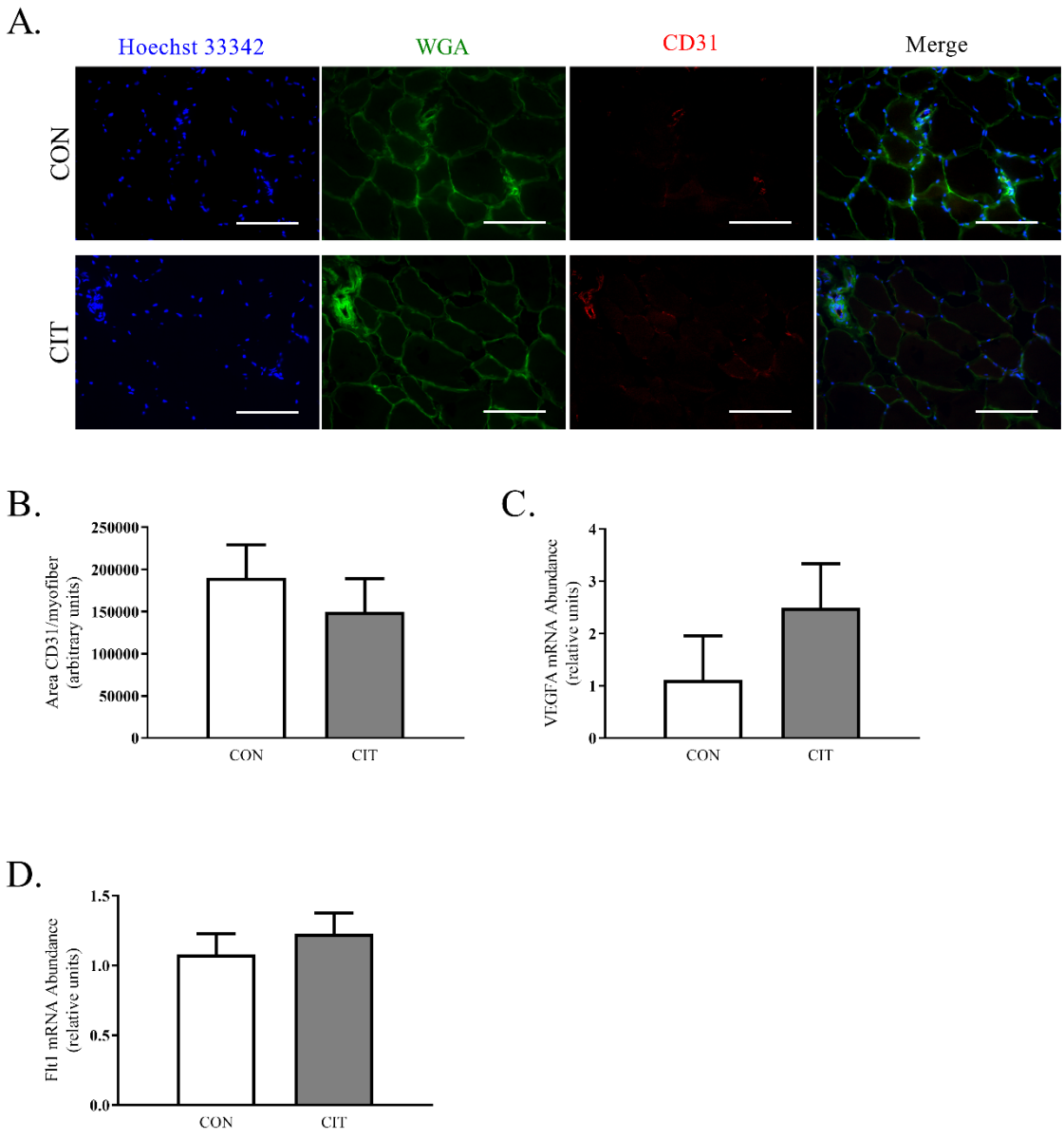

Figure 3.4. Citrulline does not impact vascular components in equine gluteus medius. Horses (n = 12) were fed urea or citrulline for 20 d prior to collection of skeletal muscle biopsies from the gluteus medius. (A) Skeletal muscle biopsies were fixed and embedded in tissue sectioning medium. Serial sections 10 µm thick were permeabilized and blocked then probed with anti-CD31. (B) Fluorescent area of CD31 was quantified and expressed

relative to the number of myofibers. Ribonucleic acid was extracted from snap-frozen biopsies, DNA removed, reverse transcribed to cDNA, and quantitative polymerase chain reaction performed. Citrulline did not affect (C) *VEGFA* or its receptor, *Flt-1*. Means and SEMS are presented. VEGFA = vascular endothelial growth factor A; Flt-1 = vascular endothelial growth factor A receptor.

CHAPTER IV

Conclusions and Future Directions

Horses have long been used in athletic events, with almost half of the 7.2 million horses today participating in regular physical activity in the form of either racing, showing, or working. Muscle damage may accompany strenuous exercise leading to response and adaptation mediated by SCs and numerous signaling pathways specific to the stimulus applied. Very little is known regarding eqSC-dependent response and adaptation to exercise. Equine SCs do respond to exercise, however, this response is not universal, as eqSC were stimulated following a short, intense bout of inclined treadmill exercise but not to exercise of a more moderate intensity and longer duration performed as a portion of this dissertation.

The factor responsible for eqSC activation remains elusive. Hepatocyte growth factor, a SC activator in rodents, did not accelerate S-phase entry of freshly isolated eqSC. Notably, HGF did increase proliferation and exert a moderately suppressive effect on differentiation and is likely a factor influencing eqSC dynamics after exercise. Though HGF may be found in circulation, it is a potent paracrine/autocrine factor found in the SC niche. Satellite cells from young horses respond to serum from exercised horses, serum from young mice rejuvenates SCs from old mice, and serum from injured mice activate SCs from non-injured mice. Recently, HGFA has been identified as the factor in injured serum that activates HGF signaling, but exogenous HGF administration does not recapitulate this process in vivo. The presence of SCs to a multitude of factors in specific quantities and perhaps phasic patterns may explain the discrepancies in the identification of SC activators. The advent of genetic models and new technologies may lead to further

understanding of growth factor regulated SC dynamics, particularly in vivo where additive effects will ultimately influence response.

In addition to further characterizing growth factor regulation of SCs, future studies should focus on defining the primary determinant of eqSC activity after exercise. In humans and rodents, SCs are active following both endurance and resistance training hypertrophic and non-hypertrophic in nature. This suggests a role for SCs beyond hypertrophy, perhaps as a mediator of the remodeling process accompanying endurance exercise. Further, defining the role of eqSC in horses with previous exposure to exercise and those that have never received training is imperative, as the concept of muscle memory may prevent application of conclusions derived from trained animals to the untrained. After defining the guiding factors for SC activity, it may prove beneficial to re-evaluate nutritional strategies to enhance both SC activity and nutrient reserves for improved performance.