EPH/EPHRIN INVOVLEMENT IN SKELETAL MUSCLE DEVELOPMENT AND REGENERATION

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have examined the Dissertation entitled

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presented by Laura L. Arnold,

a candidate for the degree of

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and hereby certify that, in their opinion, it is worthy of acceptance.

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DEDICATION

This dissertation is dedicated to my parents. My mom and dad have supported me throughout my PhD. I appreciate their encouragement and their attempts at scientific advice. I treasure my mother for dealing with late night phone calls and driving the 10 hours from Ohio to come visit me multiple times. My parents are always willing to drive to hop in the car to come help me get through tough times. I know I can always count on both of my parents for anything I need.

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I have mentored many undergraduates throughout my graduate studies. Many of them became my undergraduates after wandering into lab and looking lost at what to do. However, as the months and years go on they have become independent and have made significant contributions to my data. I look forward to seeing where they all end up.

A special thanks to my committee, I have appreciated your guidance and fitting me into your busy schedules. The tips I have learned from our meetings have shaped the way I will present my data in the future. I will keep you updated on my scientific progress.

TABLE OF CONTENTS

LIST OF ILLUSTRATIONS

CHAPTER 1

CHAPTER 2

CHAPTER 3

CHAPTER 4

AUTHOR CONTRIBUTION

CHAPTER 1: Eph/ephrin signaling during development

Laura L. Arnold and DDW Cornelison drafted and critically revised the manuscript

CHAPTER 2: Mice lacking EphA7 exhibit blunted skeletal muscle specification and commitment

Laura L. Arnold, Danny A. Stark, Sammy Zino, Rebecca Craigg, Jacqueline Ihnat, and Hannah R. Pancoast performed data collection. Laura L. Arnold, and Danny A. Stark performed data analysis and interpretation. Laura L. Arnold and DDW Cornelison designed experiments, drafted and critically revised the manuscript.

CHAPTER 3: Is EphA3 a determinate of 'fast' and 'slow' satellite cells?

Laura L. Arnold, Sammy Zino, and Jacqueline Ihnat performed data collection. Laura L. Arnold performed data analysis and interpretation. Laura L. Arnold and DDW Cornelison designed experiments, drafted and critically revised the manuscript.

CHAPTER 4: Oxidative metabolic shift effect on skeletal muscle

Laura L. Arnold, Sammy Zino, and Kyle Nix, performed data collection. Laura L. Arnold performed data analysis and interpretation. Laura L. Arnold and DDW Cornelison designed experiments, drafted and critically revised the manuscript.

CHAPTER 5: Significance and Future Directions

Laura L. Arnold drafted and critically revised the manuscript

EPH/EPHRIN INVOVLEMENT IN SKELETAL MUSCLE DEVELOPMENT AND REGENERATION

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ABSTRACT

Skeletal muscle development and regeneration is one of the best-described areas of vertebrate biology, due in large part to muscle cells' characteristic sequence of specification, determination, and differentiation. However, many questions still remain open, including the relative extent to which intrinsic lineage factors, local interactions with other myogenic cells, and systemic physiological factors affect muscle cell identity and activity.

Eph/ephrin signaling can promote proliferation or differentiation, survival or death, adhesion or deadhesion, and repulsion or attraction, depending on the molecular and cellular context. In skeletal muscle tissue, the activity of Eph/ephrins in development and regeneration is not yet fully explored. This dissertation describes experiments into the roles two different Eph proteins may play in mediating muscle development, homeostasis, and regeneration. The first, EphA7, appears to act during both muscle development and muscle regeneration in the adult to promote myogenic specification and hypertrophy. The second, EphA3, is differentially expressed by activated satellite cells residing on fast vs. slow muscle fibers, potentially in response to expression of an ephrin ligand (ephrin-A3) solely on slow myofibers. The final data chapter focuses on a mouse model in which overexpression of PGC-1a, a transcriptional coactivator that promotes mitochondrial biogenesis, induces a shift from glycolytic (typical of fast myofibers) to oxidative (typical of slow myofibers) metabolism in the skeletal muscle. We note that surprisingly, in spite of this physiological adaptation, the

expression of fast vs. slow myosin heavy chain isoforms is not significantly altered.

The focus of this work is on molecular and cellular factors affecting skeletal muscle morphogenesis and fiber type patterning during development, homeostasis and regeneration, and highlights the potential for juxtacrine interactions to direct these processes.

CHAPTER 1:

Eph/ephrin signaling during development

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ABSTRACT

Vertebrate development requires the orchestration of multiple signaling pathways, receiving and sending information to a broad array of developing cell types organized in time and space. These pathways are broadly classified as endocrine (systemic soluble signals), paracrine (local soluble signals), or juxtacrine (surface-localized signals.) One such juxtacrine signaling axis, in which cell-surface Eph receptors interact with cell-surface ephrin ligands, is known to determine cell identity, positioning, and activity in many cell types during development as well as in adult stem cell niche interactions. This chapter will briefly review Eph/ephrin signaling during development, then focus on what is currently known regarding Eph/ephrin signaling in skeletal muscle development, homeostasis, and regeneration.

Overview of Eph/ephrins

The majority of cell-cell signaling pathways studied in the context of vertebrate development, such as FGF, TGFβ, Wnt, and Hedgehog. are paracrine (a secreted ligand diffuses away from the cell that secretes it, often producing different results at different local concentrations). However, a key subset of developmental signaling pathways, often determining cell identity and/or location, are juxtacrine (requiring cell-cell contact.) The best-studied example of a juxtacrine pathway is Notch-Delta: cells expressing either Notch receptor or Delta ligand at their cell surface interact across the extracellular space, leading to changes in transcription in either or both cells. The effects of Notch-Delta signaling often segregate or differentiate cells at compartment borders or among different potential fates [reviewed in (Siebel and Lendahl, 2017)]. Similarly, the Eph/ephrin juxtacrine signaling pathway acts in almost all tissues during development, homeostasis, or disease to define and reinforce cell boundaries, promote or inhibit cell specification, and mediate cellular sorting and migration [reviewed in (Ventrella et al., 2017)]. However, the additional complexities inherent in Eph/ephrin signaling make examining and interpreting Eph/ephrin expression and activity somewhat more problematic.

Eph proteins are receptor tyrosine kinases with multiple intracellular pathways available for them to affect cell identity and activity [reviewed in (Holmberg and Frisen, 2002; Kania and Klein, 2016; Klein, 2012)]. Eph receptors are also highly promiscuous: most EphAs can bind to any of the ephrin-As, and some EphAs can

bind to an ephrinB [reviewed in (Himanen et al., 2007)]. In addition, ephrins may themselves act as 'receptors' when they bind an Eph on an adjacent cell, leading to bidirectional signaling or, in some cases, reverse signaling [reviewed in (Davy and Soriano, 2005; Miao and Wang, 2012; Murai and Pasquale, 2003)]. Finally, the specificity of Eph/ephrin interactions as well as their cellular effects are highly context-specific, with membrane localization (i.e., to lipid rafts) [reviewed in (Gauthier and Robbins, 2003)], association with other receptor tyrosine kinases (Nakayama et al., 2013), adhesion receptors (Davy and Robbins, 2000; Noren et al., 2009) or even other Ephs (Jurek et al., 2016) determining the specificity and downstream effects of Eph-ephrin interactions. Thus, while cell sorting and migratory repulsion are the most common consequences of Eph/ephrin signaling, in different contexts it can promote cellular proliferation, differentiation, survival, death, motility, or adhesion [reviewed in (Gucciardo et al., 2014)].

Eph/ephrins in development

The context in which Eph/ephrin interactions were first described was during migration of neural crest cells (Krull et al., 1997) and motor axons (Wang and Anderson, 1997) during embryonic development. As Eph-expressing cranial neural crest cells migrate laterally over the rhombomeres of the developing hindbrain into the branchial arches (Smith et al., 1997) or trunk neural crest cells migrate through the anterior half of developing somites, their paths are directed by repulsive interactions with ephrin-expressing cells defining areas into which

migration is prohibited. As Eph/ephrin expression changes over developmental time, neural crest cell migration pathways also change. Ephs and ephrins are induced very early in development in opposing regions at the prospective rhombomere boundaries (Cooke et al., 2001; Cooke et al., 2005; Irving et al., 1996) or somite boundaries (Durbin et al., 1998) to promote segmentation.

Eph/ephrin signaling remains critical throughout life not only for central nervous system adaptation (Fiederling et al., 2017) but also for multiple stem cell niches: the hematopoietic (Nguyen et al., 2016; Ting et al., 2010), intestinal (Holmberg et al., 2006), brain (Jiao et al., 2008; Khodosevich et al., 2011; Theus et al., 2010), bone (Arthur et al., 2010; Matsuo and Otaki, 2012), skin and hair (Genander et al., 2010; Genander, 2012), retinal (Fang et al., 2013), tooth (Arthur et al., 2009) and cardiac (Goichberg et al., 2011) stem cell niches all utilize Eph/ephrin signals to regulate homeostasis and regeneration. Not surprisingly, many cancers also show altered Eph/ephrin signaling [reviewed in (Chen et al., 2015; Dodelet and Pasquale, 2000; Kandouz, 2012)].

Eph/ephrins in muscle development

Myogenesis in the embryo is initiated when cells in the dermomyotome of the somite (Chevallier et al., 1977; Christ et al., 1977) are induced by local paracrine signaling (Hopwood et al., 1989) to express a member of the MyoD family of myogenic regulatory transcription factors (MRFs), which specifies them to the muscle lineage (Weintraub et al., 1991). Cells located in the dorsomedial

dermomyotome, in response to Shh from the notochord and floorplate (Borycki et al., 1999) and Wnt signals from the dorsal neural tube (Ikeya and Takada, 1998; Ott et al., 1991; Tajbakhsh et al., 1998), will upregulate the MRF myf-5. Shortly thereafter, cells of the ventrolateral dermomyotome expressing the paired-box transcription factor, Pax3, (Borycki and Emerson, 1997; Williams and Ordahl, 1994) will upregulate MyoD itself in response to Wnt signals from the dorsal ectoderm (Tajbakhsh et al., 1998). Due to MRF autoregulation, cells in both compartments will proceed to expression of both myf-5 and MyoD (Smith et al., 1994) and cells in the dorsal dermomyotome will upregulate a differentiation-specific MRF, myogenin (Rawls et al., 1995). Myoblasts of this lineage will generate skeletal muscles of the deep back (Kablar et al., 1998), while myoblasts formed in the ventrolateral lineage will undergo an epithelial-to-mesenchymal transition and emigrate away from the somite to form the muscles of the trunk and limbs (Figure 1-1). Although expression of Ephs and ephrins in the somite is well-studied in the context of somitogenesis and neural crest cell and motor axon pathfinding, there is as yet no indication that Eph/ephrins in the somite influence dermomyotome cell specification, activity, or patterning.

In order to migrate away from the somite, myoblasts must express Pax3 and c-met, which is necessary for myoblast migration (Bladt et al., 1995; Bober et al., 1994; Franz et al., 1993). The c-met ligand, HGF, is expressed by the lateral plate mesoderm in the limb buds and is sufficient to induce myoblast migration (Brand-Saberi et al., 1996). In addition, to this chemotactic effect it may also

regulate muscle patterning in the limb (Scaal et al., 1999). A role for Eph/ephrin signaling in myoblast migration during embryonic limb muscle development has not been established, although it would seem a likely mechanism and multiple Ephs and ephrins are present in the developing limb bud to regulate patterning of motor axons, vasculature and cartilage elements (Adams and Eichmann, 2010; Araujo et al., 1998; Eberhart et al., 2000; Wada et al., 2003). Ephrin overexpression studies done in the chick (Swartz et al., 2001) suggest that EphA4-expressing limb myoblasts respond repulsively to ephrin-A5; because endogenous ephrin-A5 expression is localized to the ventral half of the forming limb bud this could provide a potential mechanism for sorting subpopulations of myoblasts, but experimental loss of ephrin-A5 in the limb bud does not appear to affect motor neuron or myoblast migration (Vaidya et al., 2003; Winning and Krull, 2011).

At least two and as many as four (depending on the species studied) populations of myoblasts (Edom-Vovard et al., 1999; Hutcheson et al., 2009) are thought to emigrate from the somite to populate the limb bud. After separating into the dorsal and ventral premuscle masses, the first wave (embryonic myoblasts) will differentiate to form primary myofibers, which are smaller, often mononucleated fibers that will form a ‘scaffold’ prepatterning the muscles (Crow and Stockdale, 1986; Seed and Hauschka, 1984). Later, a second population (fetal myoblasts) will differentiate along the primary myofibers to form secondary myofibers, which are larger multinucleated fibers that will constitute the majority of muscle

fibers at birth (Page et al., 1992). It is during this stage of differentiation that the precursor to the eventual pattern of fast and slow muscle fibers is generated. However, contradictory data exist in the literature regarding whether myoblasts that will generate primary and secondary myofibers, or fast and slow myofibers, truly constitute unique populations (Condon et al., 1990; Mouly et al., 1987; Van Swearingen and Lance-Jones, 1995). As noted above for control of migration, no involvement of Eph/ephrin signaling in myoblasts lineage sorting or muscle patterning and differentiation in the prenatal limb bud has yet been observed.

During late fetal and postnatal development, muscle fibers formed during embryogenesis grow by hypertrophy. During this process, proliferating myoblasts will exit the cell cycle, upregulate myogenin and finally the fourth MRF, MRF4, (Miner and Wold, 1991; Rhodes and Konieczny, 1989) while losing expression of MyoD and myf-5. These myocytes are terminally differentiated, and will fuse into existing myofibers to provide additional myonuclei as the muscle fibers grow. The balance between myoblast proliferation and differentiation is regulated by multiple paracrine signaling pathways in the limb bud, including FGF2 (pro-mitogenic) and TGF-β (anti-mitogenic) (Christ and Brand-Saberi, 2002; Filvaroff et al., 1994; Flanagan-Steet et al., 2000; Itoh et al., 1996; Savage et al., 1993; Zappelli et al., 1996). In mouse, this process of differentiation, fusion and hypertrophy continues until mature myofiber diameters stabilize at 2-3 months of age (Pawlikowski et al., 2015). Key transcription factors, secreted signaling molecules, and adhesion-associated signals that determine the placement,

pattering, and size of specific individual muscles (Colasanto et al., 2016; Schäfer and Braun, 1999) as well as limb musculature in general (Kardon et al., 2002; Kardon et al., 2003; Kardon, 1998; McPherron et al., 1997) have been identified, but it is clear to the field that our current understanding remains incomplete. In addition to the myoblasts that will eventually differentiate and fuse to generate and grow the developing limb, trunk, and axial musculature, a unique population of myogenic cells is also generated in the somite during embryogenesis (Hutcheson et al., 2009; Lepper et al., 2009; Lepper and Fan, 2010; Murphy and Kardon, 2011; Schienda et al., 2006). To date, no Eph/ephrin involvement has been reported for this process; we report elsewhere in this thesis that EphA7 appears to act to promote muscle differentiation and hypertrophy in the postnatal limb.

The contractile function of skeletal muscle requires innervation by motor neurons, whose cell bodies reside in the spinal cord and project axons into the muscle according to cell-autonomous topographic maps (Landmesser and Morris, 1975; Landmesser, 1978; Landmesser, 2001; Laskowski and Sanes, 1987; Milner et al., 1998). Involvement of forward, reverse, and autoregulatory Eph/ephrin signals (as well as the activity of other families of contact-mediated guidance proteins) in motor axon pathfinding and neuromuscular synaptogenesis is well-established (Chadaram et al., 2007; Dudanova et al., 2012; Franz et al., 2008; Kao and Kania, 2011; Luria et al., 2008; Luxey et al., 2013; Nguyen et al., 2002; Wang et al., 1999); expression data also suggest that Eph/ephrins may act at the

neuromuscular junction itself (Lai et al., 2001). Recent work from our group, showed that ephrin-A3 expression exclusively on slow muscle fibers inhibits their innervation by fast motor neurons (which would cause them to convert to fast muscle fibers) as neuromuscular junctions are stabilized during postnatal maturation (Stark et al., 2015).

Eph/ephrin signaling during regeneration

Once adulthood is reached, adult stem cells ,satellite cells, are responsible for all postmaturation muscle hypertrophy and repair (Lepper et al., 2011; Murphy et al., 2011; Sambasivan et al., 2011). In uninjured, nonpathological adult muscle these rare, mononucleated cells are located between the cell membrane and the secreted basal lamina of multinucleate muscle fibers in a state of nonproliferative 'quiescence' (Mauro et al., 1961). Satellite cell quiescence is emerging as a much more dynamic, complex, tightly-regulated, and heterogeneous state than previously appreciated [reviewed in (Doles and Olwin, 2015; So and Cheung, 2018)]. Quiescent satellite cells are marked by their persistent expression of Pax7 (or, in some populations, Pax3) (Kassar-Duchossoy et al., 2005; Kuang et al., 2006; Relaix et al., 2005; Relaix et al., 2006) and Pax7 is necessary for their specification and/or maintenance of identity (Oustanina et al., 2004; Seale et al., 2000; Seale et al., 2004).

In response to local muscle injury, satellite cells exit the quiescent state and the sublaminar niche, re-enter the cell cycle, and initiate protein expression of MyoD

[reviewed in (Dumont et al., 2015)] (Figure 1-2). Activated satellite cells proliferate, migrate to the site of injury, and eventually upregulate myogenin, differentiate and fuse with damaged or de novo muscle fibers in response to a complex and dynamic set of secreted and matrix cues [reviewed in (Ciciliot and Schiaffino, 2010; Dumont et al., 2015; Yin et al., 2013)]. The intrinsic and extracellular pathways that regulate activation, proliferation, migration, commitment to differentiation, and fusion are still being defined and explored, and constitute one of the major areas of research in the field at this time. Similarly, the intrinsic and extracellular pathways that specify or impose heterogeneity within the population of quiescent and activated satellite cells are also an area of significant interest in the field [reviewed in (Biressi and Rando, 2010; Sambasivan and Tajbakhsh, 2015; Tierney and Sacco, 2016)].

Previous work from our group has characterized expression of Eph/ephrin mRNA and protein in adult muscle and in quiescent and activated satellite cells, which identified a host of Ephs and ephrins dynamically expressed over the course of muscle regeneration in multiple distinct cell types (Stark et al., 2011). While it was recently suggested that EphB1 signaling in satellite cells promotes proliferation and/or self-renewal over differentiation based on expression of a dominant-negative form of EphB1 in a myogenic cell line (C2C12) (Alonso-Martin et al., 2016). To date no direct role for Eph/ephrin signaling has been demonstrated in regulation of satellite cell identity or activity. In Chapter 2 of this dissertation we will present new data indicating that EphA7 promotes

commitment to myogenic differentiation in satellite cell-mediated muscle regeneration, and in Chapter 3 we will present new data suggesting that expression of EphA3 indicates heterogeneity of satellite cells that are resident on fast vs. slow myofibers.

At the conclusion of muscle regeneration, as at the conclusion of muscle development, a steady-state arrangement of patterned and functioning muscle fibers is present in each muscle, along with a population of quiescent satellite cells that are available to repair or replace the differentiated muscle fibers if and when the need arises. Among the Eph/ephrins we surveyed previously in uninjured adult muscle, we noted some with expression on subsets of differentiated muscle fibers, some with expression on quiescent satellite cells, and some with expression on nonmuscle resident cell types (Stark et al., 2011); Stark, unpublished). It is unknown as yet to what extent, if any, this steady-state expression contributes to muscle homeostasis.

Conclusions

Juxtacrine signaling, including Eph/ephrin signaling in any of its many potential modalities, directs cell specification, association, migration, identity, and patterning in multiple tissues during development, homeostasis, repair, and disease. This chapter provided an overview of Eph/ephrin signaling, myogenesis, and what is currently known of the interface between the two. The goal was to provide background and context for the subsequent chapters, in which I will

present my new data extending our understanding of how Eph/ephrin signaling contributes to skeletal muscle generation and regeneration.

FIGURES AND LEGENDS

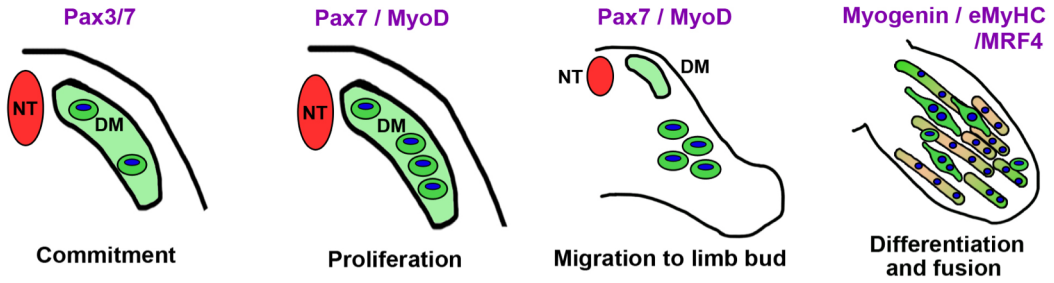

Figure 1-1 Myogenic marker expression during muscle progenitor cells progression through development

During development, the neural tube (NT) and the somite are adjacent to one another. The dermomyotome (DM) is derived from the somite and is the source for muscle progenitor cells. These muscle progenitor cells migrate from the DM to their site of differentiation and leads to fusion of muscle progenitor cells that form muscle fibers.

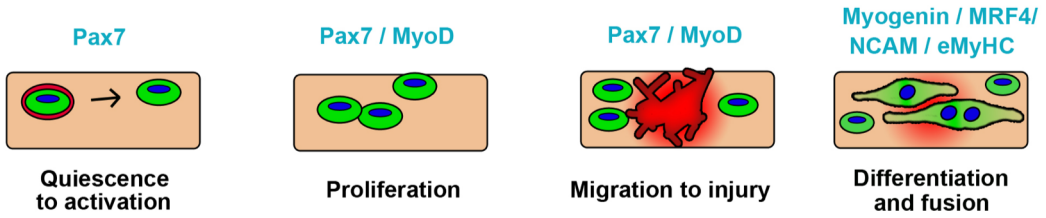

Figure 1-2 Myogenic marker expression during satellite cells progression through regeneration

Adult satellite cells are quiescent during homeostatsis until activation due to a muscle injury. The preliminary action of satellite cells is to proliferate, then satellite cells migrate to the site of injury. Upregulation of MRFs and muscle specific markers during differentiation lead to regeneration of injured muscle fibers.

CHAPTER 2:

Mice lacking EphA7 exhibit blunted skeletal muscle specification and commitment

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ABSTRACT

Skeletal muscle fibers are syncytial, multinucleated cells first formed during embryonic development by the fusion of differentiated myogenic precursor cells (MPCs). The conversion of specified, proliferating skeletal muscle precursors (myoblasts) to terminally-differentiated, postmitotic myocytes is a critical step in skeletal muscle development and repair. If this step is inhibited, there will be no functional muscle fibers, while if it is precocious, the muscle fibers generated may be insufficient for the anatomical need. We have identified a contact-dependent cell surface receptor, EphA7, which is expressed by differentiated myocytes during embryonic and adult myogenesis and is transiently expressed on nascent myofibers *in vivo*. In mice lacking EphA7, hindlimb muscles have fewer myofibers at birth, and those myofibers are reduced in size and have fewer myonuclei, as well as reduced overall numbers of MPCs, throughout postnatal life. Similarly, adult EphA7$^{-/-}$ mice have reduced numbers of satellite cells and exhibit delayed and protracted muscle regeneration. The expression pattern and loss-of-function phenotype in EphA7$^{-/-}$ mice suggests a role in terminal differentiation. Consistent with this, molecular studies of satellite cell-derived myogenic cells from EphA7$^{-/-}$ mice reveal a delay in their expression of differentiation markers. We propose a model in which EphA7 expression on differentiated myocytes promotes commitment of local myoblasts to terminal differentiation via cell-cell contact mediated signals.

INTRODUCTION

Skeletal muscle comprises approximately half of adult human body mass, and is necessary for all voluntary and involuntary movements including ambulation, posture, and respiration, making it a tissue that is critical for life. Due to its characteristic and hierarchical lineage specification, skeletal muscle is also one of the best-characterized tissues in developmental biology, and muscle stem cells (satellite cells) are a similarly well-studied adult stem cell. Myogenesis begins in the somite, where cells in the dermamyotome expressing the transcription factor Pax3 are specified to the myogenic lineage through paracrine interactions with local structures including the neural tube, surface ectoderm, and lateral plate mesoderm (Williams and Ordahl, 1994). Depending on where the cells are located within the dermamyotome, the newly-specified myoblasts will express either myf-5 or MyoD (Hirsinger et al., 1997; Stern et al., 1995; Tajbakhsh et al., 1998), which are both members of the family of bHLH myogenic regulatory transcription factors (MRFs) [reviewed in (Funk et al., 1991; Kablar and Rudnicki, 2000)]. Myoblasts located in the dorsomedial compartment of the dermamyotome will commit to terminal myogenic differentiation in three successive waves, permanently exiting the cell cycle and initiating expression of the MRF myogenin (Kahane et al., 1998b; Kahane et al., 1998b; Kahane et al., 2001; Patapoutian et al., 1995; Yee and Rigby, 1993). These myofibers will eventually give rise to the epaxial musculature of the back.

Alternatively, myoblasts located in the ventrolateral myotome will instead undergo an epithelial-to-mesenchymal transition and emigrate into the developing limb buds in response to stimulation of the HGF receptor c-met (Brand-Saberi et al., 1996; Heymann et al., 1996; Scaal et al., 1999). There, they will first proliferate in response to local FGF signals (Flanagan-Steet et al., 2000) then differentiate, again in successive waves. Primary muscle fibers will first form from $Pax3^{+}MyoD^{+}$ cells which upregulate myogenin and differentiate in response to local signals (Kardon et al., 2002). These myocytes yield short, mononucleated myofibers that are initially disordered, but later align to form a 'scaffold' to prepattern the nascent limb musculature (Lee et al., 2013). The primary myofibers will then grow by addition of differentiated myocytes. A second population of $Pax3^{+}$ and/or $Pax7^{+}$ myoblasts will then undergo extensive proliferation in response to signals from the developing bone (Bren-Mattison et al., 2011), differentiate, and fuse to form secondary myofibers between and among the primary myofibers (Duxson et al., 1989). A third population of $Pax7^{+}$ myoblasts will appear last (Hutcheson et al., 2009), some of which will give rise to stem cells in the adult (described below). Myoblast proliferation, commitment to terminal differentiation, and fusion with existing myofibers will continue through fetal and postnatal development, until the animal has reached maturity (Pawlikowski et al., 2015).

In the event of acute injury, skeletal muscle possesses a population of adult tissue-specific stem cells (satellite cells) capable of undergoing a similar lineage

progression: initially expressing Pax7 (Seale et al., 2000) and/or Pax3 (Boutet et al., 2012; Kuang et al., 2006), upon local injury satellite cells become activated from a state of quiescence and rapidly upregulate MyoD and myf-5 [reviewed in (Cornelison and Wold, 1997)]. Directed by local cues, these adult myoblasts will proliferate, migrate to the site of injury, and subsequently commit to terminal differentiation, exiting the cell cycle and upregulating myogenin expression. Their terminally-differentiated progeny will fuse with each other or an existing muscle fiber to replace the damaged muscle, while a subset of satellite cells will retain stem cell characteristics and return to quiescence.

Although our understanding of the cellular and molecular mechanisms promoting myogenic cell specification, proliferation, migration, and differentiation during development and regeneration has grown dramatically over the past several decades, many fundamental questions remain unanswered in multiple areas of the field (DDW, 2018). One such question is whether and to what extent local populations of myogenic cells communicate amongst themselves in order to coordinate the cellular activities described above in space and time within the population. To mediate local, population-level effects, juxtacrine signaling is an attractive candidate due to its properties of either generating heterogeneous populations of cells from initially homogeneous ones, or sorting heterogeneous cells from within a mixed population via differential affinities. As would be expected, multiple contact-mediated signaling interactions have already been described in the context of myogenesis,

regulating processes such as progenitor cell commitment to the myogenic lineage, myoblast differentiation and fusion, and satellite cell quiescence [reviewed in (Krauss et al., 2017)]. Here we present new data suggesting that EphA7, a component of the Eph/ephrin juxtacrine signaling axis, acts at multiple stages of myogenesis to promote specification and/or differentiation of myogenic precursor cells.

RESULTS

EphA7 expression in muscle is characteristic of terminally differentiating myocytes and nascent myofibers *in vivo* and *in vitro*

Ephs are receptor tyrosine kinases that act via juxtacrine interactions with cells presenting their ligands (ephrins) to modify cell motility, assortment, proliferation, differentiation, and survival in almost all tissues that have been examined to date (Kania and Klein, 2016; Klein, 2010; Klein, 2012). Our initial observations during an expression screen for Eph/ephrins in the context of adult skeletal muscle regeneration suggested that EphA7, while absent during muscle homeostasis, is upregulated by regenerating myofibers *in vivo* by 3 days after a barium chloride injury (Stark et al., 2015). This expression pattern raised the possibly that EphA7 signaling could play a role in events associated with terminal differentiation or maturation of muscle fibers during satellite cell-mediated muscle regeneration.

To extend our initial observations, we first examined EphA7 expression during a timecourse of acute regeneration following local barium chloride (BaCl) injury. Tibialis anterior (TA) muscles were collected at 3, 5, 7, 10, 16, and 21 days post injury (dpi), and sections were stained for expression of EphA7, laminin to identify myofiber boundaries, and embryonic myosin heavy chain (eMyHC) to identify newly-formed myofibers (Figure 1). We noted that EphA7 was consistently expressed by nascent myofibers at all timepoints examined, and that as muscle regeneration was resolved and eMyHC expression was lost EphA7 expression was also extinguished. At later timepoints (10 or 16 dpi) those myofibers with persistent EphA7 expression were consistently smaller than $EphA7^{-}/eMyHC^{-}$ myofibers; these larger fibers were presumably more mature nascent myofibers as they had centrally-located nuclei, a hallmark of regeneration.

To examine the timecourse of EphA7 expression by satellite cells (rather than myofibers), we assayed expression on explanted satellite cells in the absence (Figure 2E-H) or presence (Figure2A-D) of the host myofiber. Consistent with *in vivo* results, satellite cells do not initially express EphA7 in either context. In monoculture, expression of EphA7 is first noted 4 days after isolation in rare, dispersed cells. Upon switching to low-serum medium, which promotes differentiation, increasing numbers of cells (often in close association with one another) upregulate EphA7. Similarly, when satellite cells were isolated and cultured in association with their host myofiber, we again noted no expression of

EphA7 on satellite cells until 48 hours after isolation. Intriguingly, in this context we also noticed at 96 hours, EphA7 expression primarily on satellite cells that were in contact with multiple other EphA7^{+} satellite cells, in clusters on the surface of the myofiber.

To more rigorously define the correlation between EphA7 expression and muscle differentiation state, we assayed satellite cells *in vitro* for co-expression of EphA7 with markers of myogenic progenitors (Pax7), myoblasts (MyoD), and differentiated myocytes (NCAM, which we have previously shown is expressed exclusively of cells that have committed to myogenesis (Capkovic et al., 2008))(Figure 2I-L). We noted that expression of EphA7 and Pax7 are mutually exclusive, while EphA7 and NCAM are uniformly coexpressed, further suggesting that EphA7 is restricted to committed myocytes. MyoD, which is expressed by both proliferating and differentiating satellite cells (Cornelison and Wold, 1997), is expressed in a subset of EphA7^{+} cells, presumably those that are NCAM^{+} and have committed to terminal differentiation.

We repeated the immunohistochemical screen in developing embryos to confirm that EphA7 expression is a property of differentiating myocytes and nascent myofibers. Previously-published work suggests that EphA7 mRNA is present by e11.5 (Alonso-Martin et al., 2016), by which time myogenesis in the somite and limb bud will have already begun. When we examined mouse embryos at e9.5, we observed EphA7 protein staining in the somite (Figure 3A-B)

colocalizing with nuclear expression of MyoD. By e12.5, EphA7 expression marked myogenin expressing cells of the primary myotome (Figure 3C-D). In e15.5 embryos, EphA7 also identified differentiated, embryonic myosin heavy chain (eMyHC)-expressing limb (Figure 3E-F).

EphA7$^{-/-}$ mice display decreased myofiber size, myofiber number, myonuclear number, and progenitor cell number

Mice carrying a germline deletion of EphA7 have been characterized as having multiple nonlethal defects in the central nervous system (Clifford et al., 2014; Kim et al., 2016; Rashid et al., 2005) but no data regarding a potential phenotype in skeletal muscle have been reported. When we examined the morphology and morphometrics of limb muscle in EphA7$^{-/-}$ mice, we noted that while the overall appearance was not grossly different from wild type, they did appear smaller. When we measured the wet weight of the TA, it was consistently reduced in the EphA7$^{-/-}$ mice at all timepoints sampled (Figure 4A-B). This decrease appears to be due to both a reduction in muscle fiber number (Figure 4C-D) and in muscle fiber size (Figure 4E-F); in addition, the myofibers in EphA7$^{-/-}$ mice contained fewer myonuclei (Figure 4I-L). These reductions led us to ask if these is a decrease in the progenitor cell pool. When we quantified the number of myogenic precursor cells present in EphA7$^{-/-}$ muscles using Pax7 as a marker, we observed a reduction (Figure 4G-H) compared to wild type.

Perhaps surprisingly given the EphA7 expression we noted in the somitic, limb, and trunk musculature, we did not observe a decrease in somite size or myogenic precursor cell number in the somite of EphA7$^{-/-}$ embryos at e11.5 (Figure 5A-F). The size of nascent myofibers in the developing limb at e18.5 was also unchanged (Figure 5G-I), although we did observe a reduction in the number of myogenic precursor cells in the limbs at that time (Figure 5J-L).

Muscle regeneration in EphA7$^{-/-}$ mice is protracted and characterized by persistence of immature myofibers

To determine whether loss of EphA7 affected muscle regeneration as well as muscle development, we analyzed tibialis anterior (TA) muscles at 5, 10, 14, and 28 days after acute injury by barium chloride. Regenerated myofibers can be identified by their centrally located nuclei and/or expression of developmental isoforms of myosin heavy chain such as embryonic myosin heavy chain (eMyHC); they are also initially smaller than existing myofibers and grow by addition of differentiated myocytes until they have recovered their initial diameter. When we calculated the cross-sectional area of satellite cell-derived myofibers at each timepoint after injury we noted that while at the earliest timepoint (5 dpi), when nascent myofibers have only recently formed, there was little difference between the WT and EphA7$^{-/-}$ muscles (Figure 6A, top row). However, at later times when hypertrophy of the nascent myofibers is occurring the EphA7$^{-/-}$ muscle is characterized by persistently smaller regenerated myofibers. Regeneration has largely resolved in the WT muscle by 28 days after injury: the

average fiber size has recovered almost to initial measurements (Figure 6B) and transient expression of eMyHC has stopped (Figure 6C). In the EphA7$^{-/-}$ muscle, while the regenerated myofibers have also recovered their initial size by 28 days after injury, many of them are still immature, as indicated by their persistent expression of eMyHC. This delay in maturation is evident early in regeneration *in vivo*: overall expression of eMyHC is both higher and more prevalent in regenerated myofibers in EphA7$^{-/-}$ mice one week after injury (Figure 6D), and the regenerated EphA7$^{-/-}$ muscle contains many differentiated but unfused myocytes and small mononucleated myofibers lacking a basal lamina (Figure 6E).

EphA7$^{-/-}$ satellite cells have a delayed and protracted transition from proliferating myoblasts to differentiated myocytes *in vitro*

A simple explanation for decreased myogenic progenitor cells, and subsequent decreases in myonuclear number, myofiber diameter, and myofiber number in EphA7$^{-/-}$ muscle would be a proliferation deficit in myogenic precursor cells or myoblasts.

An alternative hypothesis could be that the transition from one myogenic specification state to the next is inhibited in the absence of EphA7. To determine whether there is a specific step in the sequence of myogenesis during which EphA7 is required, we assayed markers of activation, proliferation, and differentiation in WT and EphA7$^{-/-}$ satellite cells *in vitro*. We noted that while initial expression of Pax7 and upregulation of MyoD were not significantly

different between WT and EphA7$^{-/-}$ satellite cells cultured on their host myofibers, upregulation of myogenin expression within 48 hours after myofiber isolation did not occur in EphA7$^{-/-}$ satellite cells (Figure 7A-B). Similarly, the fraction of satellite cells in monoculture expressing of Pax7, proliferating, and expressing MyoD did not differ between WT and EphA7$^{-/-}$ cells, but terminal differentiation (as measured by expression of myogenin) was delayed (Figure 7C-D). These data led us to propose that EphA7 acts to promote terminal differentiation and myofiber maturation in satellite cell-derived myofibers.

DISCUSSSION

Skeletal muscle is unique among vertebrate somatic tissues in that skeletal myofibers, the functional unit of the tissue, are syncytial cells formed by fusion of hundreds or thousands of terminally-differentiated mononuclear cells (myocytes). As such, skeletal muscle is particularly amenable to the use of contact-mediated signaling pathways to induce and coordinate cellular activities such as commitment to differentiation [reviewed in (Krauss et al., 2017)]. Indeed, cell-cell contact and adhesion-based signals have been shown to be strongly promyogenic even in the presence of soluble factors that promote myoblast proliferation and thereby inhibit myocyte differentiation. Adhesion proteins such as cadherins, their multifunctional coreceptors Cdo and Boc, and the netrin-3 receptor neogenin are necessary and sufficient to promote robust myogenesis *in vitro* and *in vivo* [reviewed in (Krauss et al., 2005)]. It is important to note that the enhanced myogenesis brought about by these juxtacrine interactions is distinct from promotion of myocyte fusion, which has recently been shown to require a class of proteins in vertebrate muscle that do not appear to be related to classical contact-mediated signaling (Millay et al., 2013; Quinn et al., 2017; Shi et al., 2017; Zhang et al., 2017), and instead appears to derive from upregulation of MyoD-dependent transcription [reviewed in (Krauss et al., 2017)].

In this work we present data suggesting that contact-mediated signaling interactions involving EphA7 also serve to promote myogenesis during mammalian muscle development and regeneration. EphA7 is one of a large

family of receptor tyrosine kinases that are best characterized as promoting repulsion during cell migration and cell sorting at domain boundaries [reviewed in (Ventrella et al., 2017)]. However, interactions between Ephs and their ephrin ligands have also been shown to directly affect differentiation: for example, EphB2/ephrinB1 interactions within the osteoblast lineage promote osteoblast differentiation by stimulating nuclear translocation of the transcriptional coactivator TAZ, and in multiple neuronal and stem cell lineages Eph/ephrin interactions modulate cell fate choice both directly and indirectly [reviewed in (Wilkinson, 2014)]. EphA7 is a particularly likely candidate for a pro-differentiation molecule because it has been characterized as a tumor suppressor in several cancers (Nakanishi et al., 2007; Oricchio et al., 2011; Wang et al., 2005) as well as a negative regulator of proliferation through reverse signaling with Ephrin-A2 in the adult stem cell niche of the hippocampus (Holmberg et al., 2005) and hair follicle (Genander et al., 2010). Three different splice variants of EPHA7 have been discovered, which encode one full length (FL) and two truncated (T1 and T2) proteins lacking the catalytic kinase domain (Holmberg et al., 2000). Classically, EphA7 has been investigated as a guidance protein that repels ephrin-expressing neuronal axons from specific areas in development (Araujo et al., 1998; Sefton and Nieto, 1997), however cell adhesion can occur in the presence of a truncated EPHA7 splice variant (Holmberg et al., 2000). EphA7-T1/T2 works as a dominant-negative protein that blocks the dimerization of EphA7-FL, thus blocking phosphorylation and the cell signaling that results in repulsion. After the discovery that EphA7-FL did not have

an obvious forward signaling function, EphA7-T1/T2 were used in cancer therapeutics (Oricchio et al., 2011).

It is a common observation that that sparse plating of myogenic cells *in vitro* inhibits myogenic differentiation, while cells cultured at higher confluence exhibit a much higher degree of differentiation, regardless of pro-mitogenic conditions such as high serum. This has been described as a version of the 'community effect', a phenomenon first noted by John Gurdon in the context of amphibian muscle development (Gurdon, 1988). He found that single mesoderm cells, or aggregates of less than 100 mesoderm cells, will not express MyoD and differentiate into muscle even under conditions that promote myogenesis, while aggregates of 100 or more cells would differentiate efficiently (Gurdon et al., 1993); later experiments showed that the cell-cell adhesion molecule N-cadherin is responsible for at least a portion of this effect (Holt et al., 1994). Similar studies in mouse suggested that a minimum of 30-40 cells is required for myogenic differentiation (Cossu et al., 1995). As noted earlier, skeletal muscle fibers are syncytial cells formed following permanent withdrawal of myogenic cells from the cell cycle: it would make sense that before committing to such a course of action, a potential myocyte would like some assurances that if it 'takes the plunge', other differentiated cells would be available for fusion. Similarly, it seems practical for a signal conveying this information to be contact-mediated.

Based on the data presented here, EphA7 on differentiated myocytes could potentially be functioning in several different ways (Figure 8A): it could be interacting with an ephrin-A also expressed on terminally-differentiated myocytes, in which case signaling would be bidirectional because the cells are equivalent; it could be interacting with an ephrin-A on myogenic cells that are not themselves expressing EphA7 (myoblasts), in which case one could propose either forward or reverse signaling; or it could be interacting with an ephrin-A on a non-myogenic cell type that we have not yet identified, promoting either forward or reverse signaling. We propose a model in which EphA7 promotes myogenic differentiation in populations of myogenic cells containing an initially small number of stochastically-differentiated myocytes through reverse signaling to EphA7- myoblasts (Figure 8B.) In the adult case, Pax7+ satellite cells will become activated and upregulate expression of MyoD, with or without coincident expression of Pax7; some cells will experience a combination of intrinsic and extrinsic factors that induce them to differentiate, exit the cell cycle, and upregulate myogenin; these cells will also initiate expression of EphA7. Once this occurs, EphA7 on the surface of the myocyte signals to adjacent myoblasts to promote further 'synergistic' commitment, which leads to a rapidly differentiating community of cells that can subsequently fuse into myotubes *in vitro* or myofibers *in vivo*. However, in the absence of EphA7, while stochastic commitment still occurs, the accelerated synergistic commitment is lost, leading to decreased numbers of differentiated cells available for fusion. Thus, myogenesis still occurs in muscle lacking EphA7, but it progresses more slowly

and produces smaller syncytia containing fewer myonuclei.

Future studies will test this model and identify the mechanism by which EphA7 acts as a community effect factor to promote differentiation. Given that in other instances of promotion of myogenesis by cell-cell contact signals the mechanism involves upregulation of MyoD-dependent muscle gene transcription, it is intriguing to speculate that upregulation of myogenin and commitment to terminal differentiation could be a direct consequence of EphA7 reverse signaling in this context.

FIGURES AND LEGENDS

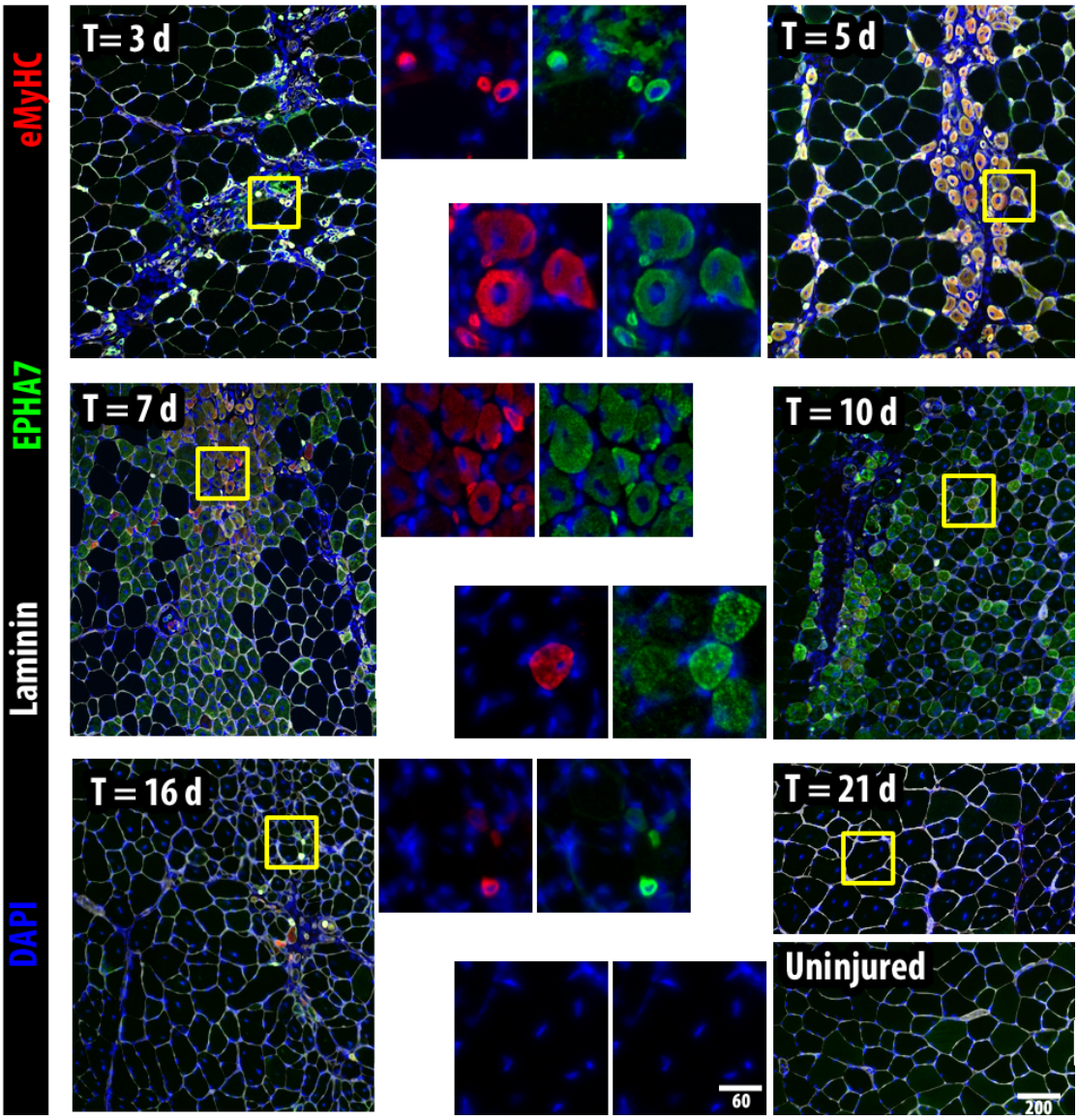

Figure 2-1 EphA7 expression parallels eMyHC expression after injury
Timecourse of sections of the TA muscle after injury by BaCl. Sections were stained for EphA7 (green), laminin (white) and eMyHC (red). Note that eMyHC expression and EphA7 expression both begin shortly after injury (3 days) and are extinguished when the injury is resolved (by 21 days.) T= days post-injury. Boxes indicate sources of color-separated insets. Scale bars are in microns.

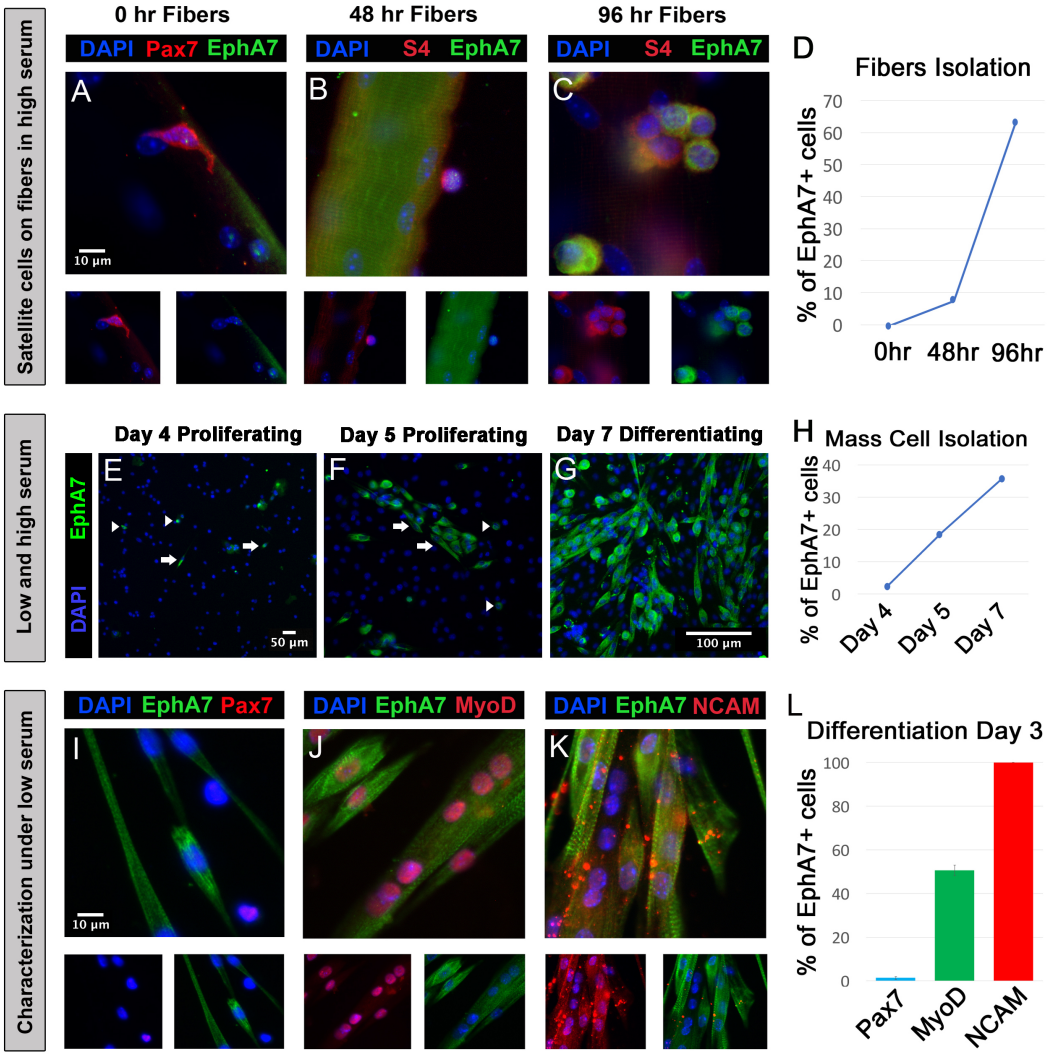

Figure 2-2 EphA7 is upregulated on satellite cells as they commit to differentiation

(Figure 2 A-D) Individual myofibers were isolated from the EDL muscle of WT mice and cultured for 0, 48, or 96hrs before fixation. EphA7 expression is absent on satellite cells of freshly-isolated myofibers, but is upregulated by 48 hours and prominent by 96 hours. Note that many EphA7+ satellite cells are in contact with other satellite cells in clusters on the myofiber (Figure-2C). (Figure 2 E-H) Similarly, expression of EphA7 on primary satellite cells in monoculture is present in the (Figure-2E). At 5 days there are clusters of EphA7 positive cells and a higher percentage of EphA7 positive cells (Figure-2F). After switching to differentiation media there is a drastic increase in EphA7 expression(Figure-2G). Indicating that as cell commit to differentiation, EphA7 expression is upregulated. Primary satellite cells were isolated, cultured for 5 days then differentiated for 3 days. Cells were stained for EphA7 (green), myogenic markers (red). Myogenic markers include Pax7 (Figure-2I) or MyoD (Figure-2J) or Neural cell adhesion marker (NCAM) (Figure2K). Manual quantification of myogenic marker cells that are EphA7 positive indicates that all cells committed to differentiation are EphA7 positive (Figure-2L).

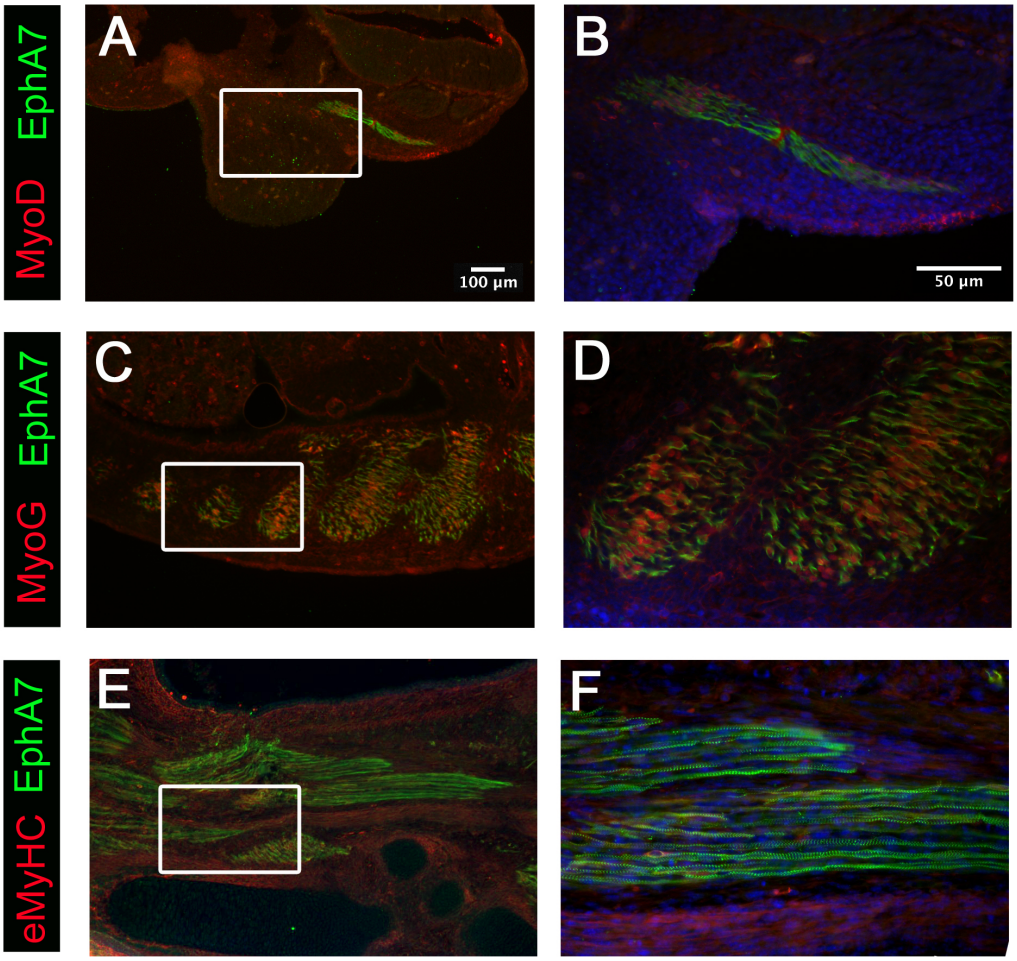

Figure 2-3 EphA7 is expressed on myogenic cells during development in the somite, body wall muscles and limb.
Embryos at different timepoints during development sectioned and stained for EphA7 and other myogenic markers. Boxed areas show increased magnification of that region of the section. Embryos staged at e9.5 days post fertilization (dpf) have expression of EphA7 on MyoD positive cells in the somites (Figure-3A/B). By e12.5 cross sections of the somite demonstrate myogenin positive cells are EphA7 (Figure3C/D). Both forelimb and hindlimb muscle at e15.5 exhibit eMyHC and EphA7 expression (Figure3E/F). Overall, EphA7 is present on myogenic cells as early as e9.5 and is expressed in the somites, body wall muscles and limb during development.

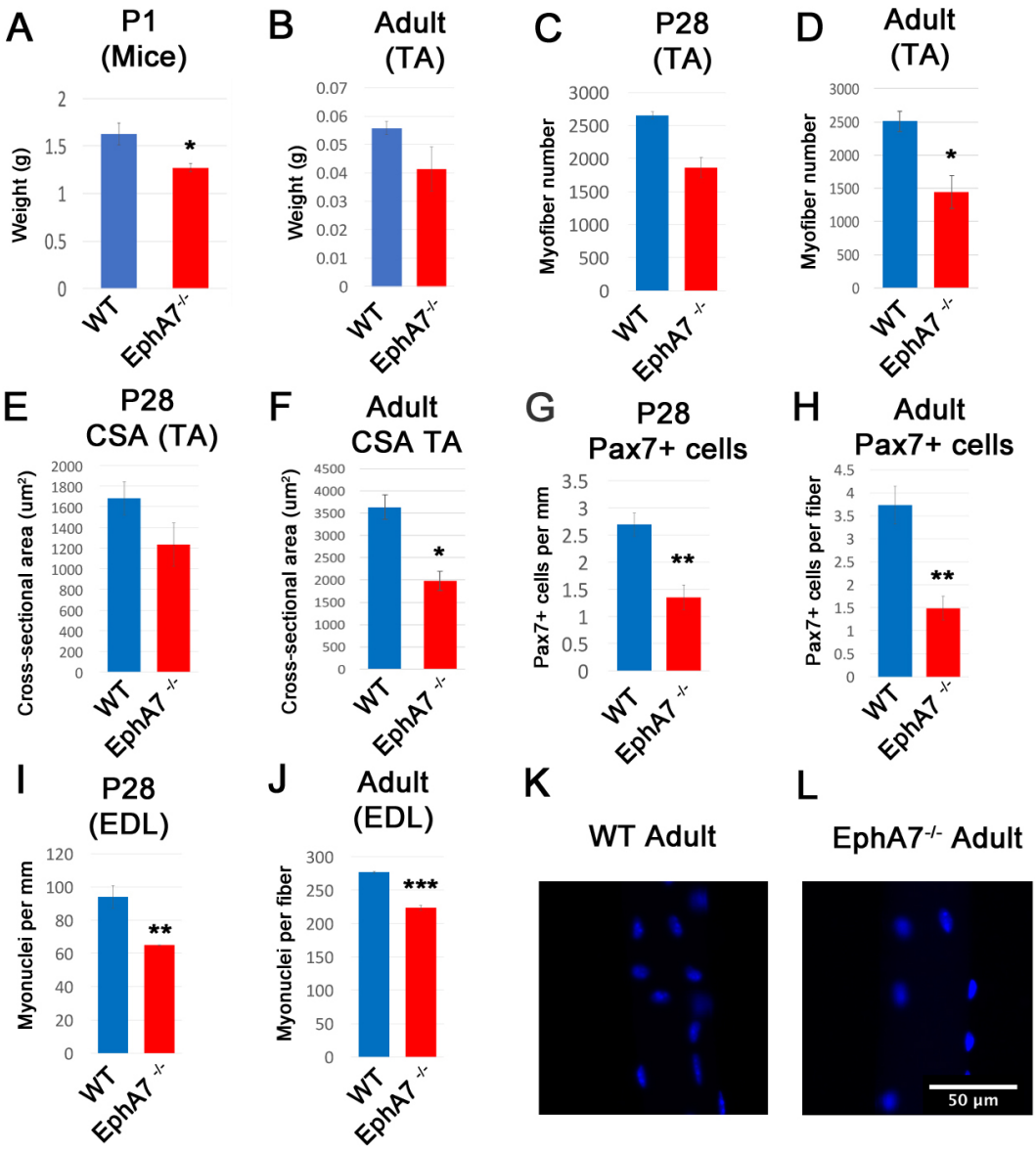

Figure 2-4 Fetal development is delayed in EphA7$^{-/-}$ mice
Myofiber counts of the TA at P28 and adult show reduction of myofibers in the EphA7 null mice (Figure 4A/B). P1 mice and adult wet weight of the TA are reduced in weight which supports myofiber reduction (Figure 4C/D). In addition, average cross-sectional area of myofibers in the TA are also reduced (Figure 4E/F). Manual counts of satellite cells marked by Pax7 are also reduced (Figure 4G/H). Manual myonuclei counts show reduced myonuclei per mm in P28 fibers and also a reduction at adult per fiber (Figure 4I/J). Myonuclei are stained by DAPI and representative images are in (Figure 4K/L).

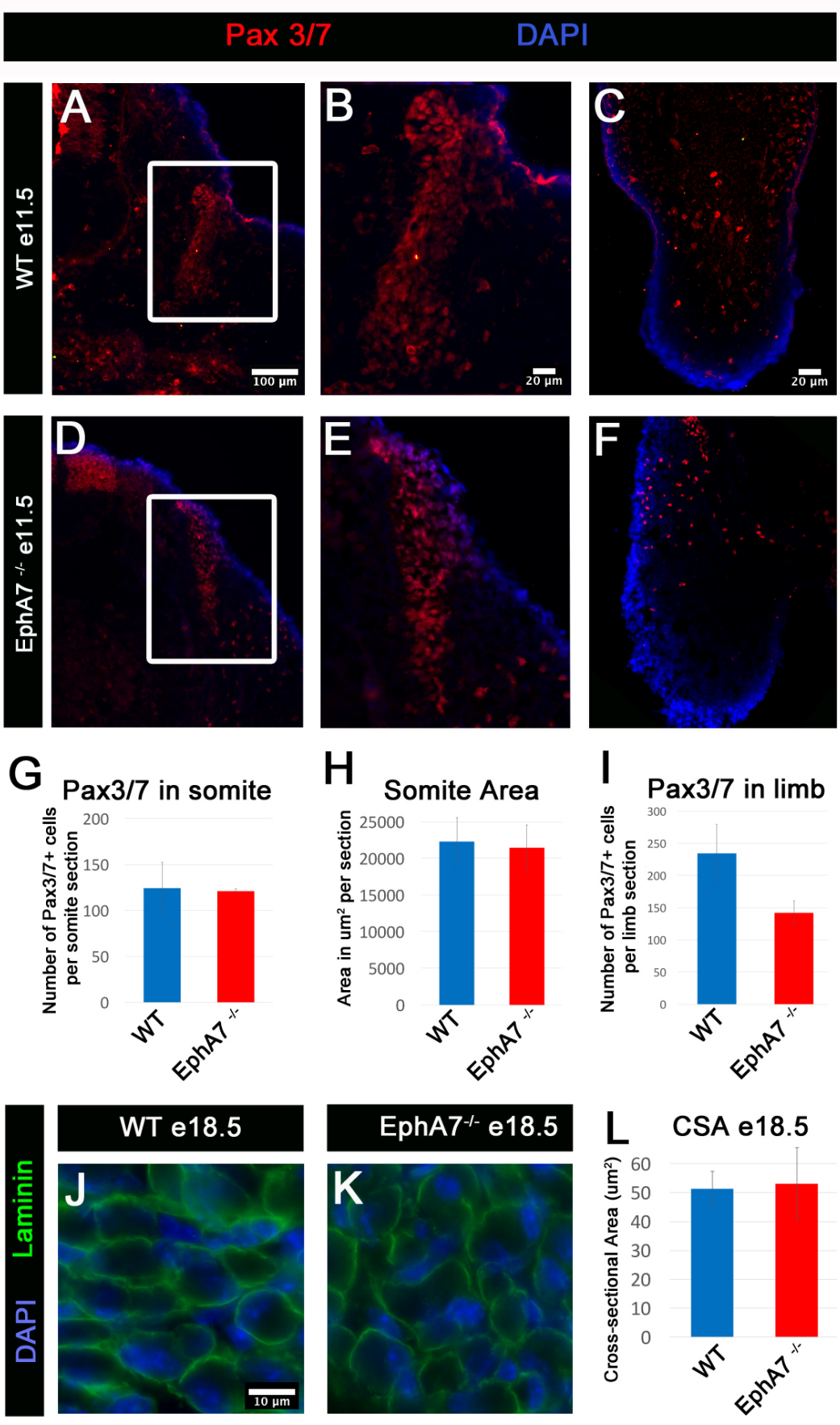
Pax 3/7
DAPI
WT e11.5
EphA7 -/- e11.5
A
B
C
D
E
F
100 µm
20 µm
20 µm
G
Pax3/7 in somite
Number of Pax3/7+ cells per somite section
200
150
100
50
0
WT
EphA7 -/-
H
Somite Area
Area in um² per section
25000
20000
15000
10000
5000
0
WT
EphA7 -/-
I
Pax3/7 in limb
Number of Pax3/7+ cells per limb section
300
250
200
150
100
50
0
WT
EphA7 -/-
DAPI
Laminin
WT e18.5
EphA7-/- e18.5
J
K
10 µm
L
CSA e18.5
Cross-sectional Area (um²)
60
50
40
30
20
10
0
WT
EphA7 -/-

Figure 2-5 Reduced number of Pax3/7 cells in EphA7$^{-/-}$ forelimb at e11.5 however no CSA difference at e18.5.

e11.5 embryos were sectioned at the forelimb level and stained with Pax3/7 antibodies. There is no difference in size of somites or number of Pax3/7+ cells at the forelimb level (Figure 5A/B/D/E). There are fewer Pax3/7+ cells in the forelimbs in EphA7$^{-/-}$ mice (Figure 5C/F). CSA of e18.5 WT and EphA7$^{-/-}$ mice have no difference (Figure 5L). Representative images are shown in (Figure 5J/K).

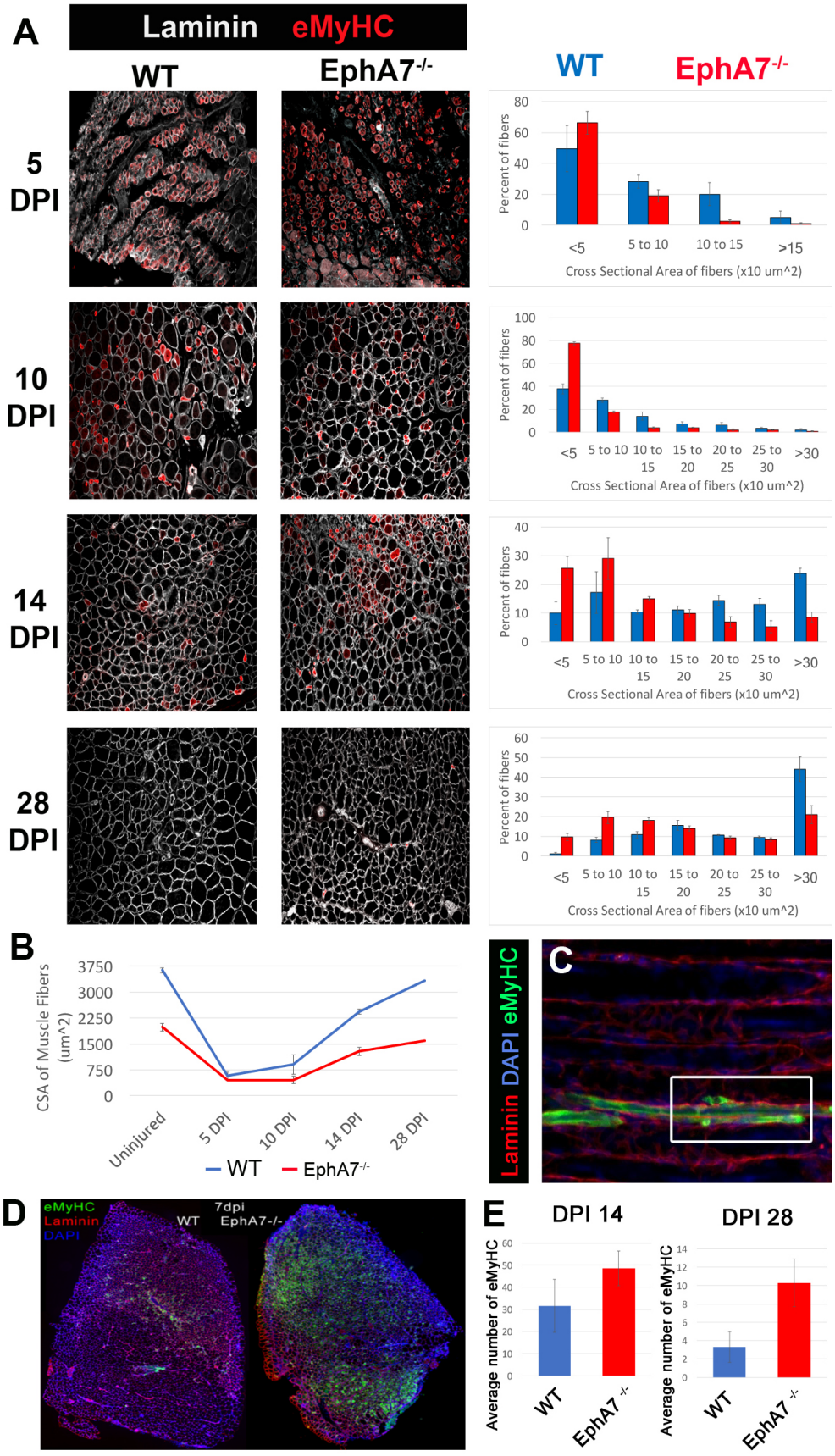
A
Laminin eMyHC
WT
EphA7-/-
5 DPI
10 DPI
14 DPI
28 DPI
WT
EphA7-/-
Percent of fibers
Cross Sectional Area of fibers (x10 um^2)
<5
5 to 10
10 to 15
15 to 20
20 to 25
25 to 30
>15
>30
B
CSA of Muscle Fibers (um^2)
Uninjured
5 DPI
10 DPI
14 DPI
28 DPI
WT
EphA7-/-
C
Laminin DAPI eMyHC
D
eMyHC
Laminin
DAPI
7dpi
WT
EphA7-/-
E
DPI 14
DPI 28
Average number of eMyHC
WT
EphA7 -/-

Figure 2-6 EphA7$^{-/-}$ mice regenerate to their baseline cross-sectional area (CSA) but have more nascent and eMyHC+ fibers

Following a timecourse of acute injury, EphA7$^{-/-}$ mouse muscle is characterized by persistence of small-caliber myofibers (A), recovery to the initial diameter of uninjured EphA7$^{-/-}$ myofibers (B), and persistence of nascent myofibers as indicated by prolonged expression of embryonic MyHC (C-E).

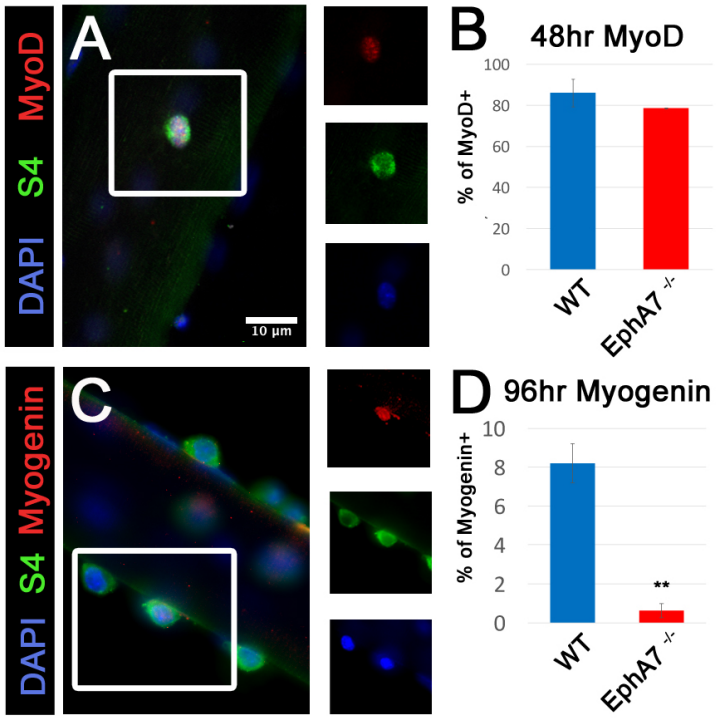

Figure 2-7 Upregulation of terminal differentiation markers is delayed in EphA7$^{-/-}$ myoblasts

While there is no significant difference in expression of a myoblast marker (MyoD) by fiber-associated satellite cells 48hrs after isolation (A-B), at 96 hours after isolation the majority of WT satellite cells express myogenin, while few EphA7$^{-/-}$ cells do (C, D).

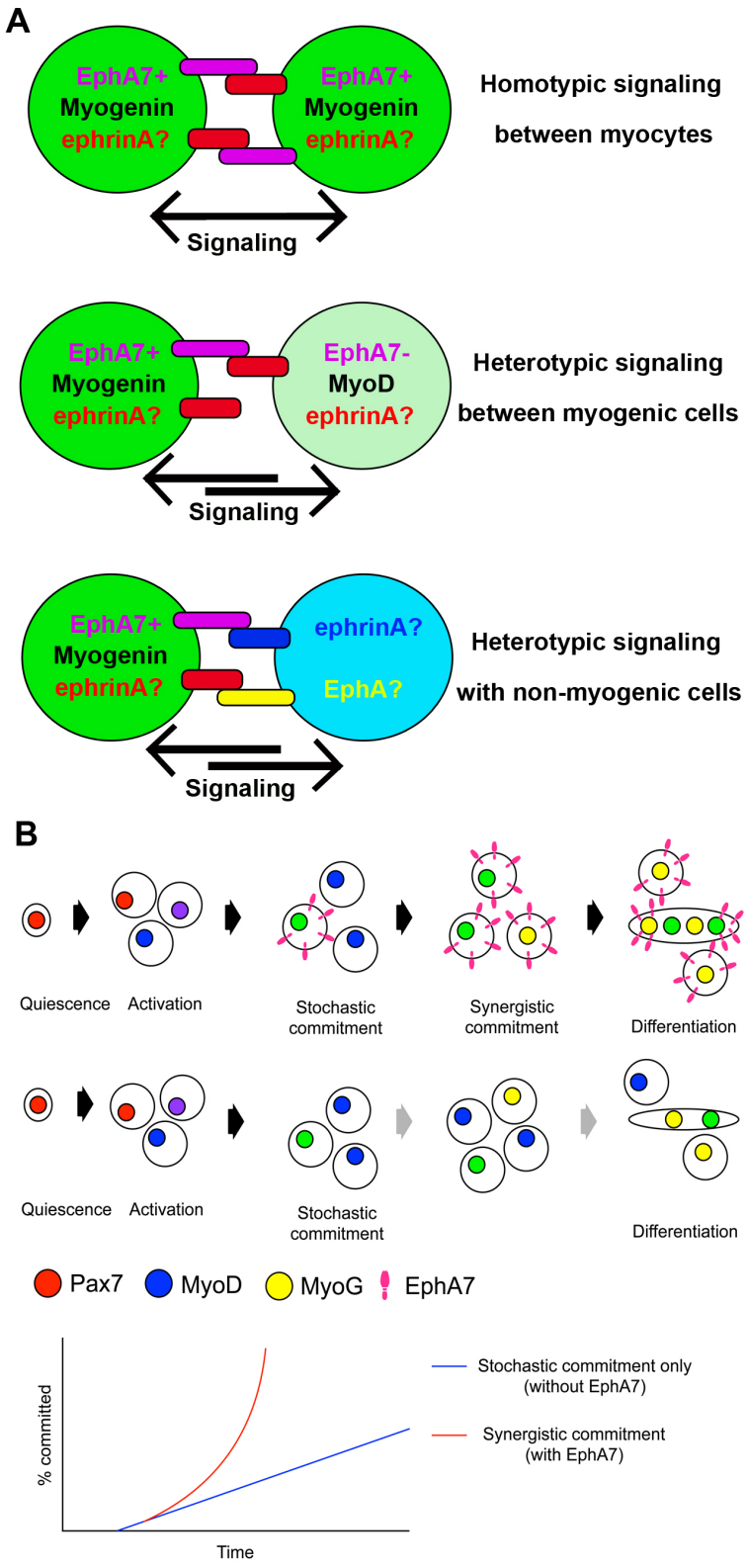

Figure 2-8 Model: EphA7 promotes myoblast differentiation via cell-cell contact. Potential signaling interactions in this system (A). Proposed model of EphA7 activity promoting myogenic differentiation via the community effect (B).

METHODS

Muscle satellite cell isolation and culture

Adult mouse myoblasts were isolated by our published methods (Capkovic, 2008). Briefly, mice were euthanized, hindlimbs removed and skinned, and muscles removed in Dulbecco's PBS. Following physical and enzymatic dissociation, cell slurries were filtered and pelleted then plated in Ham's F12 (Invitrogen) supplemented with 15% horse serum (Equitech), 5 nM FGF2, and penicillin/streptomycin (Sigma) on gelatin-coated plates. Cells were maintained at 37°C and 5% CO_2 in a humidified incubator. For differentiation, cells were washed briefly with cold DPBS and switched to Kaighn's F-12 supplemented with 2% horse serum with pen/strep.

Viable myofiber explants were isolated from EDL muscles using our published methods (Cornelison et al., 2004; Cornelison and Wold, 1997). As above, muscles were dissected, but were not physically dissociated. After collagenase digestion, free-floating myofibers were picked with a glass pipette and transferred into growth medium for culture as above.

Myofibers from neonatal mice were teased from muscles that were dissected then fixed overnight in 4% PFA.

All experiments involving mice were conducted in accordance with National Institutes of Health and University of Missouri Institutional Animal Care and Use Committee guidelines.

Antibody staining

For fluorescence immunohistochemistry of muscle sections, muscles were flash-frozen in liquid nitrogen-cooled isopentane and sectioned on a Leica cryostat. For fluorescence immunohistochemistry of mouse embryos, embryos were fixed overnight in 4% paraformaldehyde, washed in PBS, and equilibrated into 15% then 30% sucrose in PBS prior to freezing and sectioning.

For fluorescence immunocytochemistry of cultured cells, satellite cells prepared as above were replated onto gelatin-coated glass coverslips, allowed to adhere for a minimum of 2 hours, and then fixed in 4% ice-cold paraformaldehyde. Cells were blocked for 1 hour at room temperature with 10% normal goat serum with 1% Nonidet-P40 then incubated with primary antibody overnight at 4°C. For staining of adult myofiber-associated satellite cells, myofibers at the timepoints indicated in the text were fixed in 4% PFA, washed in PBS, and arranged on slides for staining.

Concentrations of primary antibodies were rabbit anti-EphA7 (Santa Cruz Biotechnology, Inc.) at 1:200; rabbit anti-laminin (Sigma-Aldrich) at 1:300; mouse anti-eMyHC (clone F1.652, Developmental Studies Hybridoma Bank) at 1:50; rat anti-CD34 (RAM34, eBioSciences) at 1:100. Secondary antibodies were raised in goat and conjugated with Alexa fluorophores (Invitrogen), and used at 1:500. Cells were washed in PBS, incubated with secondary antibody for 1 hour at room temperature, washed, and mounted using Vectashield (Vector labs) containing DAPI to visualize nuclei. All fluorescent images were acquired and processed on an Olympus BX61 upright microscope using Slidebook software (Intelligent Imaging Innovations). Digital background subtraction was used to remove signal only less than or equal to

levels present in control samples processed without primary antibody, and was applied equally to the entire field.

In vivo muscle injury

Mice were anesthetized with 5% Avertin, then 70 uL of 1.2% $BaCl_2$ was injected into the left TA. Mice were allowed to recover for 5-21 days postinjury before injured muscle were harvested for analysis.

CHAPTER 3:

EphA3 is differentially expressed by satellite cells on fast and slow myofibers

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ABSTRACT

Eph receptor tyrosine kinases and their membrane-associated ligands, ephrins, are expressed in almost every tissue and regulate multiple key processes during development, homeostasis, and regeneration, particularly in establishing tissue organization. Their diverse biological functions are achieved primarily by changing cell adhesion or promoting cell repulsion following cell-cell contact. Multiple groups have noted that myoblasts derived from fast vs. slow muscles, either in the embryo or in the adult, maintain a differential identity *in vitro* and *in vivo*, and preferentially form differentiated myotubes with a fiber type corresponding to the source of the cultured myoblasts. However, to date no candidate mediators of this self-sorting have been identified. Data from our group showing that satellite cells exhibit classical repulsive behavior in response to ephrin guidance ligands, and that one such ligand (ephrin A3) is specifically expressed by slow (MyHC 1) myofibers in adult muscle led us to hypothesize that satellite cells may also have an intrinsic fast vs. slow identity that can be identified by Eph/ephrin expression and activity. We show that satellite cells from fast (TA) and mixed/slow (soleus) muscles respond differentially to ephrin A3 *in vitro*, and that an ephrin A3 receptor (EphA3) is preferentially upregulated upon activation by satellite cells resident on fast myofibers. If this cell surface receptor is a marker of 'fast' satellite cells, it would raise the possibility that sorting interactions of satellite cells with other satellite cells, with myocytes/myofibers, or with non-muscle cell types may be a component of muscle regeneration and repatterning following acute injury. It also raises the question of how and when subpopulations of satellite cells are segregated

by fiber type during development. Ideally, it will also provide a tool to prospectively separate them for further analysis.

INTRODUCTION

How the development, regeneration, and maintenance of specific fiber types in skeletal muscle has been long standing enquiries within the field of skeletal muscle. The regeneration process is largely contributed by adult muscle stem cells, or satellite cells, which can form muscle fibers that match the original muscle composition before injury. Typically, when muscle is in homeostasis, each muscle fiber expresses one of the four myosin heavy chains (MyHC). These MyHC can be classified into two groups: fast contracting but fatigue prone, and slow contacting but fatigue resistant. MyHC Type I is expressed on the only slow fiber type, while there are three fast fiber types, MyHC Type 2a / 2x / 2b, with MyHC 2a being the slowest of the fast group and MyHC 2b being the fastest.

The success of adult skeletal muscle fibers ability to regenerate after injury is largely due to the presence of muscle stem cells, satellite cells (Lepper, Partridge et al. 2011, Murphy, Lawson et al. 2011). Normally, satellite cells are in a state of quiescence, a non-proliferating and resting state. Once a myotrauma occurs, satellite cells become activated and begin to migrate to the site of injury. There, satellite cells will proliferate, fuse with themselves or other myofibers, and differentiate to repair the damaged muscle (Hawke and Garry 2001). Studies show that chemically induced injury causes myonecrosis in skeletal muscle, which will repair itself within 21 days (Rosenblatt and Woods 1992).

It has been suggested that there are two populations of satellite cells that are preprogramed to forming fast or slow myofibers (Miller, Crow et al. 1985, Crow and Stockdale 1986, Miller and Stockdale 1986, Nikovits, Cann et al. 2001). Primary chicken satellite cells isolated from slow muscle groups show that satellite cells have a higher chance of forming a myotube that expresses slow MyHC, while satellite cells isolated from fast muscle groups will only form fast MyHC myotubes (Feldman and Stockdale 1991). This suggests that there are two different populations of satellite cells that would lend themselves to forming a specific type of MyHC myofibers. In addition, satellite cells isolated from fast or slow muscle have a different myogenic profile after 8 days removed from rabbits (Barjot, Rouanet et al. 1998). This provides evidence that the satellite cells can be classified based off their fiber type origin. Although, no markers exist to distinguish between satellite cells from different muscle fiber types, being able to sort satellite cells by fiber type would have benefits such as in cell engraftment therapies. Satellite cells that express slow MyHC have a greater chance of fusing with host myofibers than those who only express fast MyHC (Petersen and Huard 2000). There is no known molecular mechanism that can characterize if predetermined populations of satellite cells will repair fast or slow myofibers.

Eph receptors are in the receptor tyrosine kinases (RTK) family of proteins and are activated by ligands called ephrins. Both Ephs and ephrins are membrane bound and can be classified into two groups with the designation of the letter A or B. In most cases, Eph A receptors (Eph A1-8, A10) interact with ephrin A ligands (ephrin

A1-5), while Eph B receptors (Eph B1-6) interact with ephrin B ligands (ephrin B1-3) (Kullander and Klein 2002). Eph/ephrin signaling has shown to be important in the remodeling of vasculature (Brantley-Sieders and Chen 2004), bone (Matsuo and Otaki 2012), and neural plasticity (Gerlai 2001). EphA3 has specifically been shown to be increased in regeneration events such as myocardial infarction (Dries, Kent et al. 2011), spinal cord injury (Willson, Irizarry-Ramirez et al. 2002), and optic nerve injury (King, Wallace et al. 2003), thus showing its importance in other systems for regeneration. This family of proteins is also known for its role in cytoskeletal mediated repulsion during development and cell migration. We first showed Eph/ephrin presence in satellite cell migration and muscle regeneration in vitro and in vivo (Siegel, Atchison et al. 2009, Stark, Karvas et al. 2011).

EphA3 is the only EphA receptor with about 50% cell expression on activated syndecan-4 (S4) positive satellite cells by FACS (Stark, Karvas et al. 2011). With expression of ephrin-A3 on all slow MyHC fibers and a known repulsive interaction of Eph and ephrins in many contexts, we hypothesize that EphA3 may be present on satellite cells associated with fast muscle fibers. In this study, we show that EphA3 is strongly upregulated on satellite cells that reside on fast muscle origin. Satellite cells from a slow muscle origin have low to no EphA3 expression. Initial selection of fibers that are fast or slow MyHC appear to follow this pattern. Removal of satellite cells whether abrasive or voluntary results in upregulation of EphA3 on all myoblasts. Indicating, that EphA3 must be regulated by the fiber that is associated with.

RESULTS

Satellite cell expression of EphA3 is different based muscle group fiber type

With the interest of EphA3 and ephrin A3 possibly signaling to determine satellite cell commitment to a specific MyHC fate. Isolation of individual fibers and fixing them at 0, 48, and 96 hrs allows for a timecourse of satellite cell expression. Our initial examination of EphA3 expression shows no expression during quiescence and upregulation at 48hrs. Almost all myoblasts on fibers expression EphA3 at 96hrs.

As sample size increased, there is a difference in level of expression of EphA3 based on muscle origin. The EDL/Plantaris are considered fast muscle groups while the soleus is considered a slow muscle group. The soleus is about 42% slow while the EDL and plantaris is 2% or less (Stark, Coffey et al. 2015). After examination of EphA3 expression on a per fiber basis (fibers given EphA3 expression scores of Strong Positive, Weak/Mixed Positive, or Negative are shown as examples.) At 96 hours EDL fibers scored positive, while soleus fibers were weakly positive or negative. Thus, supporting that EphA3 is expressed on satellite cells associated with fast MyHC fibers (ephrin A3 negative).

The TA, like the EDL, is considered a fast muscle group, and has a vastly different ratio of fast and slow muscle fibers compared to the soleus. Comparing satellite cell Eph expression and activity between TA and soleus satellite cells could indicate if cells are repulsed by ephrin A3. Satellite cells were isolated from the TA or the soleus and cultured for four days then plated over the ephrin A3 stripes with

growth media. Satellite cells were fixed 24 hours after plating. Quantification of cell response to the ephrin or laminin were analyzed using (Stark, 2011) methods. Satellite cells from the TA are repelled from ephrin A3 stripes to a greater degree than soleus cells. This could indicate that a receptor ephrin A3 is present on satellite cells from the TA.

To test whether EphA3 is localized to myoblasts residing on slow MyHC muscle fibers we used the *myh7-CFP* mouse. The *myh7-CFP* mouse expresses CFP under the control of the MyHC I promoter (Chakkalakal, 2012). Single soleus *myh7-CFP* fibers were fixed at 0 and 48hrs after isolation and immediately sorted by hand based on CFP expression, then stained for EphA3 and scored as above. As expected, at 0hrs after isolation there is no expression of EphA3. However at 48hrs, satellite cells on fibers have low expression of EphA3 no matter what fiber type they are associated with. This data may seem to disprove our hypothesis however, when compared to 48hr fibers from the EDL and soleus the results are similar. Therefore, the 96hr timepoint is the most significant and would be a true test of our hypothesis.

<u>ephrin A3 interaction with satellite cells from slow twitch muscle groups</u>

Since, ephrin A3 is a ligand for EphA3 and has a repulsive effect on 'fast' satellite cells, we investigated expression of EphA3 on myoblasts in the ephrin A3 null mouse. The ephrin A3 null mouse has been characterized to have a decrease in the number of slow myofibers (Stark, Karvas et al. 2011). Single isolated fibers from the ephrin A3 null mouse were cultured for 0, 48, and 96hrs. Consistent with

wildtype mice, there is no expression of EphA3 during quiescence. At 48hrs after isolation, 53% of myoblasts residing on myofibers from the soleus express EphA3, which is 11% higher than WT. The myoblasts residing on myofibers from the EDL/Plantaris show a similar trend. However, by 96hrs EphA3 is present on more myoblasts on WT fibers regardless of muscle group origin. Presence of ephrin A3 on fibers may of a signal to satellite cells that are upregulating EphA3 to stop and downregulate expression. Therefore, lack of ephrin A3 on fibers allows for upregulation of EphA3 regardless of fiber type.

Lack of ephrin A3 results in higher percent of EphA3 expression in satellite cells

To see if EphA3 expression is heritable when cultured off of fibers, isolating satellite cells from a mass cell preparation which does not allow any contact of activated myoblasts with their associated myofibers could reveal if EphA3 there are subpopulations of EphA3 positive and negative cells. Satellite cells were isolated, plated on gelatin and cultured for 5 days. Then, cells were switched to differentiation media and stained for myogenic markers. MyoD and EphA3 are co-expressed in 90% of cells after 3 days in differentiation media. While, EphA3 and neural cell adhesion marker (NCAM) are co-expressed on 98% of cells. Without specific regard to muscle group origin, all myoblasts and myocytes upregulate EphA3.

With no subpopulations of EphA3 during differentiation from a mass cell isolation, this brings the questions of if isolating ‘slow’ or ’fast’ satellite cells separately would have subpopulation of EphA3 positive and negative cells. Plating

individual 24hr CFP positive (slow) and CFP negative (fast) isolated myofibers into laminin coated wells. Waiting 4 days after plating which allows for satellite cells to crawl off the myofibers, and then stained for EphA3, and S4. When comparing CFP negative (fast) fibers and CFP positive fibers (slow) we do not see any differences in EphA3 expression. By increasing the number of fibers in the wells up to 15 fibers we still see all myoblasts express EphA3 no matter the fiber type that the satellite cells originated from. There is also no difference in expression by letting the cells differentiate more.

DISCUSSION

The major finding of this chapter is that EphA3 is strongly expression on 'fast' satellite cells at 96hrs. This could have a large impact on the satellite cell field with no other marker to ever be able to indicate a satellite cells likelihood of differentiating into a specific MyHC type. Future directions of 96hr CFP sorted fibers will indicate the inheritability of EphA3 as a marker of 'fast' satellite cells.

With EphA3 expression upregulated on myoblasts and myocytes that have no association with any fiber type and upregulation of EphA3 on satellite cells that voluntarily migrate off a fast or slow myofiber, the original associated fiber is relevant. The ephrin A3 null mice have drastic increase in EphA3 expression on all satellite cells regardless of muscle origin. Therefore, it is important for ephrin A3 to be present to signal for the downregulation or stopping the upregulation of EphA3. It would be interesting to plate 'fast' satellite cells on slow MyHC fibers (ephrin A3

positive) to see if EphA3 is ever upregulated on these cells. This would test if there is an intrinsic difference between ‘fast’ and ‘slow’ satellite cells or if the environment/signaling is more critical.

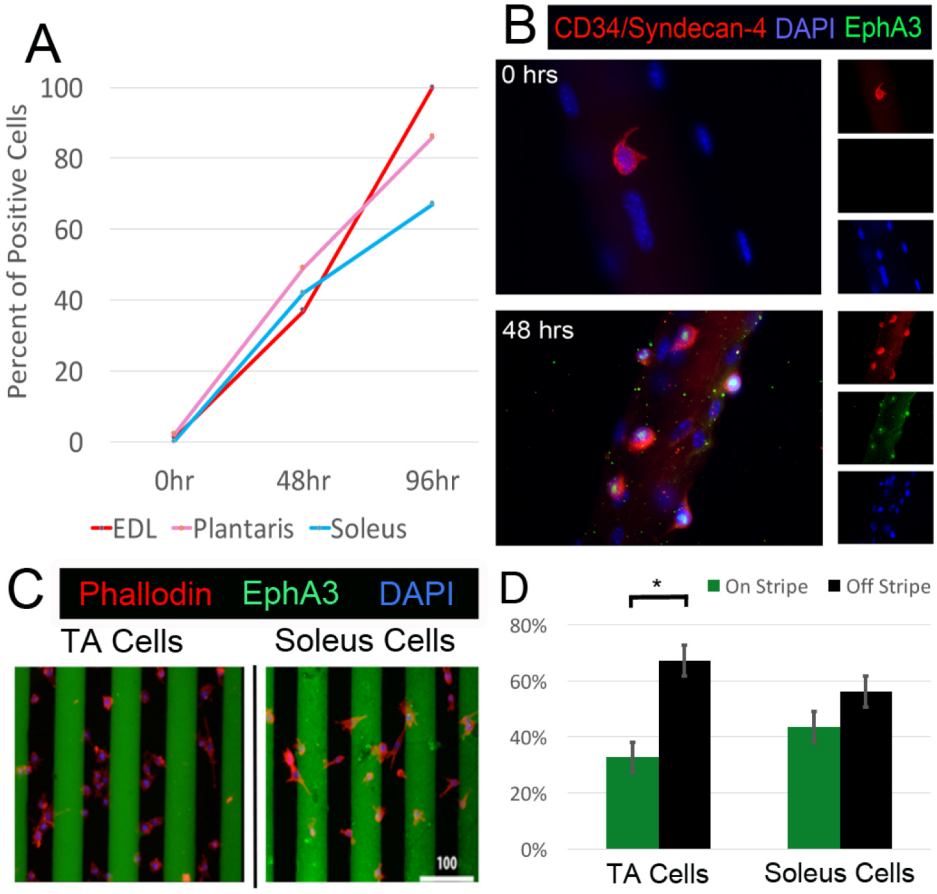
There are many additional experiments that could be done to test this hypothesis. FACS sorting of EphA3 positive satellite cells and negative satellite cells would allow for pure populations of cells to be differentiated to test if ‘slow’ or ‘fast’ satellite cells are more likely for form specific MyHC types. This experiment could also be translated into an in vivo experiment using Rosa-MTMG or H2B-GFP cells as a transplant cells into a WT mouse. This would allow counts of fusion to specific MyHC fibers and differentiation into specific MyHC of new myofibers.

months after birth. Our one timepoint of adult myonuclei counts does not lend itself to an easy explanation. It is very possible that there is a myonuclear domain phenotype present at birth which results from a developmental phenotype. It is also possible that the myonuclear domain phenotype is not present until after birth and the incorporation of progenitor cells is higher than normal after birth. It should be noted that presence of an oxidative metabolism can result in a smaller myonuclear domain (Tseng, Kasper et al. 1994). With the MCK-PGC-1a mouse having this alteration from conception, we would hypothesize that the myonuclear domain phenotype in the MCK-PGC-1a mouse would be more likely to originate from an embryonic phenotype.

Exploration of the presence of EphA3 on satellite cells attached to myofibers resulted in a dramatic increase in EphA3 expression on satellite cells in MCK-PGC-1a mice. Possibly, myoblasts residing on MCK-PGC-1a fibers receive a signal from the fiber to upregulate EphA3, or an unknown secreted factor from the fibers causes this upregulation. With Eph/ephrins being membrane bound, it is plausible that the fiber signals to the myoblasts to upregulate EphA3. We have not detected the secretion of any factors that would trigger an upregulation of EphA3. However, that does not mean that there is not any.

FIGURE LEGENDS

Figure 3-1 EphA3 upregulation on satellite cells and repulsive interaction between satellite cells and ephrin A3. Reprehensive images depicting EphA3 is not expressed during quiescence and is present on about 50% of satellite by 48hrs (Figure 1B). At 96hrs, most satellite cells express EphA3 (Figure 1A). Testing the interaction of satellite cells and ephrin A3, known to be on all slow MyHC fibers, results in satellite cells from the TA to be repulsed from ephrin A3 stripes (Figure 1C). Cells isolated form the soleus have no preference or avoidance of the ephrin A3 stripes (Figure 1D).



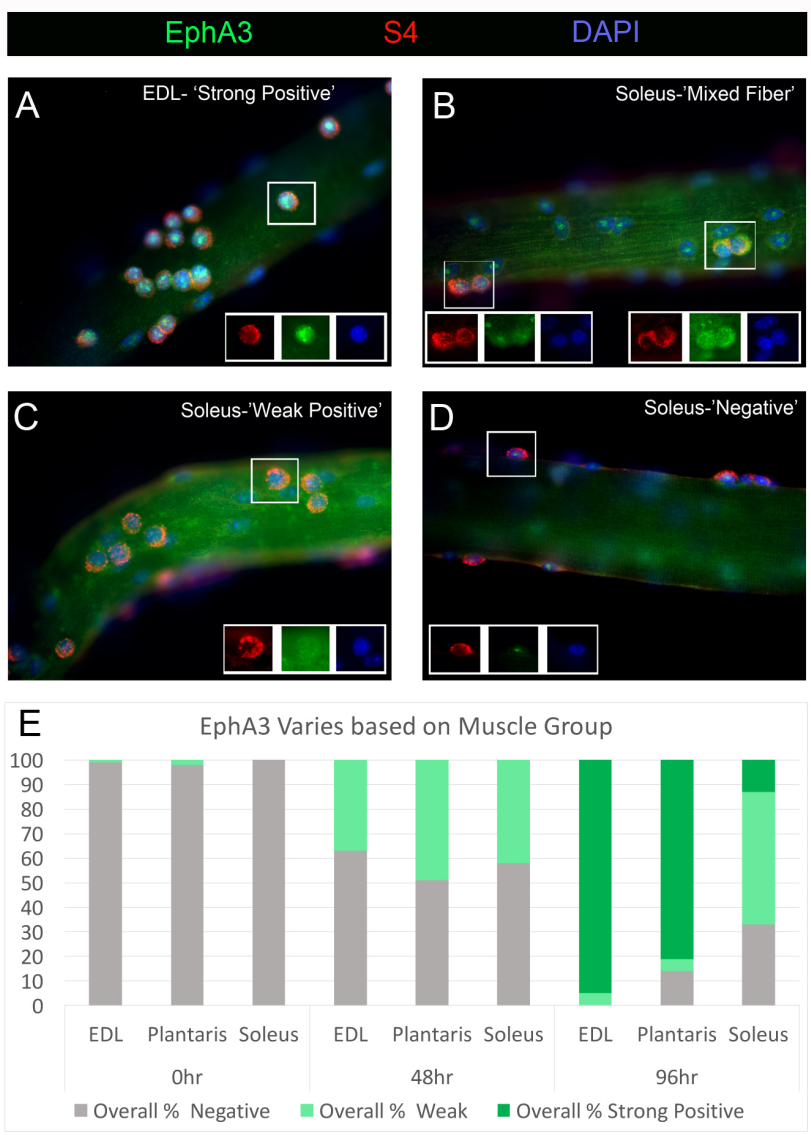
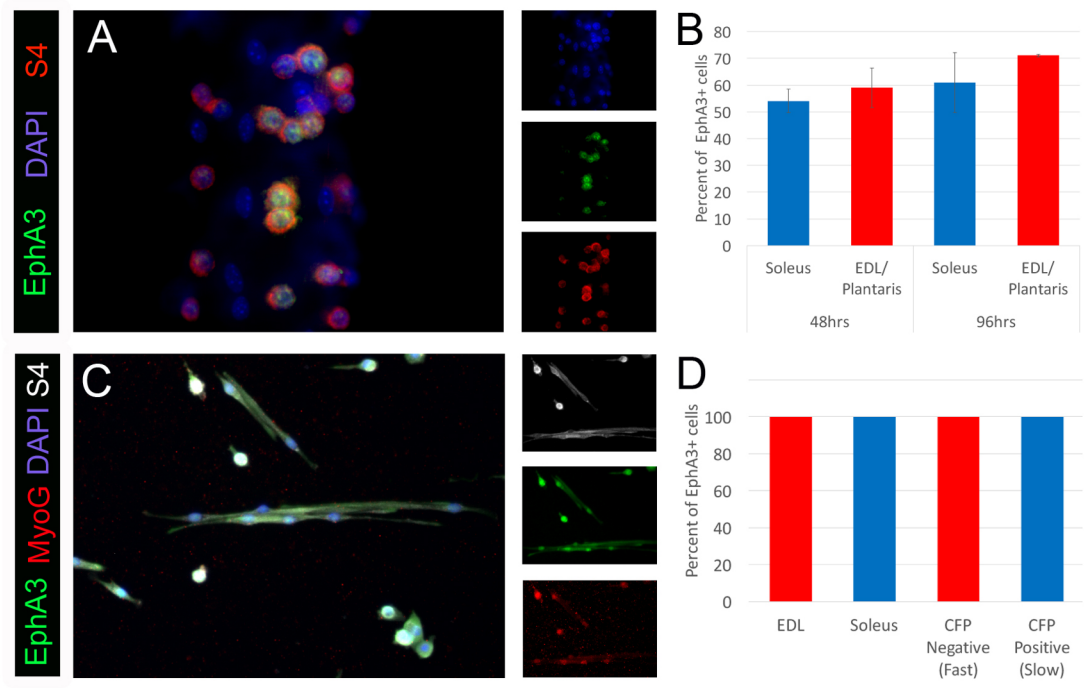

EphA3
S4
DAPI
A
EDL- 'Strong Positive'
B
Soleus-'Mixed Fiber'
C
Soleus-'Weak Positive'
D
Soleus-'Negative'
E
EphA3 Varies based on Muscle Group
100
90
80
70
60
50
40
30
20
10
0
EDL
Plantaris
Soleus
EDL
Plantaris
Soleus
EDL
Plantaris
Soleus
0hr
48hr
96hr
Overall % Negative
Overall % Weak
Overall % Strong Positive
EphA3
S4
DAPI
F
G
Percent of Cells Positive
60
40
20
0
CFP Positive (Slow)
CFP Negative (Fast)
CFP Positive (Slow)
CFP Negative (Fast)
0 hrs
48 hrs
H
I

Figure 3-2 EphA3 varies based on muscle group origin and isolation of MyHC fiber specific fibers follows same expression pattern. Culturing myofibers of 0,48, and 96hrs and staining for EphA3, sydencan-4 (S4) allows for quantification of EphA3 on satellite cells. There are distinctive levels of EphA3 correlated with muscle origin (Figure 2E). 96hr EDL fibers show strong positive expression while the soleus has mainly weak EphA3 expression levels (Figure 2A-D). Sorting fibers based off MyHC expression by myf-7-CFP mice, which have all slow fibers that are CFP positive allows for specific isolation of only fast or slow MyHC isoforms. Initial results indicate that sorted CFP fibers follow WT isolations and stains (Figure 2G). EphA3 is not expressed on quiescence satellite cells no matter fiber type origin. At 48hrs, there is low expression of EphA3 on fast and slow myofibers (Figure 2H/I). While other satellite cells remain negative at this time (Figure 2F).

Figure 3-3 ephrin A3 is required for EphA3 inhibition
Fibers isolated from ephrin A3 null mice have strong expression of EphA3 no matter muscle origin at 96hrs (Figure 3A). There is also a faster upregulation of EphA3 compared to WT at 48hrs (Figure 3B). Fibers stained for synedcan-4 (S4) mark satellite cells and manual counts of EphA3 all for percent of EphA3 positive cells. Determining whether specific fiber origin is required for EphA3 subpopulations to be defined, separation of CFP positive and negative fibers and culturing satellite cells indigenous to those individual fibers indications EphA3 expressed on all myoblasts no matter fiber type origin (Figure 3C-D).



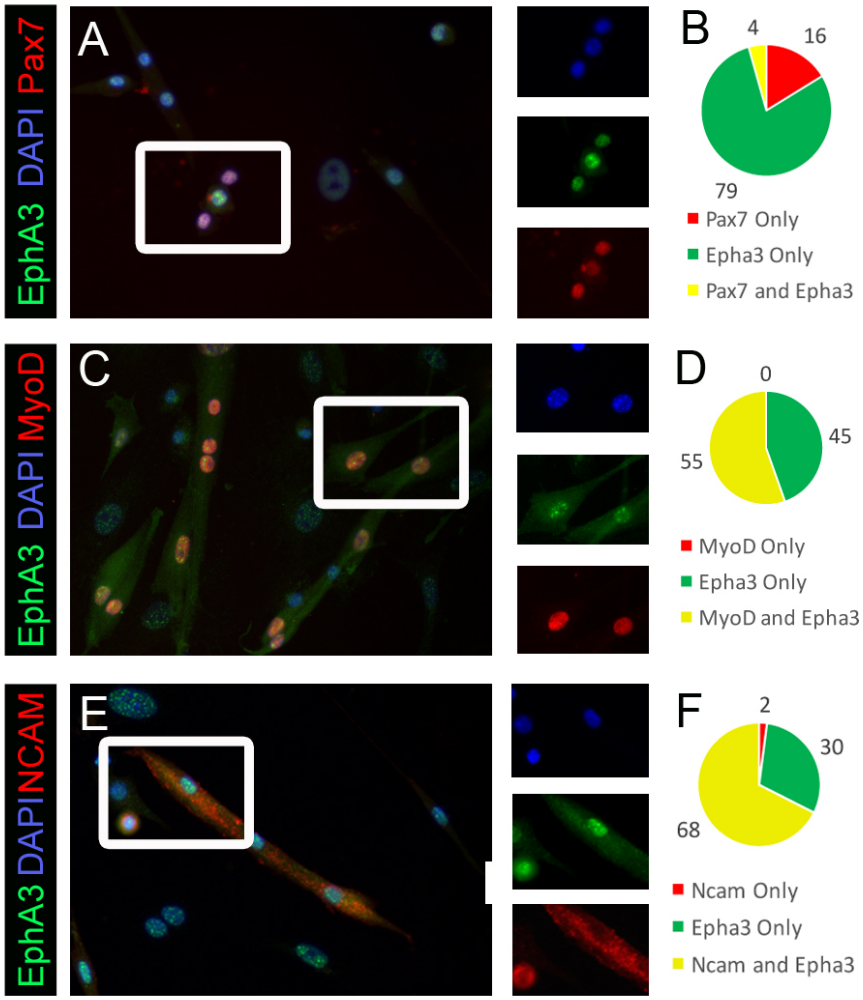


Figure 3-4 EphA3 expression is not specific to differentiated satellite cells from fast muscle groups in monoculture

Satellite cells isolated without regard for association of fiber type reveal that without contact of a myofiber there are almost no EphA3 subpopulations after 3 days in differentiation media. EphA3 is upregulated on all differentiated cells (Figure 4C-F). There is a Pax7 only population at this timepoint and would logically be the satellite cells pool that will go back into quiescence (Figure 4A-B).

METHODS

Animal Care and Use

All mice were handled and used in accordance with National Institutes of Health and Institutional Animal Care and Use Committee approved protocols.

Muscle Satellite Cell Isolation and Culture

Adult mouse myoblasts were isolated from LacZ, Myh7-CFP,ephrin A3 null mice by our published methods (Capkovic, 2008).

Viable single myofibers were isolated using our published methods (Cornelison, 1997) (Cornelison 2004).

Immunohistochemistry and Imaging

Immunohistochemistry and imaging for panels BLAH were done as previously described (Stark, Karvas et al. 2011). Concentrations of primary antibodies (Santa Cruz Biotechnology, Inc.) were rabbit anti-EphA3, 1:100, rabbit anti-Ephrin A3, 1:100, (Sigma-Aldrich) rabbit anti-laminin, 1:300, (clones BA-D5, Developmental Studies Hybridoma Bank) Mouse anit-MyHC-I 1:50. Images were collected as previously stated in (Stark, Coffey et al. 2015).

Stripe Assays

Acid-washed coverslips were coated with recombinant ephrin stripes and then laminin coated at 10ug/ml (Sigma). Coating of stripes and laminin preparation was done using our published method in (Stark, 2011). Primary satellite cells were

cultured for four days and then plated over the stripes with growth media. Satellite cells were fixed 24 hours after plating with 4% PFA. Quantification of cell response to the ephrin or laminin were analyzed using (Stark, 2011) methods.

Statistical Analysis

A minimum of three mice were used for quantification. Student T-test/sqr(n) was used to determine statistical significance.

CHAPTER 4:

Oxidative metabolic shift in skeletal muscle

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ABSTRACT

Metabolic activity in muscle fibers can affect the amount of ATP produced, which will dictate the MyHC chain expression. The amount of ATP produced will determine the muscle fibers speed of contraction and fatigability. Oxidative fibers with high ATP production, are classified as MyHC 1 and 2a, while glycolic muscle fibers have low ATP production and are MyHC 2x and 2b. The promoter, muscle creatine kinase (MCK), overexpresses PGC-1a (peroxisome proliferative activated receptor gamma coactivator 1) creating the MCK-PGC-1a mice. This mouse has a switch in oxidative metabolism in its muscle fibers which switches the muscle fiber type according to the Speigelman lab. Our lab did a comprehensive muscle fiber type comparison which revealed no such change.

INTRODCTION

Skeletal muscle is made up of different muscle fiber types, which have distinct metabolic requirements that effect the rest of the animal. These fiber types are classified as myosin heavy chain (MyHC) type 1 (slow twitch muscle) and MyHC type 2 (fast twitch muscle). MyHC type 1 has only one slow subtype of muscle, however there are 3 subtypes of MyHC 2, which are 2a, 2x, and 2b. MyHC 1 and MyHC 2a fibers use oxidative metabolism, which allows for high resistance to fatigue. MyHC 2x and 2b use glycolytic metabolism and are therefore less resistant to fatigue but can exert more force.

Oxidative fibers perform aerobic respiration, which involves oxygen and glucose in the production of adenosine triphosphate (ATP). There are more mitochondria in oxidative fibers working to produce an overall higher volume of ATP compared to glycolytic fibers. The more ATP a muscle fiber can produce, the slower it will fatigue. MyHC 1 fibers also contract slowly and use ATP at a slower rate than fast twitch muscle fibers do. Glycolytic fibers use anaerobic glycolysis to produce ATP. Glycolytic muscle fibers use ATP at a faster rate and produce less. Therefore, glycolytic muscle fibers fatigue faster than oxidative muscle fibers do.

Peroxisomes are subcellular organelles with enzymes that affect metabolic function. Peroxisome proliferator activated receptors (PPAR) are members of the nuclear hormone receptor superfamily. Activated PPARs can repress transcription through protein-protein interactions with other transcription factors. Changes in

metabolic function can affect myogenic progenitor cells, skeletal muscles and brown adipocytes which are all derived from the same lineage (Seale, Bjork et al. 2008).

PGC-1a (peroxisome proliferative activated receptor gamma coactivator 1) was discovered as a PPARy interacting protein and has been shown to regulate transcriptional function (Puigserver, Wu et al. 1998, Puigserver, Adelmant et al. 1999, Barger and Kelly 2000, Puigserver, Rhee et al. 2001). There is large group of data supporting PGC-1a regulation of mitochondrial biogenesis and cellular respiration (Puigserver and Spiegelman 2003, Kelly and Scarpulla 2004).

PGC-1a is a transcriptional activator involved in the regulation of mitochondrial production which affects the production of brown fat and muscle fiber typing (Lin, Wu et al. 2002). PGC-1a knockout mice show a decrease in body fat percentage but no fiber type change (Lin, Wu et al. 2004). However, transgenic mice with the promoter muscle creatine kinase (MCK) overexpressing PGC-1a have an increase in MyHC 1 and MyHC 2a (Lin, Wu et al. 2002). The MCK-PGC-1a mice distinctly have a red muscle physical appearance, which indicates an increase in oxidative fiber type. Previous MyHC-type quantification revealed an increase of 10% of MyHC 1 and 20% of MyHC 2a in the plantaris (Lin, Wu et al. 2002).

After more comprehensive tests into the specific types of MyHC in the MCK-PGC-1a mouse, our data shows no significant change in muscle fiber type ratio. Supporting this, there is no change in satellite cells per fiber and cross sectional area

of fibers, which is expected with a fiber type change to MyHC type 1. There is a significant increase is in myonuclei per fiber in the MCK-PGC-1a mouse.

RESULTS

For a comprehensive investigation into the fiber type quantification, isolation of the tibialis anterior (TA), extensor digitorum longus (EDL), gastrocnemius, soleus and plantaris was performed. Serial sections of these muscles were stained separately for MyHC 1, 2a or 2b with laminin, which allowed for the calculation of the percentage of each individual MyHC type. With the report of MyHC 1 being significantly higher in the MCK-PGC-1a mice compared to wildtype mice, we begin our fiber type examination. However, our quantification of MyHC 1 fibers in the soleus revealed a decrease from 42% (WT) to 34% (MCK-PGC-1a), although this is not significant (Figure 1A/B). In addition, there are no significant changes in the gastrocnemius, plantaris, TA or EDL regarding the percentage of MyHC 1 fibers compared to WT. Since this result was surprising and contradicts the Speigelman lab results, a full screen of the other MyHC types was performed. Full fiber type quantification of the gastrocnemius, plantaris, soleus, TA, and EDL show no other significant changes in MyHC 2a, 2x, or 2b (Figure 2A).

Without an increase in the number of MyHC 1 fibers, there would need to be a shift towards MyHC 2a for the red muscle appearance in MCK-PGC-1a mice. Oxidative metabolism is active in MyHC 1 and MyHC 2a fibers. The TA is characterized by having a majority of fast twitch muscle fibers, making it a glycolic

muscle. In contrast, almost all the muscle fibers in the soleus are MyHC 1 and 2a, making it an oxidative muscle (Figure 2B-G). The comparisons of the percent of oxidative muscle fibers demonstrate a slight increase in the amount of oxidative fiber type by 5% in the TA, which is not significant. The soleus also has no significant change.

Without a drastic conversion to oxidative metabolism in muscle fibers in the MCK-PGC-1a mouse, a measure of the number of satellite cells per fiber counts was collected. There are typically more satellite cells per fiber on MyHC 1 fibers compared to MyHC 2 fibers (Schmalbruch and Hellhammer 1977), and we found that there are 3.6 satellite cells per fiber in WT and 2.83 satellite cells per fiber in the MCK-PGC-1a mouse when fixed at 0hrs. Even with the slight increase in slow fibers in the MCK-PGC-1a mouse, there is no significant change in the amount of satellite cells per fiber (Figure 3A). Therefore, our fiber type data is supported by this satellite cells per fiber data.

MyHC 1 and 2a fibers are typically smaller than 2x and 2b fibers, but based off our fiber typing data there should be no change in cross sectional area (CSA) per fiber. After quantification, there no statistical difference was found between CSA of fibers in the MCK- PGC-1a mouse compared to WT (Figure 3B). Overall, the lack of any significant change in the composition of fibers, satellite cells per fiber or CSA per fiber supports our contradiction of any change in MyHC fiber type quantification in the MCK-PGC-1a mouse.

However, upon isolating individual fibers, there was a visual difference in the amount of myonuclei in MCK-PGC-1a fibers compared to WT. Myonuclei counts yielded a significant increase in the number of myonuclei per fiber in MCK-PGC-1a mice (Figure 3E). An increase in myonuclei per fiber could indicate that the satellite cells are more prone to differentiation and fuse to existing fibers faster in the MCK-PGC-1a mice. Analysis of the MCK-PGC-1a myoblasts and myocytes during differentiation showed that the numbers of cells expressing MyoD and NCAM were relatively equal to WT (Figure C/D). There does not seem to be a difference under differentiating conditions between WT and MCK-PGC-1a cells from our initial results.

With the initial idea that the MCK-PGC-1a mice have more MyHC 1 fibers, we conjectured that this would result in an increase in the amount of ephrin A3 expressing fibers (Stark, Coffey et al. 2015). Our hypothesis of ephrin A3 positive fibers repulsing EphA3 satellite cells could result in a decrease of EphA3 positive satellite cells. Quantification of EphA3 on satellite cells on 48hr fibers from the EDL/Plantaris and soleus resulted in a shocking difference between both mouse models. Satellite cells from MCK-PGC-1a mice upregulate EphA3 expression faster than WT satellite cells (Figure 4A-B). At 48hrs after isolation, 93% of myoblasts residing on the EDL and the soleus in MCK-PGC-1a mice expressed EphA3, which is almost twice as much as myoblasts from WT mice. The exact cause for the difference in EphA3 expression is unknown.

DISCUSSION

Our comprehensive study of all the subtypes of MyHC from various muscles refutes others claims of an increase in MyHC 1 fibers in MCK-PGC-1a mice. The use of immunofluorescent antibodies allows for specific quantification of the individual MyHC fibers per muscle. This method is far more accurate and specific when compared to the use of metachromic-ATPase stain in the Lin, 2002 paper, which does not allow for the specific counts of MyHC. That method only demonstrates the presence of MyHC 1 and 2a fibers and was also only performed in the plantaris and was not calculated as a percent difference. Reasonable differences between the studies could be variability between the depths that sections of muscle were taken from, which could account for some change. However, this would not completely account for the disparity between the results.

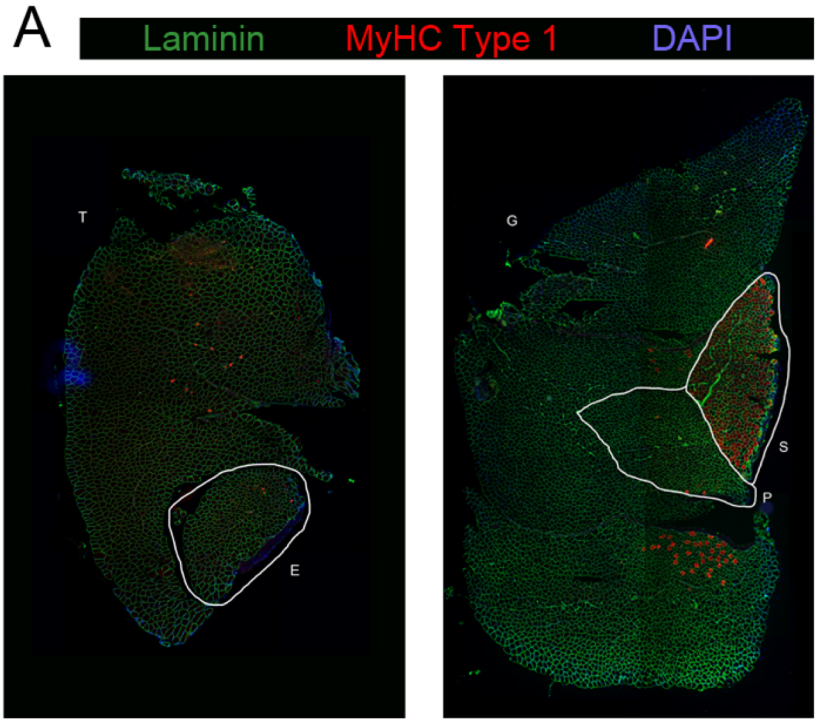
With the increase in myonuclei number per fiber but no CSA increase, the myonuclear domain hypothesis becomes relevant. The myonuclear domain hypothesis states that there is a proportional relationship between the cytoplasmic volume and nuclear number within a muscle fiber. This hypothesis suggests that during development incorporation of muscle progenitor cells results in an increase in cross sectional area or cytoplasmic volume as nuclei are added. The increased number of myonuclei in the MCK-PGC-1a fibers without CSA increase means that intrinsically there is a myonuclear domain dysfunction.

The number of muscle fibers that makes up each muscle is established at birth and does not change throughout an organism's lifetime. Hypertrophy of muscle fibers due to incorporation of myocytes expands the muscle volume and occurs for several months after birth. Our one timepoint of adult myonuclei counts does not lend itself to an easy explanation. It is very possible that there is a myonuclear domain phenotype present at birth which results from a developmental phenotype. It is also possible that the myonuclear domain phenotype is not present until after birth and the incorporation of progenitor cells is higher than normal after birth. It should be noted that presence of an oxidative metabolism can result in a smaller myonuclear domain (Tseng, Kasper et al. 1994). With the MCK-PGC-1a mouse having this alteration from conception, we would hypothesize that the myonuclear domain phenotype in the MCK-PGC-1a mouse would be more likely to originate from an embryonic phenotype.

Exploration of the presence of EphA3 on satellite cells attached to myofibers resulted in a dramatic increase in EphA3 expression on satellite cells in MCK-PGC-1a mice. Possibly, myoblasts residing on MCK-PGC-1a fibers receive a signal from the fiber to upregulate EphA3, or an unknown secreted factor from the fibers causes this upregulation. With Eph/ephrins being membrane bound, it is plausible that the fiber signals to the myoblasts to upregulate EphA3. We have not detected the secretion of any factors that would trigger an upregulation of EphA3. However, that does not mean that there is not any.

FIGURE LEGENDS

Figure 4-1 MyHC Type 1 is not significantly altered in MCK-PGC-1a mice. Cross sections of the TA/EDL (Fig. 1A-left) and gastrocnemius/plantaris/soleus (Fig. 1A-right) stained with laminin (green), and MyHC type 1(red). Quantification of the percent of MyHC Type 1 positive fibers manually counted with ImageJ in WT and MCK-PGC-1a sections (Fig. 1B).



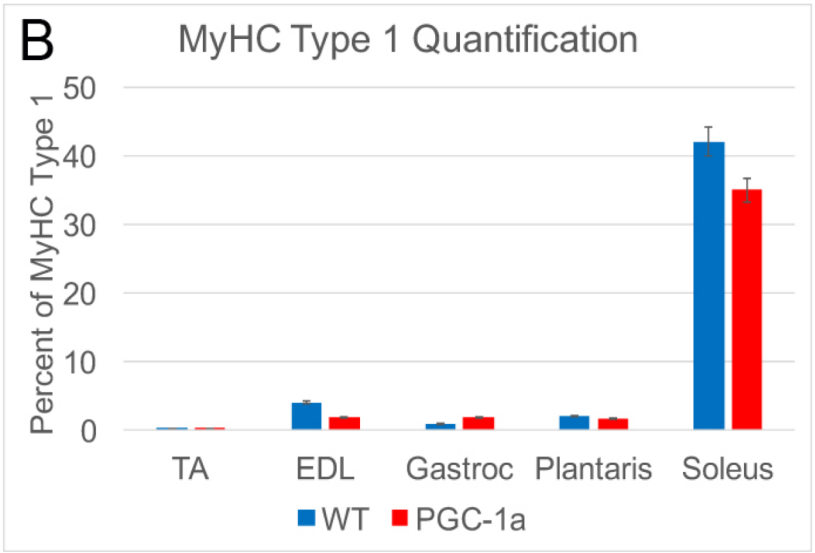


Figure 4-2 No significant change in the frequency of other MyHC isoforms is detected in MCK-PGC-1a mouse hindlimb muscle

Cross sections of the TA/EDL (Fig. 2B/C) and gastrocnemius/plantaris/soleus (Fig. 2E/F) stained with laminin (green), and MyHC type 1 or 2a (red). Quantification of the percent of individual MyHC fibers manually counted with ImageJ in WT and MCK-PGC-1a sections (Fig. 2A). MyHC Type 2x was quantified as the difference of the total number of fibers from the total MyHC of Type 1/2a/2x. Oxidative and glycolytic quantification were based on addition of MyHC type 1 and 2a for oxidative (Fig. 2G) and MyHC type 2x and 2b for glycolytic (Fig. 2D).

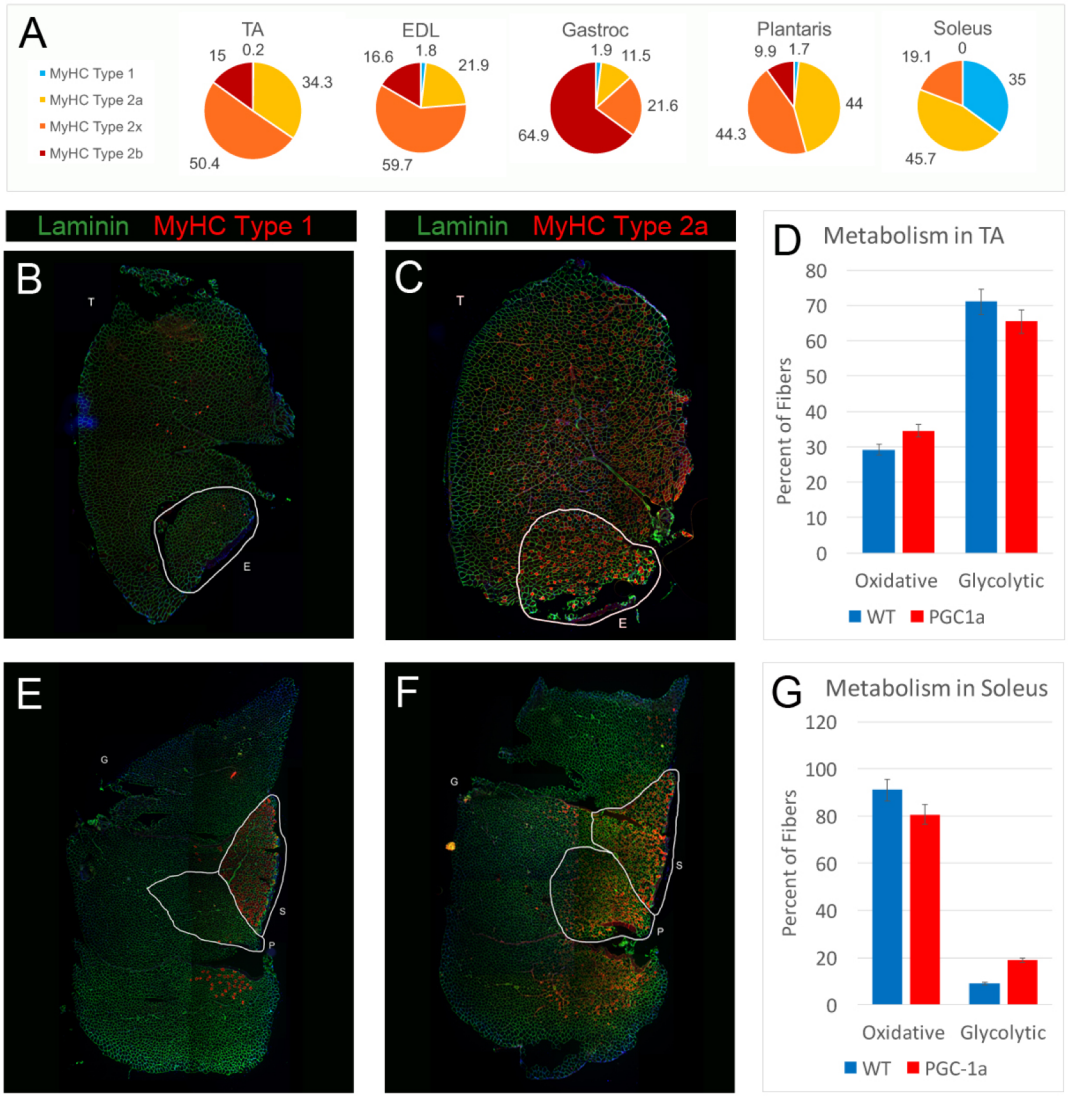


Figure 4-3 Myonculei per fiber are increased in MCK-PGC-1a mouse hindlimb muscle but satellite cell counts, CSA, and myogenic marker expression are not altered

Isolation of single myofibers from MCK-PGC-1a and WT mice were fixed at 0hrs. Pax7 antibodies were used to mark quiescent satellite cells (Fig. 3A). Myonuclei marked by DAPI were individually counted manually from single myofibers. Increase in average myonuclei per fiber were calculated as higher in MCK-PGC-1a mice with representative images (Fig. 3E/F). Cross sections of the TA were stained with laminin antibodies to outline the myofibers. ImageJ was used to quantify the cross-sectional area (CSA) of each myofiber (Fig. 3B). Satellite cells isolated from the hindlimb were cultured in differentiation media for 6 days before immunofluorescent antibodies of NCAM or MyoD were used to determine the commitment of cells (Fig. 3C/D). Manual counts of the number of cells were used to get percent counts. There is no difference in CSA, MyoD or NCAM expression in MCK-PGC-1a mouse.

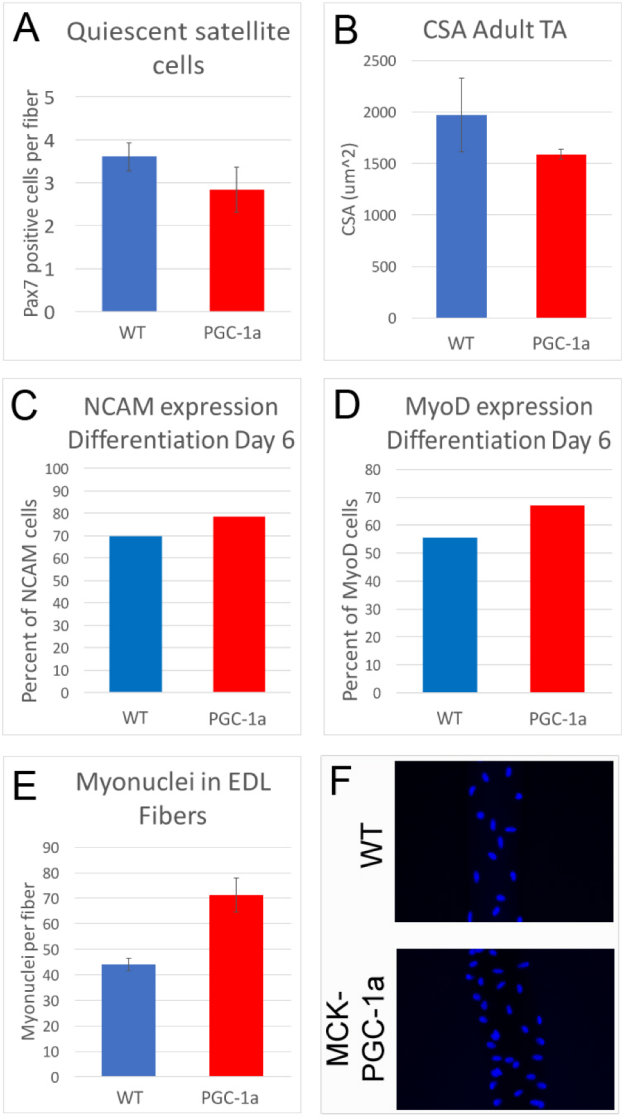
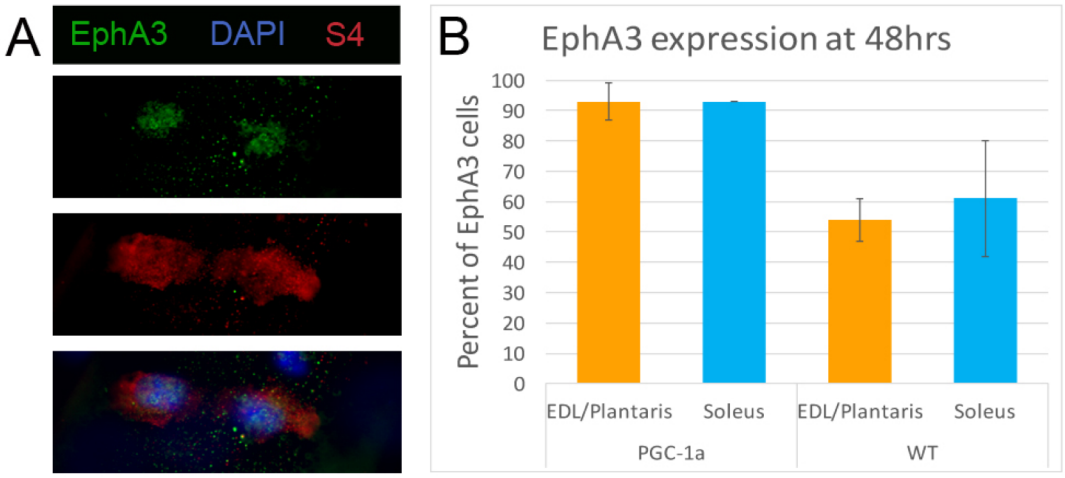


Figure 4-4 EphA3 expression is increased on satellite cells from MCK-PGC-1a mouse myofibers compared to WT 48hrs after myofiber isolation
Isolated individual myofibers were cultured for 48hrs then fixed and stained for EphA3 (green) and syndecan-4 (S4) (red) (Fig. 4A). Quantification of syndecan-4, marking satellite cells, positive cells that are also EphA3 positive results in percent of EphA3 positive satellite cells at 48hrs (Fig. 4B).



METHODS

Animals

All experimental procedures involving the use of mice were carried out with The University of Missouri's Animal Care and Use committee. MCK-PGC-1a mice were acquired from Jackson labs.

Cell Culture

Primary satellite cells were harvested from the hind limb muscles of mice that were at least 3 months of age. Muscles were dissected out, minced, and digested with 4000mg/uL collagenase in F-12 (Ham) for 1 hr in a waterbath at 37°C. The samples were vortexed every 10min. Filtration eliminated muscle debris and filtered cells were pelleted at 2000rpm for 5min. Cells were plated in growing media (F-12 (Ham), 15% horse serum, 1% penstrep) for 4 days then switched to differentiation media (F-12 (Kaighn's), 2% horse serum, 1% penstrep) for 2 days. Primary myoblasts were grown on gelatin coated slides for staining. The cells were fixed with 4% paraformaldehyde for 15 min, then washed 3 times with PBS.

Isolation and culture of single myofibers

Single myofibers were isolated as previously described (Siegel, Atchison et al. 2009). Fibers were harvested from the EDL, plantaris, and soleus muscles of mice that were at least 3 months of age.

Immunocytochemistry

Cells or sections were blocked for 1 hour at room temperature with 10% normal goat serum with 1% Nonidet-P40 then incubated with primary antibody overnight at 4°C. Cells were washed, incubated with secondary antibody for 1 hour at room temperature, washed again, and mounted using Vectashield (Vector Labs). All images were taken on an Olympus BX61 upright microscope using Slidebook6 (3i) software.

The primary antibodies used were EphA3, NCAM (Santa Cruz Biotechnology), Pax7, MyoD, MyHC1, MyHC2a, MyHC2b (Developmental Studies Hybridoma Bank).

Sample preparation and cryosections

Muscles from mice were dissected and placed on cork in a bed of optimal cutting temperature media (OCT). Samples were then placed into super-cooled isopentane then submerged in liquid nitrogen for several minutes. Samples were cyrosectioned at 20um and then stained as described above.

Statistical analysis

Muscles from a minimum of three mice were used for fiber type quantification. A two-tailed student's *t* test for two samples of equal variance was used to calculate p-values.

CHAPTER 5:

Significance and Future Directions

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CONCLUDING REMARKS

Eph/ephrin signaling have been studied for a long time in other systems, however our knowledge of the involvement of these RTKs is very basic in skeletal muscle. There are sure to be many novel discoveries that will come from research in this area. The work from our lab before this dissertation has shown that Eph/ephrins modify satellite cell motility and differentiation patterning. In addition, that ephrin A3 is present only ever on slow twitch muscle fibers and plays a role in innervation. In this dissertation, we have shown that EphA's play a role in satellite cell heterogeneity (EphA3) and differentiation (EphA7). Before this work, there has been very little work done on Eph/ephrins in skeletal muscle.

EphA7's involvement in skeletal muscle development

With the drastic lack of knowledge of roles for Eph and ephrins during skeletal muscle development, regeneration and homeostasis, this chapter adds identification of EphA7 involvement in differentiation during development. The formation of limb skeletal muscle has different stages that include embryonic and fetal development. From our first investigation, there is no difference in CSA at e18.5, however by P1 there are weight and CSA differences that carry through fetal development and are never recovered during adulthood. The fusion of muscle progenitor cells during fetal development is directly related to CSA and myonuclei numbers. Therefore, we have identified that EphA7 is involved the fusion of muscle progenitor cells during fetal development. We also show preliminary evidence for a delay in differentiation in satellite cells during adulthood. Our model suggests that

lack of EphA7 causes a delay in differentiation which could result in a decrease in fetal development.

Heterogeneity in satellite cells

Satellite cell heterogeneity and its effects on differentiation into specific fiber types is largely unknown. The discovery of EphA3 present on satellite cells that reside on muscle fibers from fast twitch muscle groups is one of the first steps to finding a marker. EphA3 downregulation seems dependent on the presence of ephrin A3. Culturing satellite cells off their associated muscle fibers results in expression of EphA3 on all satellite cells no matter fiber type origin. This would seem that the muscle fiber is signaling to the satellite cells to downregulate EphA3 on slow muscle fibers. When isolating muscle fibers from the ephrin A3 null mice there is significant upregulation of EphA3 on all muscle fibers, demonstrating that ephrin A3 is downregulating EphA3 on satellite cells associated with slow muscle fibers. Further work, needs to establish whether EphA3 and ephrin A3 determine satellite cell differentiation into specific fiber types. However, the groundwork of EphA3 being the first marker for 'fast' satellite cells is present in this dissertation.

Future directions

More basic research needs to be done with the EphA3 before more higher level experiments are attempted. The results of this dissertation in relation to EphA3 upregulation and what is affecting EphA3 expression should be of foremost importance. The need to finish the CFP MyHC separation of individual fibers and

culture for 96hrs is critical. Afterwards, the loss of the ephrin A3 having such a dramatic effect on EphA3 expression is interesting. Engraftment of 'fast' satellite cells plated on slow muscle fibers before and after EphA3 is upregulated would be of interest. Would EphA3 never be upregulated on 'fast' satellite cells if they are associated with a slow muscle fiber? Subsequent experiments could involve engraftment, differentiation assays, MyHC type assays, and muscle progenitor lineage tracing. All of these experiments are currently not viable without a marker for 'fast' or 'slow' satellite cells.

A mechanistic approach would be very enlightening for the EphA7 project. There are known pathways that differentiation of satellite cells occur through and it would be shocking to not see EphA7 interacting with any of these known proteins. The mechanism that EphA7 may be involved with during development may be different than during adult regeneration. Overexpression and inhibition experiments of EphA7 could allow for some mechanistic insight. Would overexpression of EphA7 increase differentiation but deplete the satellite cell pool? Another noteworthy experiment would be repeated injury on the EphA7$^{-/-}$ mice. Knowing that EphA7$^{-/-}$ mice have reduced satellite cells, would repeated injury result in depletion of the satellite cell pool or reduction CSA due to trying to maintain the satellite cell pool?

The satellite cell field is presented with a protein family that is mainly unstudied. Our lab is pioneering the research in this area with the discovery signaling roles for ephrin A3/EphA8, EphA7, and EphA3 in satellite cells. There are sure to be

other specific roles for Ephs and ephrins in muscle development and regeneration. One aspect that our lab will expand on is a signaling mechanism for these established roles of Eph/ephrins. Eph and ephrins are known to signal through a variety signaling pathways and play many different roles. Figuring out specific pathways could allow for better understanding in this area which may lead to therapeutic applications of research in this area.

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VITA

I was born on May 6th, 1991 in Cleveland, OH. Sports are an integral part of our family. When I was little my parents would drive my brother, Doug Arnold, and I around town to different soccer tournaments. Around when we entered middle school my brother and I both started becoming more serious about soccer. We both played on travel soccer teams and now traveled outside the state for different tournaments. When I was in high school I even traveled to England to play soccer. I was fortunate to have a great team to play on and my parents who were willing to sacrifice their free time. I played as the goal keeper of the Olympic State Team and won other state and national awards.

I believe that playing soccer instilled in me how to preserve through situations that are unpleasant. There were games that I remember playing on a boys' soccer team where I was the goalie and we had more than 10 goals scored on us. It is easy for a goal keeper to think that all the goals scored are your fault. Some of them could be but most of them shouldn't be only the goal keeper's fault. This was my first interaction with failure and I had many moments while playing that were stressful and hostile. I would like to think that this experience helped prepare me to deal with the many failures every graduate student faces.

I went on to play at Bowling Green State University on a scholarship. This made my undergraduate decision of where to attend very easy. As I continued through undergrad, I started working in the lab of Dr. Ray Larsen. He was very nice and personally taught me several bacterial genetic techniques. This was my first experience in a research lab and I started to realize that I enjoyed it more than

playing soccer. I enjoyed doing experiments, thinking, getting results and figuring out what is wrong if the experiment failed. The more time I spent in lab the more I thought that this is what I want to do. I spoke with the graduate students and they all said that I should apply to graduate programs. I knew that I wanted to work in a more translational area and with a different model. I liked working with bacteria but I was more interested in health-related research.

I applied to Mizzou and decided to attend since the biological sciences department had a variety of professors that had cool research. I rotated through Dr. D Cornelison's lab and after a few weeks I realized that I wanted to come in on the weekends and stay late at lab because I thought my research was that interesting. I knew that this was the kind of environment that I wanted to be in since I knew graduate school would require lots of hours. I spent a lot of time being mentored by Dr. Danny Stark while he was in lab. He was a main graduate student mentor since I was working on a similar project to his. After he graduated, Dr. Dane Lund helped fill the void and taught me other techniques but also lab maintenance so I would be ready when he graduated. I have spent many hours in lab without these two men however, I need to move on to first step in a more independent scientific path.

This where I begin my scientific career, I am leaving from my PhD with a great skill set and I look forward to learning new techniques and new topics. I am grateful for everyone's guidance throughout my life and will try and make all of you proud.