An Asymmetric Jam2/Par Complex Renews Muscle Stem Cells by Localized p38alpha/beta MAPK Signaling

Andrew A. Troy
University of Colorado at Boulder, airtroys@gmail.com

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An Asymmetric Jam2/Par Complex Renews Muscle Stem Cells

By Localized p38α/β MAPK Signaling

by

Andrew Adam Troy

B.S., University of California, Davis

A thesis submitted to the Faculty of the Graduate School of the University of Colorado in partial fulfillment of the requirement for the degree of Doctor of Philosophy

Department of Molecular, Cellular and Developmental Biology

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This thesis entitled:
An Asymmetric Jam2/Par Complex Renews Muscle Stem Cells By Localized p38α/β MAPK Signaling
written by Andrew Adam Troy
has been approved for the Department of Molecular, Cellular and Developmental Biology

________________________________________________________
Professor Mark Winey (Thesis Committee Chair)

________________________________________________________
Professor Bradley Olwin (Thesis Advisor)

Date________________

The final copy of this thesis has been examined by the signatories, and we find that both the content and the form meet acceptable presentation standards of scholarly work in the above mentioned discipline.
Troy, Andrew Adam (Ph.D, Molecular, Cellular and Developmental Biology)

An Asymmetric Jam2/Par Complex Renews Muscle Stem Cells by Localized p38α/β MAPK Signaling

Thesis directed by Professor Bradley Bruce Olwin

Abstract

Skeletal muscle is maintained and repaired by satellite cells. Satellite cells are quiescent in uninjured muscle but activate, proliferate and repair the muscle after injury. The quiescent satellite cell population is renewed during the injury repair, but how and when this happens is unclear. Recently, several subpopulations of satellite cells have been described with an enhanced capacity for self-renewal raising the possibility that there is a subset of satellite cells dedicated to maintaining the quiescent satellite cell population. I find that all satellite cells activate in response to injury and, after the first division, quiescent satellite cells reappear. I show that these quiescent cells are generated by a subpopulation of satellite stem cells that divide asymmetrically in response to muscle injury. During this division, Jam2 establishes asymmetry by recruiting the Par complex to sites of cell-cell or cell-fiber junctions and only one daughter cell receives the Jam2/Par complex. The Par complex associates with Tiam1 and promotes the activation of p38α/β MAPK through a novel signaling pathway, promoting proliferation and commitment to myogenesis in only one daughter. The Jam2 expressed in this daughter cell promotes quiescence in the adjacent daughter cell through the Jam2 extracellular domain. This asymmetric division results in an active myoblast and a quiescent cell that retains the satellite stem cell identity. Thus, the asymmetric activation of p38α/β MAPK by the Par complex and the lateral regulation of
quiescence by Jam2 ensures the self-renewal of the satellite stem cell population. The timing of this asymmetric division illustrates a novel link between the initial response to muscle injury and satellite cell self-renewal. This is potentially a mechanism for satellite cells to gage the severity of the muscle injury and organize the satellite cell response accordingly.
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Chapter 1: Introduction
Skeletal Muscle Satellite Cells

Satellite Cells Maintain And Repair Skeletal Muscle

Skeletal muscle consists of syncytial muscle fibers that span the length of the muscle. Structural and motor proteins are arranged within muscle fibers so that the fiber constricts lengthwise upon depolarization, generating the muscle contractions that are responsible for vertebrate movement (Farah and Reinach, 1995). Properly functioning muscle tissue is necessary for vertebrates to find food, avoid predation, reproduce and perform many other behaviors fundamental for survival. Thus, it follows that there is a strong evolutionary pressure upon animals to ensure that skeletal muscle is maintained in working condition and that function is rapidly restored if the muscle is damaged.

Indeed, injured skeletal muscle has the remarkable ability to fully regenerate (Carlson and Faulkner, 1983). Muscle fibers are terminally differentiated and cannot proliferate to replace damaged fibers. Instead, myonuclei are replaced by a population of single nucleate cells distributed throughout the muscle tissue, termed satellite cells (Schultz and McCormick, 1994). Satellite cells are rare in uninjured muscle and are named for the niche they occupy, along the outside of muscle fibers, between the basal lamina and the myofiber membrane (Figure 1.1A) (Mauro, 1961; Schultz et al., 1978).

While the majority are quiescent in uninjured muscle, satellite cells rapidly activate in response to muscle damage (Jones et al., 2005) and undergo their first division within 48 hours following muscle injury (Cornelison et al., 2001). Satellite cells then enter a period of rapid proliferation, forming a population of committed muscle precursor cells termed myoblasts (Figure 1.1B) (Schultz and McCormick, 1994). Eventually, myoblasts exit the cell cycle and
Figure 1.1: Satellite cells repair damaged muscle. In uninjured muscle, satellite cells reside quiescently between the basal lamina (yellow) and the muscle fiber (A, red/pink). Upon muscle injury, satellite cells activate and proliferate to produce a population of committed myoblasts (B, red). Myoblasts differentiate and fuse to repair the injured muscle, providing new myonuclei (blue, elongated). In the repaired muscle, quiescent satellite cells again reside between the muscle fiber and the basal lamina (C).
terminally differentiate, fusing with the muscle fibers, replenishing myonuclei and repairing the muscle (Schultz and McCormick, 1994). After muscle repair, quiescent satellite cells are found between the basal lamina and muscle fibers, as in the uninjured muscle (Figure 1.1C) (Schultz, 1984), demonstrating that quiescent satellite cells are replaced or renewed following muscle injury.

Quiescent Satellite Cells Are Poised To Rapidly Respond To Muscle Injury

The majority of quiescent satellite cells express the transcription factors, Pax7 (Olguin and Olwin, 2004) and Myf5 (Beauchamp et al., 2000), which regulate the maintenance of satellite cell identity and commitment to myogenesis. Additionally, quiescent satellite cells express the growth factor receptors, c-Met (HGF receptor) (Cornelison and Wold, 1997) and FGFR1 (Cornelison et al., 2000), and the heparan sulfate proteoglycans, Syndecan-3 and Syndecan-4 (Figure 1.2A) (Cornelison et al., 2001). As both HGF and FGF-2 are released by the muscle and surrounding matrix upon muscle injury and promote satellite cell activation (Yablonka-Reuveni and Rivera, 1994; Yablonka-Reuveni et al., 1999; Kästner et al., 2000; Anderson and Pilipowicz, 2002; Tatsumi et al., 1998; Tatsumi and Allen, 2004; Tatsumi et al., 2006), expression of c-Met and FGFR1, as well as Syndecan-3 and Syndecan-4 which facilitate the binding of FGF its receptors (Rapraeger et al., 1991), enable satellite cells to detect and respond to an injury.

Quiescence is not a default, dormant state for satellite cells in the absence of stimulation. Microarrays performed on satellite cells in quiescence show that almost as many genes are transcriptionally down regulated upon activation as are up regulated, suggesting that satellite
Figure 1.2: Growth factor and p38α/β MAPK signaling promote satellite cell activation. Quiescent satellite cells express FGFR1, c-MET, Syndecan-4 and Syndecan-3 and prevent activation through the degradation of MyoD mRNA (A). The release of HGF and FGF-2 during muscle injury results in the stabilization of MyoD mRNA via p38α/β MAPK signaling and the increase in transcription factors that promote satellite cell activation (B).
cells are actively maintained in quiescence (Hausburg, Thesis). TTP is an RNA-destabilizing protein that positively regulates satellite cell quiescence. TTP is active in quiescent satellite cells and targets transcripts containing AU-rich elements, like MyoD mRNA, promoting degradation and blocking translation. As MyoD promotes both satellite cell proliferation and myogenesis (Montarras et al., 2000; Cornelison et al., 2000), preventing MyoD activity would represent one mechanism to ensure satellite cells remain in quiescence. Upon satellite cell activation, p38α/β MAPK phosphorylates TTP, deactivating TTP and allowing the translation of MyoD and other TTP targets (Figure 1.2B, Melissa Hausburg, Thesis).

It is interesting that genes that promote activation and myogenesis are actively transcribed during quiescence. Negative regulating MyoD at translation rather than transcription would allow the rapid production of MyoD protein upon muscle injury. Thus, quiescent satellite cells expend energy to remain on the brink of activation, allowing a rapid response to muscle injury.

*Phosphorylation Of p38α/β MAPK Promotes Satellite Cell Activation In Response To Injury*

Muscle injury results in the release of growth factors and the subsequent activation of satellite cells. Injured muscle synthesizes nitric oxide, which results in the release of HGF from the extracellular matrix which results in the activation of the HGF receptor, c-Met, expressed by quiescent satellite cells (Figure 1.2B, 1.3) and promoting the exit of satellite cells from quiescence (Tatsumi et al., 1998; Anderson and Pilipowicz, 2002; Tatsumi and Allen, 2004; Tatsumi et al., 2006). Additionally, FGF-2 is expressed by muscle and the surrounding tissue upon injury (Kästner et al., 2000), which drives satellite cell activation and proliferation (Figure
Figure 1.3: Transcription factors and cell signaling regulate myogenesis. The height of the triangles indicates the expression of transcription factors at points of the timeline below the graph. The direction of the colored arrows and their position relative to the timeline above indicates the impact of that protein on the progression of satellite cells toward myogenesis. Black T-lines and arrows between the colored arrows indicate the interaction between myogenic signaling molecules.
The binding of FGF-2 to its receptor is facilitated by the proteoglycan, Syndecan-4 (Steinfeld et al., 1996), and loss of Syndecan-4 in satellite cells results in the inability of satellite cells to activate (Cornelison et al., 2004). The earliest detectable marker of satellite cell activation is the phosphorylation of p38α/β MAPK, which occurs only minutes after muscle injury (Jones et al., 2005). Active p38α/β MAPK phosphorylates and inhibits TTP, stabilizing MyoD mRNA (Figure 1.2B) (Melissa Hausburg, Thesis), and stabilizes SMAD4 to promote the transcription of the myogenic transcription factor, Myf5 (Hsu et al., 2011). MyoD and Myf5 then promote satellite cell activation, proliferation and commitment to myogenesis (Montarras et al., 2000; Ustanina et al., 2007; Gayraud-Morel et al., 2007). Blocking p38α/β MAPK activity with a pharmacological inhibitor prevents satellite cell proliferation or differentiation (Jones et al., 2005), maintaining satellite cells in a quiescent-like state. Thus, p38α/β MAPK acts as a molecular switch to drive the transition of satellite cells from quiescence to activation (Figure 1.2).

MyoD, Pax7, Myf5 And Myogenin Regulate Myogenic Progression

Once active, satellite cells become proliferative myoblasts which will eventually differentiate into skeletal muscle. The progression of satellite cells from quiescence to proliferation and differentiation is regulated, in part, by the expression patterns of myogenic transcription factors.

MyoD is a strong muscle regulatory factor that not only promotes the myogenic commitment of satellite cells, but is sufficient to drive myogenesis when over-expressed in non-
muscle cells as well (Tapscott et al., 1988). While MyoD protein is absent in quiescent satellite cells (Olguin and Olwin, 2004), its transcript and protein are expressed as little as 3 hours after p38α/β MAPK activation (Cornelison and Wold, 1997; Jones et al., 2005) and is maintained through both proliferation and terminal differentiation (Olguin and Olwin, 2004). MyoD is necessary for both the proliferation and differentiation of myoblasts as MyoD null satellite cells show defects in both behaviors (Cornelison et al., 2000; Montarras et al., 2000). In proliferating myoblasts, MyoD protein is regulated in a cell cycle specific manner where MyoD protein levels are highest in G1 and lowest in S-phase (Kitzmann et al., 1998). G1 expression of MyoD is necessary for progression through the cell cycle as MyoD positively regulates the transcription of Cdc6, allowing entry into S-phase (Zhang et al., 2010).

While MyoD is necessary for myoblast proliferation (Montarras et al., 2000; Cornelison et al., 2000), the over-expression of MyoD induces cell cycle exit through the up regulation of p21 (Lassar et al., 1989; Guo et al., 1995) and differentiation through the expression of Myogenin and other muscle specific genes (French et al., 1991; Prody and Merlie, 1991; Ishibashi et al., 2005; Deato et al., 2008). MyoD achieves these contradictory effects by targeting distinct sets of genes and regions of chromatin in proliferating or differentiating myoblasts (Cao et al., 2010). The regulation of MyoD binding partners (French et al., 1991; Ordentlich et al., 1998; Page et al., 2004; Deato et al., 2008), the phosphorylation of MyoD by cdk-1 and cdk-2 (Kitzmann et al., 1999), the repression or reduction of MyoD protein and activity (Langley et al., 2002; Yamane et al., 2004; Olguin et al., 2007) and the synergistic or repressive activity of other transcription factors expressed at specific stages of myogenesis all influence whether MyoD targets and activates genes that will promote proliferation or genes that will induce
differentiation. Thus, multiple signaling pathways in satellite cells signal through MyoD to influence whether the cell enters or remains in a proliferative state and whether the cell commits to differentiation.

Like MyoD, Myf5 is sufficient to induce myogenesis in many cell types by promoting the transcription of genes necessary for myoblast differentiation (Braun et al., 1989; Arnold et al., 1992; Li and Capetanaki, 1993). However, despite its similarity to MyoD, Myf5 does not promote myoblast differentiation. Myf5 is expressed in the majority of quiescent satellite cells (Beauchamp et al., 2000) and is up regulated upon activation (Cornelison and Wold, 1997). Myf5 expression is maintained during proliferation, where Myf5 protein levels fluctuate in a cell cycle dependent manner due to the ubiquitination and degradation of Myf5 during mitosis (Lindon et al., 1998; Kitzmann et al., 1998; Doucet et al., 2005), and is lost at the onset of myogenesis (Mangiacapra et al., 1992; Lindon et al., 1998).

Myf5 null satellite cells are delayed in activation, display reduced proliferation and precociously differentiate, suggesting a role for Myf5 in promoting myoblast proliferation and preventing differentiation (Montarras et al., 2000; Ustanina et al., 2007; Gayraud-Morel et al., 2007). Additionally, Myf5 expression is associated with the repression of myogenesis by HGF (Yamane et al., 2004) and is necessary for myoblasts to resist differentiation when cultured at low density (Lindon et al., 2001), further illustrating the role of Myf5 in maintaining satellite cells in a committed, proliferative state.

The paired box transcription factor, Pax7, is expressed in both quiescent and proliferating myoblasts, but not during differentiation (Olguin and Olwin, 2004). Pax7 negatively regulates the myoblast transition from proliferation to differentiation as over-expression of Pax7 inhibits
myoblast differentiation, either maintaining satellite cells in a prolonged proliferative state (Zammit et al., 2006; McKinnell et al., 2008; Collins et al., 2009) or inducing cell cycle exit into a quiescent like state (Olguin et al., 2007; McFarlane et al., 2008). Additionally, Pax7 null muscle has a greatly reduced number of satellite cells and, as a result, reduced ability to generate or regenerate muscle (Seale et al., 2000; Oustanina et al., 2004), highlighting the importance of Pax7 in the maintenance or specification of quiescent satellite cells.

Pax7 regulates myogenesis by controlling the expression of Myf5 and MyoD. Pax7 promotes Myf5 transcription through the recruitment of a histone methyl transferase to the Myf5 locus (McKinnell et al., 2008) and negatively regulates MyoD protein by promoting its proteasome mediated degradation (Olguin et al., 2007). While MyoD is necessary for both myoblast proliferation and differentiation, over-expression of MyoD induces myoblast differentiation (Lassar et al., 1989), suggesting that the level of MyoD protein may influence whether it promotes proliferation or differentiation. Reducing the level of MyoD protein might be the mechanism by which Pax7 promotes the proliferative effects of MyoD and inhibits myoblast differentiation.

Results from other labs suggest an opposite role for Pax7 in the regulation of MyoD. Ectopic expression of a dominant negative Pax7 mutant, where the DNA binding region of Pax7 is fused to the Engrailed transcriptional repressor domain, reduces MyoD protein and transcript (Relaix et al., 2006; Collins et al., 2009), suggesting that Pax7 transcriptional activity is necessary for MyoD expression. These contradictory effects of Pax7 could reflect a mechanism by which Pax7 maintains low levels of MyoD to promote proliferation. However, the dominant negative Pax7 mutants contain the Pax7 homeobox domain, which is responsible for promoting
the degradation of MyoD. As MyoD promotes its own transcription (Lassar et al., 1989), an alternative explanation for the reduced levels of MyoD mRNA and protein is that overexpressing the Pax7 homeobox domain induces the degradation of MyoD.

The expression pattern of the myogenic regulatory factor, Myogenin, is opposite that of Pax7 and Myf5 as Myogenin is expressed exclusively in differentiating myoblasts (Olguin and Olwin, 2004; Olguin et al., 2007). Myogenin acts with MyoD to promote the transcription of myogenic genes (Cserjesi and Olson, 1991; Naidu et al., 1995; Ji et al., 2009; Li et al., 2009), driving the transition of myoblasts to terminally differentiated muscle cells. Thus, the timing of Pax7, Myf5, MyoD and Myogenin expression leads to different combinatorial effects of these transcription factors, regulating the quiescence, proliferation and differentiation of myoblasts (Figure 1.3).

Multiple Signaling Pathways Regulate Satellite Cell Behavior

Satellite cell behavior is regulated by both the injury environment as well as communication among satellite cells. This occurs through multiple pathways which, in turn, regulate the transitions from quiescence to activation or from proliferation to differentiation. The growth factors HGF and FGF-2 are released from both the surrounding, injured tissue as well as other satellite cells (Hannon et al., 1996; Kästner et al., 2000; Sheehan et al., 2000; Tatsumi et al., 2006) and induce satellite cell activation (Tatsumi et al., 1998; Yablonka-Reuveni and Rivera, 1994; Yablonka-Reuveni et al., 1999; Kästner et al., 2000; Jones et al., 2001). After activation both growth factors continue to regulate myoblast behavior as HGF \ directs satellite cell migration (Siegel et al., 2009) and both FGF-2 and HGF prevents differentiation (Olwin and
Hauschka, 1986; Clegg et al., 1987; Allen and Boxhorn, 1989; Gal-Levi et al., 1998), maintaining satellite cells in a prolonged state of proliferation.

The MAP kinase, p38α/β, also has multiple functions during myogenesis. While p38α/β MAPK is necessary for satellite cell activation and proliferation (Jones et al., 2005), p38α/β MAPK has also been reported to both positively (Lluís et al., 2005; Delling et al., 2000; Lovett et al., 2010) and negatively (Page et al., 2004; Kook et al., 2008; Hsu et al., 2011) regulate MyoD transcriptional activity and differentiation. These opposing activities of p38α/β MAPK are likely due to the presence of different targets or cross talk with differing pathways depending on the stage of myogenesis.

Notch1 signaling promotes the cell cycle entry of quiescent satellite cells while blocking the myoblast transition from proliferation to differentiation. Notch is a transmembrane receptor that binds membrane bound ligands in adjacent cells, facilitating communication between cells in direct contact (Buas and Kadesch, 2010). Upon activation, Notch is cleaved, releasing its intracellular domain which then enters the nucleus and regulates gene transcription (Buas and Kadesch, 2010). In skeletal muscle, Notch1 promotes the entry into the cell cycle of quiescent satellite cells by inhibiting the transcription of CDK inhibitors (Conboy et al., 2003; Carlson et al., 2008). At later stages of myogenesis, Notch1 blocks the transition to differentiation (Kopan et al., 1994; Conboy and Rando, 2002) by negatively regulating the expression (Buas et al., 2010) and transcriptional activity of MyoD (Ordentlich et al., 1998) as well as reducing p38 activation by up regulating the p38 MAPK phosphatase, MKP-1 (Kondoh et al., 2007).

There are multiple isoforms of Notch and mechanisms to regulate its activation, allowing Notch signaling to play multiple roles in myogenesis. For instance, the Notch isoform, Notch3 is
associated with the self-renewal of satellite cells, as Notch3 is expressed primarily in an uncommitted, progenitor-like subpopulation of satellite cells (Kuang et al., 2007). Notch signaling is further regulated by the heparan sulfate proteoglycan, Syndecan-3, which complexes with Notch in satellite cells, promoting Notch cleavage and activation, thereby regulating satellite cell proliferation (Pisconti et al., 2010). Thus, the signaling pathways regulating myogenesis have many points of regulation and promote different cell fates depending on the specific context of the cell.

**Adult Stem Cells**

*Stem Cells Repair Adult Tissue*

The continued maintenance and repair of a tissue for the adult life of an organism is not a problem unique to skeletal muscle. Typically, tissue specific populations of stem cells produce the committed cells that will eventually differentiate to generate new tissue (Giebel and Bruns, 2008; Doe, 2008; Zhang et al., 2009; van der Flier and Clevers, 2009). However, stem cells cannot produce progeny uniformly committed to differentiation, otherwise the stem cell population would be lost. Instead, to both repair the tissue and to ensure that the stem cell population persists for the lifetime of the animal, stem cells divide infrequently to produce rapidly dividing, committed ‘transient amplifying cells’ as well cells that retain the stem cell phenotype. Due to their unique role and behavior, numerous methods have been developed to allow the detection or isolation of stem cells. The adult stem cells of many tissues can be identified by the expression of stem cell markers such as Sca-1 (Holmes and Stanford, 2007) and Abcg2 (Ding et al., 2010). Abcg2 is a transmembrane transporter which allows the rapid efflux
of Hoechst dye from the stem cell, providing another means by which to isolate adult stem cells. When cells purified from many different tissue types are treated with Hoechst dye and subsequently sorted based on Hoechst dye fluorescence, stem cells sort into the ‘side population’ (SP) due to the ability of Abcg2 expressing stem cells to quickly clear the dye from the cell (Ding et al., 2010).

Stem cells can also be identified due by the long term retention of thymidine analogs such as BrdU (Potten and Morris, 1988; Braun and Watt, 2004; Potten, 2004; Ma et al., 2004). BrdU is incorporated into DNA during S-phase where it can be later detected, providing insight into the proliferative history of that cell. When a cell that has been labeled with BrdU subsequently divides in the absence of BrdU treatment, the BrdU signal is diluted as more, unlabeled DNA is synthesized. Because stem cells cycle infrequently, they retain high levels of BrdU for long periods of time, allowing the identification and isolation of stem cells from the surrounding, frequently dividing cells. It has also been argued that long-term BrdU retention is due to the asymmetric segregation of the template strands of DNA, allowing the stem cell to inherit stem cell specific patterns of DNA methylation or to avoid the accumulation of DNA replication errors (Potten et al., 2002; Lansdorp, 2007).

The Par Complex Directs The Asymmetric Divisions Of Stem Cells

Stem cells must divide to generate both transient amplifying cells and stem cells. One mechanism by which stem cells accomplish this is by undergoing asymmetric divisions (Giebel and Bruns, 2008; Doe, 2008; Zhang et al., 2009; van der Flier and Clevers, 2009). The asymmetric division of adult stem cells produces daughter cells committed to different fates; one
daughter cell becomes a transient amplifying cell while the other retains the stem cell identity (Knoblich, 2008).

The stem cell niche is instrumental in regulating asymmetric divisions. The initial asymmetry in the dividing cell is created through the polar interaction between the stem cell and its niche (Fuchs et al., 2004). Additionally, once the stem cell has divided, only one daughter cell remains in contact with the niche, which then continues to reinforce the stem cell identity in only one daughter cell (Fuchs et al., 2004). The stem cell niche intracellularly regulates asymmetric division through the recruitment of the evolutionarily conserved Par complex (Knoblich, 2008).

The Par complex, which consists of the core proteins Partitioning Defective 3 (Par-3), Partitioning Defective 6 (Par-6) and an atypical Protein Kinase C (Suzuki and Ohno, 2006), is recruited to specific regions of the cell through interactions between Par-3 and transmembrane proteins, such as members of the Junctional Adhesion Molecule (Jam) family (Ebnet et al., 2001). The association between Par-3 with these membrane proteins is due to the mutual binding of PDZ domains found in each protein (Ebnet et al., 2001). Through its additional PDZ domains, Par-3 then recruits other members of the Par complex. Reciprocal signaling between members of the Par complex positively reinforces its polar localization and restricts the localization of other signaling complexes to the opposite pole of the cell (Figure 1.4A).

The primary mechanism by which Par-3 directs asymmetric signaling is by recruiting Par-6. Par-6 acts as an adaptor protein, binding Rac1, Cdc42 and aPKC (Johansson et al., 2000; Yamanaka et al., 2001; Garrard et al., 2003; Peterson et al., 2004). Through a series of feedback loops, these proteins signal to stabilize the Par complex and recruit more Par-3 to the membrane (Suzuki et al., 2002; Schonegg and Hyman, 2006; Hutterer et al., 2004). The binding of Par-6 to
Figure 1.4: The Par complex regulates asymmetric division. A diagram depicts the interactions between members of the Par complex and between the Par complex and other proteins. Double lines indicate binding, arrows indicate positive regulation, T-lines indicate repression and an * indicates regulation by phosphorylation (A). These interactions generate feedback loops resulting in the localization of signaling complexes to the opposite poles of the dividing cell (B).
aPKC activates aPKC kinase activity and this stimulatory effect of Par-6 upon aPKC is amplified by the binding of Cdc42 to Par-6 (Yamanaka et al., 2001; Garrard et al., 2003; Peterson et al., 2004). Tiam1, a guanine nucleotide exchange factor that activates Rac1, is recruited by Par-3 and, together with Cdc42, promotes Rac1 signaling (Nishimura et al., 2005) which in turn positively regulates aPKC activity and the maturation of the Par complex (Figure 1.4A) (Mertens et al., 2005).

Par complex asymmetry is maintained through the mutual inhibition of Lgl and aPKC. Lgl is phosphorylated by aPKC, which deactivates Lgl and prevents its localization to the membrane, thereby restricting Lgl to the opposite side of the cell (Betschinger et al., 2003; Plant et al., 2003; Betschinger et al., 2005). In turn, Lgl disrupts Par complex stability by competing with Par-3 for binding to Par-6 and aPKC (Yamanaka et al., 2003). Numb, an inhibitor of Notch, is another target of aPKC. Phosphorylation of Numb prevents its accumulation at the cell membrane resulting in the localization Numb opposite to the Par complex (Smith et al., 2007). Thus, Lgl and Numb accumulation is mutually exclusive with the Par complex. Conversely, the Par complex positively regulates the localization of the Crb3/Pals1/PATJ complex which in turn, reinforces the stability of the Par complex (Straight et al., 2004). Thus, through positive and negative feedback loops, the Par complex establishes mutually exclusive signaling domains at each pole of the cell (Figure 1.4B).

The activity of the Par complex is regulated in a cell cycle specific manner by the mitotic kinase, Aurora-A (Wirtz-Peitz et al., 2008). Aurora-A phosphorylates Par-6, promoting aPKC activity. The Par complex then signals through Cdc42 to orient the plane of division so that only one daughter inherits the Par complex and remains in association with the stem cell niche (Gotta
et al., 2001; Fuchs et al., 2004; Lee et al., 2006; Hao et al., 2010). The asymmetric inheritance of Lgl and Numb by one daughter cell and the inheritance of the Par and Crb3 complexes and continued association with the stem cell niche of the other daughter cell leads to distinct signaling and eventually distinct fates between the daughter cells (Fuchs et al., 2004; Suzuki and Ohno, 2006; Knoblich, 2008).

**Jam2 Recruits The Par Complex To Cell-Cell Junctions**

Jam2 is a transmembrane protein that localizes to cell-cell junctions through the interaction of extracellular IG-like domains with other cell junction proteins. Through the binding of its intracellular PDZ domain with Par-3, Jam2 recruits the Par complex to sites of cell-cell interaction to direct cell polarity (Ebnet et al., 2003; Gliki et al., 2004; Sacharidou et al., 2010).

Extracellularly, Jam2 interacts with Jam3 (Arrate et al., 2001), α4β1 integrin (Cunningham et al., 2002; Ludwig et al., 2009) and α2β1 integrin (Sacharidou et al., 2010) along with forming homodimers with other Jam2 molecules expressed on adjacent cells (Table 1) (Cunningham et al., 2000). Interaction between Jam2 and Jam3 is necessary for the proper localization of Jam3 (Lamagna et al., 2005) and regulates the binding activities of both proteins. Binding to Jam2 disrupts Jam3 homodimers, freeing Jam3 to bind to α(M)β2 integrin and facilitate leukocyte adhesion (Lamagna et al., 2005). In turn, Jam2 requires interaction with Jam3 to bind to α4β1 and facilitate the adhesion of T-cells to the vascular endothelium (Cunningham et al., 2002). Through these extracellular and intracellular interactions, Jam2 functions in a variety of cell types to regulate the stability of cell-cell interactions (Liang et al., 2002; Ludwig et al.,
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2009), the maturation of junction complexes (Ebnet et al., 2003; Lamagna et al., 2005), paracellular permeability (Aurrand-Lions et al., 2001), transendothelial migration (Palmeri et al., 2000; Aurrand-Lions et al., 2002; Johnson-Léger et al., 2002; Ludwig et al., 2005) and cellular polarity (Table 1) (Gliki et al., 2004; Sacharidou et al., 2010).

Jam2 appears to play an additional role in the maintenance of stem cell identity. Jam2 is highly expressed in embryonic, neural and hematopoietic stem cells but is rapidly lost upon differentiation (Sakaguchi et al., 2006). However, it is unclear whether Jam2 is involved in the interaction between stem cells and their niche, the regulation of asymmetric divisions or whether Jam2 performs another function in the regulation of stem cell fate.

**Muscle Stem Cells**

*Satellite Cells Self-Renew*

After the repair of a muscle injury, quiescent satellite cells resume their position between the muscle fiber and the basal lamina (Schultz, 1984) and are capable of reactivating and repairing future injuries (Hall et al., 2010). Self-renewal appears to be the primary mechanism by which satellite cells are regenerated as satellite cells transplanted into an injured host muscle contribute to both the host muscle and the host quiescent satellite cell population (Collins et al., 2005; Sacco et al., 2008; Tanaka et al., 2009; Hall et al., 2010). Additionally, a subset of satellite cells cultured *in vitro* resist differentiation, exit the cell cycle and become Pax7+/MyoD-.
quiescent satellite cells found *in vivo*. Thus, it appears that satellite cells act as muscle stem cells, generating myoblasts while ensuring their own self-renewal.

‘Satellite cell’ is a descriptive term, encompassing all mononuclear cells between the muscle fiber and basal lamina, and, as such, satellite cells may not be functionally homogenous. Indeed, numerous reports of satellite cell heterogeneity suggest that a portion of satellite cells may comprise a dedicated stem cell population. When mice are labeled with the thymidine analog, BrdU, perinatally, a subset of satellite cells retain BrdU label for up to 10 weeks after treatment (Shinin et al., 2006), although it is unclear if this is due to slow or no progression through the cell cycle or due to biased segregation of DNA strands during division.

Another subpopulation of satellite cells sort into the ‘side population’ (SP) during Hoescht dye efflux due to the membrane transporter activity of Abcg2, a characteristic of the adult stem cells in other tissues (Tanaka et al., 2009; Ding et al., 2010). Additionally, these ‘satellite-SP cells’ express the stem cell markers, Sca-1 and Abcg2 (Tanaka et al., 2009). The satellite-SP cells self-renew more efficiently than standard satellite cells as isolating and transplanting satellite-SP cells into an injured muscle results in a greatly increased contribution to the host quiescent satellite cells compared to non-SP satellite cells (Tanaka et al., 2009).

Quiescent satellite cells display variation in the expression of Myf5. A subset of quiescent, Pax7-expressing satellite cells have never expressed Myf5 as a demonstrated by the lack of recombination in satellite cells expressing the Cre gene from the Myf5 locus (Beauchamp et al., 2000). As Myf5 promotes commitment to myogenesis (Braun et al., 1989), it is possible that the lack of Myf5 is indicative that these satellite cells comprise an uncommitted satellite cell progenitor population. This conclusion is supported by transplanting Myf5 negative satellite cells.
into an injured muscle where, like the satellite-SP cells, Myf5 negative cells display an enhanced capacity for self-renewal (Kuang et al., 2007). Thus, the presence of the satellite-SP cell and Myf5 negative populations may indicate that satellite cells contain self-renewing muscle stem cells.

Satellite Cells Divide Asymmetrically

Upon activation, satellite cells continue to display heterogeneity in both the expression of myogenic regulatory transcription factors and the pattern of cell cycle progression. Directly following muscle injury, the majority of satellite cells are proliferative and are Pax7+/MyoD+. However, within 3 days of injury, satellite cells vary in their behavior as some lose Pax7 expression and begin to differentiate while others maintain Pax7 expression and resist differentiation, remaining proliferative or exiting the cell cycle to generate the mitotically quiescent, Pax7+/MyoD- ‘reserve cells’ (Olguin and Olwin, 2004; Zammit et al., 2004). It is not known whether this variation in commitment to myogenesis in cultured satellite cells reflects the behavior of a preexisting muscle stem cell population or is a result of the signaling and organization of equivalent myoblasts into differentiating, proliferative and quiescent populations.

A role for asymmetric division in the generation of myoblast diversity appears likely as multiple labs have observed satellite cell divisions yielding different daughter cells. The Myf5-satellite cells are capable of dividing asymmetrically to generate a Myf5+ daughter cell that becomes a proliferative myoblast and a daughter cell that retains the Myf5- phenotype (Kuang et al., 2007). However, it is not clear what the mechanism and timing of these asymmetric divisions are and what role, if any, they play in the generation of the quiescent, reserve cells observed in
culture. A small percentage of the BrdU-retaining satellite cells, described in the previous section, generate clones in culture that contain a single BrdU+ satellite cell among its BrdU-sister cells (Shinin et al., 2006). This suggests that some satellite cells asymmetrically segregate template DNA during mitosis. This conclusion is supported by the sequential labeling of satellite cells with the thymidine analogs, CldU and IdU, subsequent to the first division after injury (Figure 1.5A). Half of all labeled satellite cells divide to generate daughter cells that asymmetrically inherit the labeled DNA strand (Conboy et al., 2007). The frequency at which satellite cells selectively segregate DNA strands is much greater than would be expected if this reflected a mechanism for muscle stem cell self-renewal. It is likely that the asymmetric inheritance of DNA strands instead facilitates the generation of both a proliferative myoblast and a differentiating myoblast daughter cell by either regulating daughter cell fate through the segregation of methylated DNA or preventing the proliferative daughter cell from accumulating DNA replication errors.

A smaller subset of satellite cells retains DNA label when pulsed with BrdU during the first division injury and subsequently cultured without BrdU for multiple rounds of division (Shinin et al., 2006; Tanaka et al., 2009). While long term BrdU retention is often attributed to the selective retention of the template DNA strands by one daughter cell (Potten et al., 2002), it is unlikely that this explanation applies during this labeling paradigm. BrdU is incorporated into the newly synthesized, non-template strand of DNA and the long term retention of BrdU in this case would indicate the selective inheritance of the initial non-template strand of DNA (Figure 1.5B). The observation that this pattern of label retention is displayed by the stem cell marker expressing ‘satellite-SP cells’ suggests an alternative explanation (Tanaka et al., 2009). The
Figure 1.5: Satellite cells retain labeled DNA. Satellite cells were sequentially labeled with CldU (A, green) and IdU (A, red) to demonstrate the retention of the unlabeled template DNA strand (A, black). Two potential explanations for the retention of BrdU (B, C, green) when treated during the first division after injury and subsequently cultured without BrdU are the asymmetric segregation of the newly synthesized DNA strand (B) or cell cycle exit following the first division (C).
satellite-SP cells have an increased capacity for cell renewal (Tanaka et al., 2009) and may retain BrdU simply due to an early exit from the cell cycle into quiescence (Figure 1.5C).

To understand the role of these potential muscle stem cell populations in the renewal of quiescent satellite cells, I analyzed the cell cycle kinetics of satellite cells following injury. While all satellite cells divide in response to injury, a subset of satellite cells resist differentiation and exit the cell cycle after the first division. These cells are Pax7+/MyoD-, suggesting they represent the generation of quiescent satellite cells. I find that quiescent satellite cells are generated by the asymmetric division of satellite-SP cells to yield one Pax7+/MyoD+ myoblast and one Pax7+/MyoD-, quiescent satellite-SP cell. The asymmetric inheritance of a Jam2/Par complex drives this process by modulating satellite cell activation and quiescence via intracellular signaling through p38α/β MAPK and extracellular signaling by Jam2 between daughter cells.
Chapter 2: Satellite-SP Cells

Divide Once Following Muscle Injury
Introduction

Satellite cells are a population of cells that are responsible for the maintenance and repair of skeletal muscle (Schultz and McCormick, 1994). Quiescent satellite cells are defined by their position in uninjured muscle, where they reside between the muscle fiber and basal lamina (Mauro, 1961). In response to muscle injury, satellite cells activate and divide within 48 hours, subsequently proliferating to a large population of myoblasts. These myoblasts then differentiate and fuse to repair the muscle (Schultz and McCormick, 1994).

Progression from quiescence to myogenesis is regulated, in part, by the transcription factors Pax7, MyoD and Myogenin (Olguin and Olwin, 2004; Olguin et al., 2007; Weintraub et al., 1991; Brunetti and Goldfine, 1990). Pax7 is expressed in quiescent and proliferating satellite cells and prevents myogenesis through the regulation of MyoD (Olguin and Olwin, 2004; Olguin et al., 2007). MyoD is absent in quiescent satellite cells, but rapidly induced upon activation (Cornelison et al., 2001) where MyoD is required for cell cycle entry (Zhang et al., 2010). MyoD is expressed in both proliferative and differentiating myoblasts where MyoD levels are regulated in a cell cycle dependent manner (Kitzmann et al., 1998) and MyoD promotes myogenic commitment (Weintraub et al., 1991). Pax7 and Myogenin expression are mutually exclusive, where Myogenin inhibits Pax7 expression (Olguin et al., 2007) and promotes terminal differentiation (Brunetti and Goldfine, 1990).

After the muscle is repaired, quiescent satellite cells again occupy their niche along the muscle fiber (Schultz, 1984), but how and when they are renewed during injury is unclear. The capacity of transplanted satellite cells to occupy the host satellite cell niche after injection into an injured muscle suggests that satellite cells are capable of self-renewal (Collins et al., 2005;
Tanaka et al., 2009; Sacco et al., 2008; Hall et al., 2010). However, the term satellite cell is a descriptive, not functional term and several labs have described subpopulations of satellite cells with stem cell characteristics (Olguin and Olwin, 2004; Zammit et al., 2004; Shinin et al., 2006; Kuang et al., 2007; Sacco et al., 2008; Tanaka et al., 2009). These subpopulations of satellite cells express stem cell markers (Tanaka et al., 2009), display long-term BrdU retention (Tanaka et al., 2009; Shinin et al., 2006), comprise a population of Pax7+/MyoD- cells that resist differentiation in culture (Olguin and Olwin, 2004; Zammit et al., 2004), and display increased self-renewal when injected into an injured muscle (Kuang et al., 2007; Sacco et al., 2008; Tanaka et al., 2009). These reports raise the possibility that satellite cells are not equivalent and may be composed of at least two distinct populations, the canonical satellite cell that repairs muscle and a satellite stem cell that maintains the quiescent satellite cell population.

The relationship between these potential progenitor populations and their role in muscle repair and satellite cell renewal is unknown. I show that satellite cells universally activate and divide in response to muscle injury. However, after the first division, a subset of satellite cells loses MyoD expression and exits the cell cycle. This subset of cells is similar to previously described progenitor populations as it expresses the stem cell markers, Sca1 and ABCG2, retains BrdU and is Pax7+/MyoD-. I propose that a distinct population of satellite cells activates in response to injury and ensures the renewal of the quiescent satellite cell population, illustrating a novel link between muscle injury and satellite cell self-renewal.
Results

*BrdU-Retaining Cells Activate And Divide In Response To Injury*

Satellite cells may be comprised of at least two distinct populations; one population that repairs muscle injury and one population that ensures the renewal of quiescent satellite cells. Several subpopulations of satellite cells have been described that display stem cell characteristics or an increased capacity for self-renewal (Olguin and Olwin, 2004; Zammit et al., 2004; Shinin et al., 2006; Kuang et al., 2007; Tanaka et al., 2009) suggesting the existence of a satellite cell progenitor population. I attempted to identify these populations and determine their relationship to each other and their role in renewing the quiescent satellite cell pool. To identify the previously described BrdU-retaining satellite cells (Shinin et al., 2006; Tanaka et al., 2009), I labeled explanted satellite cells with BrdU during the first division and quantified BrdU retention in undifferentiated cells after 5 and 6 days in culture (Figure 2.1A).

Mass cultured satellite cells respond to explant into culture as they would to an injury. Cultured satellite cells activate and divide within the first 48 hours after injury and enter a subsequent period of rapid proliferation. The numbers of cells expressing Pax7, a marker of undifferentiated satellite cells, increases rapidly after day 2 in culture (Figure 2.1B, C). The number of Pax7+ cells begins to decrease after day 4 as satellite cells begin to differentiate (Olguin et al., 2007) and, by day 6, Pax7+ cells make up only a small percentage of the total cells (Figure 2.1B, D). Treatment with BrdU during the first division labels all cells in agreement with previous reports (Zammit et al., 2004; Tanaka et al., 2009) demonstrating that all cells cycle upon explant into culture. However, a subsequent 3-day chase without BrdU reveals a Pax7+ population of BrdU-retaining cells (13%, Figure 2.1E). The percentage of BrdU-retaining Pax7+
Figure 2.1: A subset of Pax7+ satellite cells retain BrdU. Pax7+ primary myoblasts initially increase in number in culture (B, C) before declining as cells commit to differentiation and lose Pax7 expression (B, D). Pulse labeling with BrdU for the first 2 days of culture (A) reveals a subset of Pax7+ cells that retain BrdU after 5 (E) or 6 days (F) in culture. Error bars represent standard error of the mean.
cells increases at day 6 (37%, Figure 2.1F) as the Pax7+/BrdU- cells commit to terminal differentiation and lose Pax7 expression (Olguin and Olwin, 2004; Olguin et al., 2007). Thus, it appears that all satellite cells enter the cell cycle in response to muscle injury. However, following the first division after injury, a subset of cells retains BrdU, demonstrating that these cells either cycle slowly or withdraw entirely from the cell cycle (Shinin et al., 2006).

To determine whether the BrdU-retaining cells have withdrawn from the cell cycle or cycle slowly after the first division, I treated satellite cell explants with the mitotoxin, AraC (1β-arabinofuranosylcytosine) at 1-day intervals followed by a 1-day chase without AraC to eliminate proliferating cells (Figure 2.2A). Satellite cells treated with AraC for the first 24 hours following plating fails to kill all cells, indicating three possibilities. First, some cells may not have yet entered S-phase; second, some cells may have terminally differentiated or third, a subset of cells is quiescent or cycles slowly (Figure 2.2B). However, AraC treatments from day 1 to 2 or day 2 to 3 eradicate all cells (Figure 2.2B, C), demonstrating that all satellite cells enter the cell cycle upon explant into mass culture. In contrast, ~40% of Pax7+ cells survive AraC treatments from day 3 to 4 or day 4 to 5 (Figure 2.2B, D). This suggests that these AraC-resistant cells divide once in response to injury before exiting the cell cycle.

To unequivocally demonstrate that the AraC-resistant cells initially divide, I labeled satellite cells with BrdU for the first 2 days in culture followed by AraC treatments from day 3 to 4 or day 4 to 5 post-explant (Figure 2.3A). The cells were then fixed and scored for BrdU retention. The majority of the Pax7+, AraC-resistant cells are BrdU+ after either 5 days (Figure 2.3B, C) or 6 days (Figure 2.3B, D) in culture. These data demonstrate that the AraC-resistant cells and BrdU-retaining cells are the same population. The AraC-resistant, BrdU-retaining
Figure 2.2: A subset of Pax7+ satellite cells withdraw from the cell cycle after the first division. All satellite cells divide in response to injury as AraC treatments from day 1 to 2 or day 2 to 3 (A) eliminate all Pax7+ satellite cells (B, C). A Pax7+ population of satellite cells survives treatments after the first division (B, D) demonstrating that while all cells divide initially in response to injury, a subset exit the cell cycle after the first division. Error bars represent standard error of the mean.
Figure 2.3: AraC-resistant cells divide then exit the cell cycle. AraC-resistant cells retain BrdU when pulsed with BrdU until day 2 (A) and treated with AraC from day 3 to 4 (B, C) or day 4 to 5 (B, D) and followed by a one day chase (A). Error bars represent the standard error of the mean.
population appears to divide initially in response to injury before withdrawing from the cell cycle.

*A Subset Of Myofiber-Associated Satellite Cells Divides Once In Response To Injury*

Explanting satellite cells into culture removes them from their niche and imposes an artificial environment that may affect cell behavior. Therefore, I asked if satellite cells cultured in association with the myofiber exhibit behavior similar to satellite cells in mass culture. To distinguish between satellite cells and myonuclei, I used anti-Syndecan-4 antibodies, which mark all satellite cells (Cornelison et al., 2001). To distinguish between cells that were AraC-resistant due to differentiation and cells that were AraC-resistant due to quiescence, I labeled with Pax7 or Myogenin, which exclusively label non-differentiating cells or cells that have committed to differentiation, respectively. Satellite cells on intact myofibers proliferate with a time course similar to mass culture. Pax7+/Myogenin- satellite cells first appear in doublets after 2 days in culture (Figure 2.4A, B, D). Maintaining Pax7 expression, cells continue to divide rapidly with cell cycle times no greater than 10 to 12 hours (Figure 2.4A, D, E). By day 4 in culture, large clusters of satellite cells have formed that are heterogeneous for Myogenin and Pax7 expression (Figure 2.4A, C, D). At day 5, the numbers of satellite cells have decreased as they migrate off the myofiber or differentiate and fuse with the myofiber (Figure 2.4A, D, F).

I performed pulse-chase AraC treatments, similar to those performed on mass cultured cells (Figure 2.5A) and scored for expression of Pax7 and the differentiation marker, Myogenin. All myofiber-associated satellite cells cycle initially as AraC treatments from either day 1 to 2 or day 2 to 3 eliminate virtually all cells (Figure 2.5B, C, E, F). Later treatments reveal at least two
Figure 2.4: Myofiber-associated satellite cells proliferate and differentiate in culture. Satellite cells culture attached to myofibers first appear in doublets by day 2 in culture where they express Syndecan-4 and Pax7, but not Myogenin (A, B, D). Subsequently, they rapidly proliferate (A, D, E) and by day 4, have formed large clusters heterogeneous for Myogenin expression (A, C, D). At day 5, Syndecan-4+ cells decrease as the majority down regulate Pax7, express Myogenin and fuse with the fiber (A, D, F). Error bars represent the standard error of the mean from replicate experiments (A) or between myofibers in a single experiment (D).
Figure 2.5: A subset of myofiber-associated cells are AraC-resistant after the first division. All myofiber-associated cells divide in response to injury as AraC treatments (A) during the first division eliminate nearly all Syndecan-4+ (B, C) and Pax7+ (E, F) satellite cells. Later treatments reveal both a differentiating Myogenin+ (B, D) and an uncommitted Pax7+ (E, G) AraC-resistant population. Error bars represent the standard error of the mean between fibers within a single experiment.
distinct AraC-resistant populations, a differentiating Pax7-/Myogenin+ population (Figure 2.5B, D, E, G) and an uncommitted Pax7+/Myogenin- population (Figure 2.5B, D, E, G).

It is possible that the AraC-resistant population cycles very slowly and is able to avoid the 1-day AraC treatments. Additionally, some leukemia cells develop AraC resistance through the down regulation of membrane bound AraC transporters, preventing AraC from entering the cell (Stam et al., 2003). To ensure that the AraC-resistant satellite cells survive AraC treatment due to quiescence and not to slow cell cycle progression or by drug resistance, I treated myofibers with from day 2 to 4 with AraC concentrations ranging from 100 pM to 100 µM (Figure 2.6A). Increasing the treatment time and concentrations of AraC did not eliminate the AraC-resistant population suggesting that the AraC-resistant cells are truly quiescent. Treating myofibers with 100 µM AraC for 2 day intervals yielded similar results to previous experiments; no satellite cells survived treatments during the first division (Figure 2.6B, C) while a subset of undifferentiated cells survived after the first division (Figure 2.6B, D).

Incorporation of BrdU can be genotoxic (Morris, 1991) and AraC is reported to indirectly introduce chromosomal aberrations (Park et al., 1991; Sekizawa et al., 2007), which could cause cells to divide slowly and possibly survive AraC treatments. To independently verify that a small percentage of satellite cells divide only once in culture, I marked cells with carboxyfluorescein diacetate succinimidyl ester (CFDA-SE). CFDA-SE is cell-permeant until cleaved by intracellular esterases at which point it becomes fluorescent and can only be diluted by subsequent cell divisions. This allows an accurate estimation of proliferation by measuring relative fluorescent intensity (Figure 2.7A) (Lyons, 1999). A 15-minute incubation of myofibers with CFDA-SE at day 2 labels all Syndecan-4+ cells, to varying degrees, as well as the myofiber
Figure 2.6: A subset of satellite cells survives 2 day AraC treatments at increased concentration. A subset of satellite cells survives 2 day AraC treatments at concentrations ranging from 500 pM to 100 µM (A). Treatment with 100 µM AraC for 2 days eliminates all satellite cells during the first division (B, C) but both Myogenin+ and Myogenin- resistant populations arise during later treatments (B, D). Error bars represent the standard error of mean between myofibers in a single experiment (A) or between replicate experiments (B).
Figure 2.7: A subpopulation of Pax7+ satellite cells retains CFDA-SE fluorescence. CFDA-SE is internalized by all cells and only diluted by subsequent divisions (A). A pulse with CFDA-SE labeled all Syndecan-4+ cells to varying degrees as well as the myofiber (B). A small number of CFDA-SE retaining cells were observed after chases of 1 day (C), 2 days (D) and 3 days (E).
itself (Figure 2.7B). The varied labeling of satellite cells and the background fluorescence of the labeled fiber prevent the accurate scoring of cell cycle exit by CFDA-SE fluorescence. However, I observed that the dye is quickly diluted below the fluorescence of the myofiber in the majority of Pax7+ satellite cells after 1 day (Figure 2.7C), 2 day (Figure 2.7D) or 3-day chases (Figure 2.7E) without CFDA-SE. However, a small number of Pax7+ cells retain green fluorescence at each time point, suggesting that they have not divided. BrdU retention, AraC resistance and CFDA retention verifies that an undifferentiated population of satellite cells exits the cell cycle after the first division and becomes quiescent.

_AraC-Resistant Cells Are Pax7+/MyoD- And Express Satellite-SP Markers_

AraC treatments demonstrated that the BrdU-retaining cells (Shinin et al., 2006) divide once in response to injury before exiting the cell cycle. This is similar to the Pax7+/MyoD- “reserve cells” that exit the cell cycle and resist differentiation in culture (Olguin and Olwin, 2004). To determine if the AraC-resistant / BrdU-retaining cells are the same population as the reserve cells, I treated myofibers with AraC from day 2 to 4 and scored MyoD expression. At 4 days, the majority of satellite cells on intact myofibers are MyoD+ (Figure 2.8A, C) in agreement with previous reports (Cornelison et al., 2001). In contrast, MyoD is undetectable in the majority of AraC-resistant cells (Figure 2.8B, C), while a small percentage exhibits low but detectable MyoD, suggesting that the BrdU-retaining cells and reserve cells comprise the same population.

The satellite-SP cells are another satellite cell subpopulation that displays progenitor characteristics (Tanaka et al., 2009). These cells express the stem cell markers Lymphocyte antigen 6 complex, locus A (Sca1) and ATP-binding cassette, subfamily G, member 2 (Abcg2),
Figure 2.8: AraC-resistant cells have reduced MyoD expression. 97% of myofiber-associated satellite cells express MyoD after 4 days in culture (A, C). However, after AraC treatment from day 2 to 4, the majority of surviving cells have little or no MyoD expression (B, C). Error bars represent the standard error of mean.
display long-term BrdU-retention and engraft to the satellite cell position more efficiently when injected concurrently with muscle injury (Tanaka et al., 2009). To test for a relationship between satellite-SP cells and the AraC-resistant cells, I scored AraC-resistant cells for Sca1 and ABCG2 expression. On untreated myofibers, Sca1+ (Figure 2.9A, B, C) and ABCG2+ cells (Figure 2.10 A, B, C) comprise only a small percentage of Syndecan-4+ cells after 4 days in culture. In marked contrast, 50% of the AraC-resistant Syndecan-4+ cells are Sca1+ (Figure 2.9A, D, E) and 65% are ABCG2+ (Figure 2.10A, D, E) indicating that the AraC-resistant population and the satellite-SP population overlap.

*AraC-Resistant Cells Maintain Long Term Pax7 Expression In Culture.*

To determine whether the AraC-resistant cells remain quiescent indefinitely or if they regenerate the myoblast population after AraC treatment, I treated cultured myofibers with 100 µM AraC from day 2 to 4 and allowed a 4-day recovery (Figure 2.11A, B). I found small numbers of both Pax7+/MyoD+ (Figure 2.11A) and Pax7-/MyoD+ (Figure 2.11B) satellite cells. However, I could find no Pax7+/MyoD- satellite cells and the frequency and spacing of the cells did not indicate substantial proliferation following the AraC treatment.

Reducing the concentration and duration of the AraC treatment produced similar results. Only sparsely distributed Pax7+/MyoD+ (Figure 2.11C) and Pax7-/MyoD+ (Figure 2.11D) satellite cells remained at day 8 after a 13 µM AraC treatment from day 3 to 4. The fibers became knotted during both long term culture experiments, presumably due to satellite cell fusion, making it difficult to accurately score satellite cell numbers.
Figure 2.9: AraC-resistant cells express Sca1. 3% of myofiber-associated satellite cells express Sca1 after 4 days of culture (A-C). However, ~50% of satellite cells are Sca1+ after an AraC treatment from day 2 to 4 (A, D, E). Error bars represent the standard error of the mean.
Figure 2.10: AraC-resistant cells express ABCG2. 8% of myofiber-associated satellite cells express the satellite-SP marker, ABCG2, after 4 days in culture (A-C). Following AraC treatment from day 2 to 4, ~67% of surviving cells express ABCG2 (A, D, E).
Figure 2.11: AraC-resistant cells maintain Pax7 expression and aberrantly differentiate in longterm culture. Rare Pax7+/MyoD+ (A) and Pax7-/MyoD+ (B) myofiber-associated cells are found at day 8 after 100 µM AraC treatment from day 2 to 4. Similar numbers of both Pax7+/MyoD+ (C) and Pax7-/MyoD+ (D) cells are found at day 8 following a 13 µM AraC from day 3 to 4. Mass cultured cells are universally Pax7+/MyoD+ at day 8 after AraC treatment from day 3 to 4. These cells exhibit both typical (E) and atypical morphologies (F) as well as aberrant differentiation (G) and fusion (H, I) despite continued Pax7 expression.
In the absence of the myofiber, AraC-resistant cells universally express both Pax7 and MyoD at day 8 (Figure 2.11E-I). While the combination of Pax7 and MyoD expression is typical of proliferating myoblasts, it is unlikely these cells are proliferative as they are generally found in isolation or very small groups (Figure 2.11E-G). While some AraC-resistant cells have the small, round morphology standard for uncommitted satellite cells (Figure 2.11E), others have flattened (Figure 2.11F) or elongated (Figure 2.11G) despite their continued Pax7 expression. Even more striking is the fusion and production of myotubes by Pax7+ cells (Figure 2.11H, I). Typically Pax7 marks quiescent or proliferating satellite cells and is never expressed during differentiation (Olguin and Olwin, 2004; Olguin et al., 2007). It is not clear whether the fused Pax7+ cells were generated by the clonal expansion of an AraC-resistant cell or resulted from the migration of several cells toward each other.

It is possible that satellite-SP cells only activate in response to a signal exclusively expressed in the early stages of injury. Potentially, this could be a mechanism to limit these cells to a single division in response to injury. To mimic the signaling immediately following injury, I isolated extract from freshly crushed muscles. Attempting to reactivate AraC-resistant cells on myofibers, I treated fibers with AraC from day 3 to 4 followed by treatment with crushed muscle from day 5 to 6. At day 8, to overcome the difficulty of scoring myofiber-associated cells after long-term culture, I scored all satellite cells expressing any combination of Syndecan-4, Pax7 or MyoD. While I observed a minor, but dose dependent, expansion of satellite cells in response to the crushed muscle extract (Figure 2.12A), there was no indication of the exponential proliferation characteristic of expanding myoblasts. It is possible that divisions in response to the crushed muscle extract are slow, similar to the first division in response to injury. Additionally,
Figure 2.12: AraC-resistant cells proliferate in response to crushed muscle extract. AraC treatment from day 3 to 4 followed by crushed muscle extract from day 5 to 6 results in a dose dependent increase in number of myofiber-associated satellite cells scored by Pax7, MyoD and Syndecan-4, at day 8 (A). Addition of 800 µg/ml crushed muscle extract from day 5 to 6 does not rescue the atypical morphology (B) or aberrant fusion (C) seen in mass cultured AraC-resistant cells at day 8.
AraC-resistant cells may require a signal from the non-SP satellite cells or the recently damaged fiber to generate a fully proliferative myoblast population. Treatment of mass cultured satellite cells with concentrations of crushed muscle extract as high as 800 µg/ml failed to rescue the atypical morphologies and aberrant differentiation of the AraC-resistant cells after 8 days in culture (Figure 2.12B, C).

My results support a model where there are two distinct populations of satellite cells (Figure 2.13). While both populations activate and divide in response to injury, the satellite-SP cells divide only once. After this division, satellite-SP cells return to quiescence and remain a future source of quiescent satellite cells for the muscle while the non-SP satellite cells proliferate and repair the muscle (Figure 2.13).

Discussion

Satellite cells appear to be a heterogeneous population. Several labs have described subpopulations of satellite cells with different cell cycle kinetics, abilities to resist differentiation or capacities to occupy the quiescent satellite cell position after transplant (Olguin and Olwin, 2004; Zammit et al., 2004; Shinin et al., 2006; Kuang et al., 2007; Tanaka et al., 2009). Thus, there may be a distinct population of satellite cells responsible for the maintenance of the quiescent satellite cell pool. To differentiate between these possibilities, I asked if there was a population of satellite cells that did not activate in response to muscle injury. Surprisingly, after injury, all satellite cells activate and divide, but, while the majority of satellite cells continues to proliferate, a subset divides only once and then returns to quiescence. Furthermore the BrdU retention, loss of MyoD and expression of satellite-SP markers displayed by the AraC-resistant
Figure 2.13: A subset of satellite cells divide only once in response to injury. Diagram depicts a model where satellite cells are not equal. While all satellite cells activate and divide initially in response to injury, non-SP satellite cells (red) rapidly proliferate and repair the muscle while satellite-SP cells (green) reenter quiescence (elongated circle). It is unclear whether all progeny of satellite-SP cells become quiescent after the first division or whether some contribute to the myoblast population.
cells implies that they are very similar to previously described populations of progenitor-like satellite cells (Shinin et al., 2006; Olguin and Olwin, 2004; Zammit et al., 2004; Tanaka et al., 2009). These results support a model where a progenitor population maintains quiescent satellite cells, but suggests a surprising mechanism. The progenitor population appears to divide and self-renew as a direct response to injury (Figure 2.13). Dividing in response to injury could be a mechanism to expand the progenitor pool and replace quiescent cells killed during the muscle injury or to provide an additional source of myoblasts.

While AraC-resistant cells divide only once in culture, these cells may be capable of subsequent divisions *in vivo* during injury repair. Our culture conditions are optimized to maintain satellite cells in a proliferative state and are unlikely to mimic the satellite-SP cell microenvironment. The limitations of studying the later stages of muscle regeneration in proliferating conditions are underscored by the behavior of AraC-resistant cells in long-term culture. Culturing quiescent cells under strong proliferative conditions could explain why AraC-resistant cells express MyoD but do not divide and maintain Pax7 expression through differentiation. While it is possible that altering our culture conditions would allow these cells to become fully quiescent *in vitro*, *in vivo* experiments need to be performed to truly measure the capacity of these cells to expand and self-renew in a real injury environment.

Interestingly, AraC-resistant cells are not often found in doublets. This implies that the progeny of satellite-SP cells may not all become quiescent after the first division. If this is the case, it means that satellite-SP cells contribute to both the proliferative myoblast and quiescent satellite cell populations. If so, coupling activation to satellite-SP cell self-renewal would be an efficient way for satellite-SP cells to assess the extent of muscle repair required and generate the
appropriate numbers of quiescent satellite cells and proliferating myoblasts. Moreover, limiting division to the early stages of injury would allow the progenitor cells to avoid the DNA replication errors and oxidative stress that a rapidly proliferating cell likely endures, lengthening the proliferative lifespan of the muscle (Renault et al., 2002).

**Experimental Procedures**

*Mice*

Mice were housed in a pathogen-free facility and all procedures and protocols were approved by the Institutional Animal Care and Use Committee at the University of Colorado. Mice were female B6D2F1 (The Jackson Laboratory) between 3 and 5 months of age.

*Cell Culture*

Primary satellite cells and myofibers were prepared as previously described (Cornelison et al., 2004). Primary satellite cells were cultured on gelatin coated plates and myofibers were cultured unattached to the plate. Cells were cultured under growth conditions (F12-C + 15% horse serum + 20 ng/ml bFGF) at 37°C under 5% CO₂ and 6% O₂. Media was changed daily and unattached primary cells were centrifuged and added back to the culture dish. BrdU (Sigma Aldrich) and CFDA-SE (Invitrogen) were added to a final concentration of 10 µM. AraC (Sigma Aldrich) was added a final concentration of either 13 µM or 100 µM unless stated otherwise. To prepare crushed muscle extract, hindlimb muscle was placed in PBS (1mL PBS/gram of tissue). Each piece of muscle was crushed with forceps and slowly agitated at 4°C for 2 hours. Solution was centrifuged at 3500 RPM and supernatant was centrifuged at 13,000 x g. Supernatant was
run through a 0.2 micron filter and stored at -20°C. Unattached cells were centrifuged onto slides at 200 rpms and fixed.

*Immunofluorescence*

Immunofluorescence was performed as previously described (Tanaka et al., 2009). Primary antibodies and dilutions: rat polyclonal anti-BrdU (Serotec) at 1:100, mouse monoclonal Pax7 (Developmental Hybridoma Bank at Iowa University) at 1:5, rabbit polyclonal MyoD (C-20, Santa Cruz Biotechnology) at 1:800, chicken anti-Syndecan-4 at 1:1500 and mouse monoclonal anti-myogenin (F5D) (Cusella-De Angelis et al., 1992) at 1:3. Secondary antibodies (Invitrogen) conjugated with Alexa Fluor 488, Alexa Fluor 546, Alexa Fluor 555, Alexa Fluor 594 and Alexa Fluor 647 were used at 1:1000 dilution. Myofiber-associated cells were scored by either cells per fiber or cells per millimeter of fiber length. Numbers of primary satellite cells were normalized to untreated day 2 numbers. Background fluorescence was set to the secondary only control or fluorescence of the myofiber. Blue represents DAPI staining unless stated otherwise.
Chapter 3: The Par Complex

Regulates The Asymmetric Division

Of Satellite-SP Cells
Introduction

Stem cells self-renew by undergoing asymmetric divisions (Knoblich, 2008) where they produce daughter cells committed to distinct fates. A committed daughter cell will proliferate and repair the tissue while the other daughter cell retains the stem cell identity (Lin, 2008). The Par complex, consisting of Partitioning Defective 3 (Par-3), Partitioning Defective 6 (Par-6) and an atypical Protein Kinase C (Suzuki and Ohno, 2006), generates asymmetry in the dividing cell and orients the plane of division so that each daughter cell will inherit different signaling molecules (Knoblich, 2008). These signaling molecules promote a separate fate in each daughter cell (Knoblich, 2008). Thus, stem cells can produce committed progeny while ensuring that the stem cell population is not lost.

There are subpopulations of satellite cells that share many characteristics with stem cells, including the ability to divide asymmetrically. A subset of satellite cells that do not express the marker of myogenic commitment, Myf5, can divide asymmetrically to produce a committed, Myf5 expressing daughter cell and an uncommitted, Myf5 negative daughter cell (Kuang et al., 2007). By dividing asymmetrically, these cells ensure their own self-renewal. However, it is not clear how this asymmetric division occurs and how the daughter cells adopt different fates. The satellite-SP cells are another population of self-renewing satellite cells (see chapter 2). Satellite-SP cells activate in response to injury, but divide only once before down regulating MyoD, becoming quiescence and retaining the satellite-SP cell identity.

I asked if the satellite-SP cells divide asymmetrically. I show that the Par complex is asymmetric in a subset of dividing satellite cells and that MyoD is asymmetrically expressed in a subset of doublets after the first division. The observation that the Par complex member, PKC\(_\lambda\),
is necessary for myogenesis leads me to propose that satellite-SP cells divide asymmetrically in response to muscle injury. During this division, the Par complex is asymmetrically inherited by one daughter cell. This daughter cell will express MyoD and commit to myogenesis while the other daughter cell loses MyoD expression and becomes quiescent, renewing the satellite-SP population.

Results

A Subset Of Satellite Cells Asymmetrically Regulates MyoD During The First Division

MyoD is expressed in satellite cells shortly after activation and retained by the majority of satellite cells through the early stages of differentiation (Cornelison et al., 2001). However, satellite-SP cells divide only once after muscle injury before down regulating MyoD and becoming quiescent. If an asymmetric division produces these MyoD- cells, I would expect to see doublets with asymmetric MyoD expression after the first division. I measured the levels of nuclear MyoD fluorescence relative to cytoplasmic background in doublets on myofibers 48 hours after injury. A doublet was scored as asymmetric if it contained one MyoD- cell and one cell with average or greater MyoD expression. The majority of doublets express equivalent levels of nuclear MyoD after the first division (Figure 3.1A, B), However, 12% of doublets displayed asymmetric MyoD expression (Figure 3.1A, C). Many doublets that did not meet my criteria for asymmetry still contained one MyoD- cell and one cell with detectable MyoD suggesting that I may have been too stringent in my scoring (Figure 3.1A). The observation that MyoD- cells are found in asymmetric doublets after the first division implies that these cells are generated by an asymmetric division.
Figure 3.1: A subset of doublets asymmetrically express MyoD. The majority of satellite cell doublets display equivalent levels of MyoD immunofluorescence 2 days after injury (A, B). However, a subset of doublets are asymmetric as they contain a MyoD- cell and a cell with higher than average MyoD expression (A, C). Doublets with symmetric MyoD are shown in green, doublets with asymmetric MyoD are shown in red and doublets containing a MyoD- cell that were not scored as asymmetric are shown in blue.
The Par Complex Is Asymmetrically Distributed In Satellite Cells

To determine if the genes canonically involved in asymmetric division are regulated during satellite cell activation, we examined changes in mRNA levels during the first 2 days after injury. We performed Affymetrix gene chip arrays on Syndecan-4+ satellite cells isolated by FACS from uninjured Tibialis Anterior muscles and at 12, 24 and 48 hours post-muscle injury (Tanaka et al., 2009). The Affymetrix array data was then queried for the Gene Ontology (GO) terms, cell polarity (Figure 3.2A) and planar cell polarity (Figure 3.2B). We found that members of the Par complex, including Par-3, Par-6, PKC\(\lambda\) and Cdc42 are transcriptionally regulated during the first division (Figure 3.2A). Additionally, members of the non-canonical Wnt pathway involved in planar cell polarity (Figure 3.2B), which has previously been implicated in the symmetric division of satellite cells (Le Grand et al., 2009), are also regulated.

If the Par complex is involved in the asymmetric divisions of satellite-SP cells in response to injury, I would expect to find Par complex proteins asymmetrically distributed in satellite-SP cells. I examined the expression and distribution of Par-3, PKC\(\lambda\) and Cdc42 immunofluorescence at the time of the first division after muscle injury (36 hours). I used the satellite-SP cell marker, Sca1, to look for differences in the expression and localization of the Par complex between satellite-SP cells and non-SP satellite cells. While Par-3 immunofluorescence is not detectable in the majority of satellite cells (Figure 3.3A-C), I observed that Par-3 is expressed and asymmetrically distributed in a small number of non-SP satellite cells (Figure 3.3D, Sca1-). However, in satellite-SP cells (Sca1+), I frequently observed Par-3 localized asymmetrically (Figure 3.3E-H). I noted that Par-3 was present in individual Sca1+ cells (Figure 3.3E-G) and in Sca1+ cells clustered with other satellite cells (Figure 3.3H).
Figure 3.2: Members of the Par complex are transcriptionally regulated during division. Multiple genes show regulation when heat maps derived from Affymetrix expression data of Syndecan-4+ cells isolated from uninjured (UI) muscle or muscle 12, 24 and 48 hours post-injury are queried for gene ontology (GO) annotations for cell polarity (GO:0007163, A) and planar polarity (GO:0001736, B).
Figure 3.3: Par-3 is asymmetric in satellite-SP cells. Par-3 immunofluorescence is not detected in the majority of satellite cells at 36 hours (A-C). However, asymmetric Par-3 is present in a small number of Sca1- cells (D) and frequently detected in Sca1+ satellite cells (E-H). Par-3 is detected in both individual Sca1+ satellite cells (E-G) as well as Sca1+ cells in clusters of satellite cells (H).
PKC\(\lambda\) is an atypical protein kinase C that binds Par-3 and Par-6 to regulate polarity and asymmetric division. Unlike Par-3, PKC\(\lambda\) is expressed in the majority of Syndecan-4+ cells (Figure 3.4A, B). In Sca1- cells, PKC\(\lambda\) is usually symmetrically distributed (Figure 3.4C, D) but is asymmetrically localized in a subset of cells (Figure 3.4E). Conversely, I frequently observed asymmetric PKC\(\lambda\) in Sca1+ cells (Figure 3.4F-H).

The small GTPase, Cell division control 42 (CDC42) interacts with the Par complex to establish cell polarity (Lin et al., 2000) and regulates PKC\(\lambda\) activity (Yamanaka et al., 2001). Satellite cells regulate Cdc42 transcript during the first division (Figure 3.1A) and express Cdc42 protein in most myofiber-associated Sca1- (Figure 3.5A) and Sca1+ (Figure 3.5B) satellite cells. While the Cdc42 antibody was inconsistent, I did observe polar Cdc42 in some satellite cells (Figure 3.5C). The presence and asymmetry of members of the Par complex in satellite-SP cells during the time of the first division supports a role for the Par complex in the asymmetric division of satellite-SP cells.

The Timing Of The First Satellite Cell Division Is Asynchronous

The Par complex is involved in many polarity processes apart from asymmetric division such as migration (Pinheiro and Montell, 2004; Solecki et al., 2006) and cell-cell adhesion (Suzuki et al., 2001). Live cell imaging performed in our lab has demonstrated that myofiber-associated satellite cells are highly mobile (Galati, unpublished data) suggesting that asymmetric Par-3, Cdc42 and PKC\(\lambda\) could be a result of migration rather than asymmetric division. To ensure that I observed asymmetry associated with division and not migration, I wanted to exclusively analyze mitotic cells. While satellite cells are reported to synchronously divide 36
Figure 3.4: PKCλ is asymmetric in satellite-SP cells. PKCλ is present in the majority of satellite cells 36 hours after injury (A, B) where it is evenly distributed in the majority of Sca1-satellite cells (C, D) but occasionally asymmetric (E). The majority of Sca-1+ satellite cells distribute PKCλ asymmetrically at 36 hours after injury (F-H).
Figure 3.5: Cdc42 is asymmetric in satellite cells. Cdc42 is expressed in both Sca1- (A, C) and Sca1+ (B) satellite cells at 36 hours where it is sometimes distributed asymmetrically (C).
hours after injury (Cornelison et al., 2001), I had rarely observed mitotic cells on myofibers at this time (Figure 3.2 - 3.7).

To increase the number of mitotic cells on myofibers, I paused cells in cytokinesis with the actin ring inhibitor, Blebbistatin. Treatment with Blebbistatin as early as from 30 to 34 hours in culture yielded pre-mitotic (Figure 3.6A, single cell, two centrosomes marked by γ-tubulin), mitotic (Figure 3.6B, condensed DNA, two centrosomes) and post-mitotic cells (Figure 3.6C, doublet, each cell with a single centrosome) on myofibers suggesting that some cells divide as early as 30 hours after injury. To verify this, I scored the number of pre-mitotic (Figure 3.6D, E), mitotic (Figure 3.6D, F) and post-mitotic satellite cells (Figure 3.6, D, G, doublets scored as 1) on myofibers between 30 and 33 hours in culture. While only ~3% of satellite cells were mitotic at any time, over 20% of satellite cells have formed doublets by 30 hours (Figure 3.6G).

I noted that the centrosomes were often located together in the center of the cell rather than at the poles of the cells paused with Blebbistatin (Figure 3.6B), which suggests Blebbistatin may disrupt polarity. Therefore, I attempted to optimize the preparation and the time I fixed myofibers to maximize the number of naturally mitotic cells. It is possible that satellite cells activate at different times during muscle dissection leading to asynchrony in the timing of the first division. To ensure that satellite cells activated simultaneously, I prepared and cultured myofibers at a pH of 6.5, which has previously been shown to prevent satellite cell activation (Tatsumi et al., 2006). I allowed satellite cells to activate in synchrony by transferring them to edia at pH 7.2 after 6 hours. However, I found no clear time point where the frequency of doublets increased or single cells declined drastically between 30 and 38 hours after activation (Figure 3.7A-D). Due to the increasing number of groups of 3 or more cells (Figure 3.7C, D), it
Figure 3.6: Satellite cells divide as early as 30 hours in culture. Some satellite cells divide as early as 30 hours in culture as there are pre-mitotic (A), mitotic (B) and post-mitotic (C) satellite cells when satellite cells are paused in cytokinesis with Blebbistatin treatment from hour 30 to 34. 20% of satellite cells have formed doublets by 30 hours as revealed by scoring untreated fibers for pre-mitotic (D, G, single cell, two centrosomes), mitotic (E, G, condensed DNA, two centrosomes) and post-mitotic cells (F, G, doublet, each cell with a single centrosome, scored as one).
Figure 3.7: The first satellite cell division is not synchronous. No time between 20 and 38 hours shows a substantial decline in individual satellite cells (A, D) or increase in doublets (B, D). The increasing number of satellite cells in groups of 3 or more (C, D) suggests satellite cells doublets may arise by either division or migration. As early as 27h in culture, individual cells (E), mitotic cells (F, H, pH3+) and doublets (G) are present showing that some satellite cells divide as early or earlier than 27h after injury. Scoring the percentage of pH3+ satellite cells from 27-42h (H-O) demonstrates that there is greater synchrony between cells on a single fiber than between any time point (O).
is possible that scoring the number of doublets does not accurately reflect the rate of division due to cell migration.

I then scored myofibers exclusively for mitotic satellite cells marked by phospho-Histone 3 (pH3) between 27 and 42 hours after release from low pH. Again, even as early as 27 hours after activation, I could detect single (Figure 3.7E), mitotic (Figure 3.7F) and doublet cells (Figure 3.7G). I observed a similar percentage of mitotic cells at every time point (Figure 3.7H-O) further supporting the conclusion that satellite cells are not synchronous in their first division. Interestingly, there was a greater synchrony between satellite cells on a single fiber that at any time (Figure 3.7O).

Par-3 And PKC λ Are Polar In A Subset Of Mitotic Satellite Cells During The First Division

To confirm that the Par complex is present in dividing satellite cells, I scored mitotic satellite cells displaying phospho-Histone-3 (pH3) immunoreactivity (Figure 3.8A, C-E, Figure 3.9A-D) at 36 hours after injury. Par-3 protein is not detectable in 74% of dividing satellite cells (Figure 3.8A, B) but, in the remaining 26%, Par-3 is asymmetric in 13% (Figure 3.8A, D, E) and symmetric in 13% of dividing cells (Figure 3.8A, C). PKC λ is expressed and uniformly distributed in the majority of dividing satellite cells (Figure 3.9A, B) and asymmetric in 16% of dividing cells (Fig 3.9A, C, D). I noted that Syndecan-4 immunoreactivity parallels both PKC λ and Par-3 staining. Syndecan-4 is uniform when PKC λ (Figure 3.9B) and Par-3 (Figure 3.8C) appear symmetric, but is asymmetric when either PKC λ (Figure 3.9C, D) or Par-3 is asymmetric (Figure 3.8D, E). Since the percentage of cells exhibiting asymmetric PKC λ and Par-3 immunoreactivity are similar, it is likely that Syndecan-4, PKC λ and Par-3 colocalize and are
Figure 3.8: Par-3 is asymmetric in a subset of dividing satellite cells. While the majority of mitotic satellite cells (pH3) do not express Par-3 (A, B), Par-3 is present and symmetric in 13% (A, C) and asymmetric 13% (A, B, E) of dividing cells on myofibers after 36 hours of culture.
Figure 3.9: PKCα is asymmetric in a subset of dividing satellite cells. The majority of mitotic satellite cells (pH3) show a symmetric distribution of PKCα (A, B), while PKCα is asymmetric in 16% of dividing satellite cells (A, C, D) on myofibers cultured for 36 hours.
asymmetric in the same subset of dividing satellite cells.

The Par complex is typically anchored to the cell membrane by binding the PDZ domains of membrane proteins (Suzuki and Ohno, 2006) and the interaction with a specific niche serves to orient the asymmetric division and reinforce the different fates of the daughter cells (Knoblich, 2008). However, the observation that cells resist AraC-treatment when cultured without contact with the myofiber argues that either satellite cells can asymmetrically divide without fiber contact or that the AraC-resistant satellite cells in mass culture are not a product of asymmetric division. To determine if satellite cells are capable of asymmetric division when removed from the fiber, I examined Par-3 distribution between 30 and 48 hours in mitotic cells in mass culture. Dividing cells that had attached to the gelatin coated coverslip in culture displayed symmetric Par-3 relative to the plane of division (Figure 3.10A-C). I noted Par-3 was often localized along the metaphase plate, but in a manner that would be inherited by both daughters (Figure 3.10A, B). Surprisingly, when examining mitotic satellite cells that had not yet attached to the plate, I frequently found that Par-3 was asymmetrically localized to regions of cell-cell interaction (Figure 3.10D, F, G) and sites of contact with pieces of fiber (Figure 3.10E). Importantly, after 3 days in culture, I no longer observed asymmetric Par-3 in non-adherent satellite cells (Figure 3.10H). Par-3 was often localized to the nucleus in non-dividing, non-adherent cells at all time points (Figure 3.10D, H). Par-3 is reported to localize to the nucleus in response to double strand DNA breaks (Fang et al., 2007) and it is possible the centrifugation of the non-adherent cells onto slides may have caused DNA damage and elicited this response. The asymmetric Par-3 between non-adherent cells implies that cell-cell interactions or junctions with pieces of fiber could orient asymmetric divisions in mass culture.
Figure 3.10: Par-3 is asymmetrically localized to the cell-cell junctions of non-adherent satellite cells. While adherent satellite cells in dispersed culture from 30-48h divide symmetrically in respect to Par-3 (A-C), mitotic, non-adherent satellite cells asymmetrically localize Par-3 to sites of cell-cell (D, E-G) or cell-fiber (E) interaction. Asymmetric Par-3 was not observed in dividing, non-adherent cells at 3d.
PKCλ Is Required For MyoD Activity And Myogenesis

If the Par complex regulates the fates of satellite-SP daughter cells, altering activity of the Par complex should alter myogenesis. Atypical protein kinase C is essential for the Par complex to regulate cell polarity and asymmetric division (Tabuse et al., 1998; Lin et al., 2000). There are two atypical protein kinase Cs expressed in mice, PKCλ and PKCζ. As array data indicates that satellite cells express PKCλ and not PKCζ (Figure 3.2A) and Western blots detect PKCλ, but not PKCζ, in the MM14 satellite cell line (Figure 3.11A, B) we altered PKCλ expression in MM14 cells and satellite cells. Co-transfection of MM14 cells with a PKCλ antisense construct and plasmid expressing βgal either eliminates or reduces detectable PKCλ immunoreactivity (Figure 3.11C). Activity of a MyoD-Gal4 transcriptional reporter is reduced when cotransfected with the antisense PKCλ plasmid into either MM14 cells (Figure 3.11D) or primary satellite cells (Figure 3.11E) where the MyoD reporter is normally robustly active (Figure 3.11D-F). These data indicate that PKCλ is required for MyoD transcriptional activity.

To further understand the role of PKCλ in myogenesis, we examined the effect of reduced PKCλ on differentiation. MM14 cells differentiate normally after the transfection of the sense PKCλ plasmid (Figure 3.12A, C, D) but transfection with the antisense PKCλ construct reduces differentiation as measured by fusion (Figure 3.12B, C) or myosin heavy chain expression (Figure 3.12B, D). To further assess the requirement for PKCλ in myogenic differentiation, we measured the activity of a muscle-specific reporter in MM14 cells and explanted satellite cells in the presence of the antisense PKCλ plasmid. We found that reduction or elimination of PKCλ dramatically inhibits muscle-specific reporter activity in MM14 cells (Figure 3.12E) and explants of primary satellite cells (Figure 3.12F), indicating that PKCλ plays a positive role in myogenic
**Figure 3.11: PKCλ is necessary for MyoD transcriptional activity.** Western blots detect specific PKCλ bands (A), but not PKCξ (B), in the absence of a blocking peptide in lysates from MM14 cells grown under proliferating (P) or differentiating (D) conditions. Cotransfection of a construct expressing an antisense PKCλ RNA a plasmid expressing βgal reduces or eliminates PKCλ expression in MM14 cells. Transfection of a plasmid expressing an antisense PKCλ RNA reduces MyoD transcriptional activity in MM14 cells (D) and primary satellite cells (E) co-transfected with the MyoD-Gal4/pFR-Luciferase reporter system when compared to transfection of either an empty vector or sense PKCλ plasmid. MM14 cells were either mock transfected, transfected with individual components of the MyoD-Gal4/pFR-Luciferase reporter system or both plasmids and the relative levels of luciferase activity measured indicating the Gal4 reporter system effectively measures MyoD-dependent transcription (F).
Figure 3.12: PKCλ expression and activity is required for myogenesis. Cotransfection of the antisense PKCλ plasmid reduces fusion (B, C) and myosin heavy chain expression (B, D) in MM14 cells while transfection with a sense PKCλ plasmid does not (A, C, D). Similarly, activity of a muscle-specific promoter (MSP) is reduced when MM14 cells (E) and primary cells (F) are transfected with the antisense PKCλ construct (AS) but not in the presence of a sense PKCλ (SE) or empty vector (Control). Muscle specific promoter activity is reduced in MM14 cells transfected with increasing amounts of a plasmid expressing a kinase inactive PKCλ (kiPKCλ) in a dose dependent manner (G).
differentiation. Increasing amounts of a plasmid expressing a kinase-inactive form of PKCλ reduced muscle-specific reporter activity confirming that the kinase activity of PKCλ is required for myogenesis (Figure 3.12G). A functional role for PKCλ in satellite cell myogenesis suggests that the asymmetric inheritance of PKCλ and the rest of the Par complex during division would result in asymmetric fates between daughter cells. The Par complex would promote myogenesis MyoD transcriptional activity and myogenesis in the daughter cell that received it during division. The other daughter cell would have reduced MyoD activity and possibly withdraw from the cell cycle to renew the quiescent satellite-SP cell population.

Discussion

The satellite-SP population divides only once in response to injury before withdrawing from the cell cycle. Interestingly, the quiescent, AraC-resistant cells are rarely found in doublets, despite the initial division. This suggests that the daughter cells generated by this single division in response to injury are not equivalent in their mitotoxin resistance and possibly not equivalent in their retention of the satellite-SP cell identity. To see if the satellite-SP cells divide asymmetrically, I looked at the distribution of the Par complex in satellite cells. The Par complex is involved in the asymmetric divisions of other stem cells and, if the satellite-SP cells do divide asymmetrically, they should display asymmetric distribution of the Par complex. The asymmetric localization of the Par complex during the first division after injury suggests that a subset of satellite cells do divide asymmetrically.

The member of the Par complex, PKCλ, is necessary for both MyoD transcriptional activity and for myogenesis. MyoD promotes its own transcription (Lassar et al., 1989) and the
reduced MyoD transcriptional activity may break a positive feedback loop result in the down
genregulation of MyoD by the satellite-SP cells. MyoD promotes the commitment of satellite cells to
myogenesis and satellite cells lacking MyoD show reduced proliferation and differentiation
(Montarras et al., 2000). Thus, a cell lacking the Par complex and PKCl would have reduced
MyoD, possibly resulting in reduced commitment, proliferation and differentiation. Therefore, if
the localization of the Par complex is restricted to only one daughter cell during division, I would
expect the daughter cells to differ in their commitment to myogenesis. The daughter cell that
inherits the Par complex would also inherit MyoD activity and progress through myogenesis
normally. The daughter cell that does not receive the Par complex or PKCl would be MyoD-,
would not progress through the cell cycle or differentiate, and would possibly enter quiescence
and renew the satellite-SP population (Figure 3.13).

Niche interaction is often necessary for the polarization of the Par complex and I
expected that culturing satellite cells in the absence of the myofiber would abolish asymmetry. I
was surprised to find that, while adherent cells divided symmetrically, non-adherent cells
frequently asymmetrically localized Par-3 to sites of cell-cell interaction or interaction with
pieces of fiber in non-adherent mitotic cells. Satellite cells that are slow to adhere in culture
possess a greater ability to survive and engraft when transplanted into host muscle (Qu-Petersen
et al., 2002). This similarity with the satellite-SP population suggests that satellite-SP cells and
the non-adherent cells comprise the same population. It is possible that quiescent satellite-SP
cells form stronger junctions with the myofiber or other satellite cells and hence, are slow to
release and bind to the culture dish. However, this does not rule out the possibility that prolonged
contact with a severely damaged piece of fiber or another cell in suspension simultaneously
Figure 3.13: Model of self-renewal of satellite-SP cells. A model depicting satellite-SP cell self-renewal. Satellite-SP cells divide asymmetrically in response to injury where one daughter cell receives the Par complex and the other does not. The daughter cell that receives the Par complex becomes a proliferative myoblast while the cell that does not receive the Par complex becomes quiescent and retains the satellite-SP cell identity.
delays attachment and promotes asymmetric division in any satellite cell.

How the Par complex achieves its asymmetric distribution and by what mechanism it regulates satellite cell fate is not clear. In other cells, the Par complex asymmetrically regulates Notch signaling through the localization of the Notch antagonist, Numb. As Notch signaling has an established role in promoting satellite cell proliferation (Conboy et al., 2003) and could be a potential mechanism by which the Par complex promotes the asymmetric division of satellite-SP cells.

**Experimental procedures**

**Mice**

Mice were housed in a pathogen-free facility and all procedures and protocols were approved by the Institutional Animal Care and Use Committee at the University of Colorado. Mice were female B6D2F1 (The Jackson Laboratory) between 3 and 5 months of age.

**Cell Culture**

Primary satellite cells and myofibers were prepared as previously described (Cornelison et al., 2004). Primary satellite cells and MM14 cells were cultured on gelatin coated plates and myofibers were cultured unattached to the plate. Cells were cultured under growth conditions (primary cells: F12-C + 15% horse serum + 20 ng/ml bFGF; MM14 cells: F12-C + 15% horse serum + 0.3 to 2.5 nM bFGF depending on concentration every 12h) or differentiation conditions (MM14 cells: F12-C + 5% horse serum) at 37°C under 5% CO₂ and 6% O₂. Media was changed daily and unattached primary cells were centrifuged and added back to the culture dish. Unless stated otherwise, all pictures are of myofiber-associated cells 36 hours after injury. Blebbistatin
was added at a final concentration of 80 µM. Unattached cells were centrifuged onto slides at 200 rpms and fixed.

**Immunofluorescence**

Immunofluorescence was performed as previously described (Tanaka et al., 2009). Primary antibodies and dilutions were: mouse monoclonal Pax7 (Developmental Hybridoma Bank at Iowa University) at 1:5, rabbit polyclonal MyoD (C-20, Santa Cruz Biotechnology) at 1:800, chicken anti-Syndecan-4 at 1:1500, rat monoclonal anti-pH3 (Sigma Aldrich) at 1:500, rabbit polyclonal anti-pH3 (Millipore) at 1:250, rabbit polyclonal anti-PKCl (Santa Cruz Biotechnology) at 1:50, rabbit polyclonal anti Par-3 (Upstate) at 1:250, rabbit polyclonal anti-β-galactosidase (Sigma Aldrich) at 1:250, mouse monoclonal anti-myosin heavy chain (MF20, (Bader et al., 1982)), chicken polyclonal anti-β-galactosidase antibody (Abcam) at 1:500, mouse monoclonal Sca1 (BD Pharmingen) at 1:50, mouse monoclonal γ-tubulin (Sigma) at 1:100, and mouse monoclonal Cdc42 (Molecular Probes) at 1:50. Secondary antibodies (Invitrogen) conjugated with Alexa Fluor 488, Alexa Fluor 546, Alexa Fluor 555, Alexa Fluor 594 and Alexa Fluor 647 were used at 1:1000 dilution. Myofiber-associated cells were scored by either cells per fiber or cells per millimeter of fiber length. Nuclear levels of MyoD fluorescence were measured in SlideBook 4.0 by subtracting the average cytoplasmic intensity (determined by Syndecan-4) from the average nuclear intensity (determined by DAPI). Doublets containing one daughter cell with negative nuclear staining and one daughter cell with staining one standard deviation greater than the negative cell were scored as asymmetric. Single cells containing two centrisomes marked by γ-tubulin were scored as pre-mitotic, cells with condensed DNA, as visualized with
DAPI, or positive for phospho-Histone 3 were scored as mitotic and pairs of cells in contact, each containing a single centrosome were called post-mitotic and scored as one. Any cell with in 1 cell diameter of 2 or more cells was scored as a ‘3+’ group of cells. Fusion was scored by number of nuclei in myotubes. Background fluorescence was set to the secondary only control or fluorescence of the myofiber. Confocal images were displayed as 3D reconstructions or slices through a 3D reconstruction generated in Volocity. Blue represents DAPI staining unless stated otherwise.

*Arrays*

Arrays were generated for the injury time course as previously described (Pisconti et al., 2010).

*Transfection*

Expression vectors encoding sense and antisense PKCα fragments are described elsewhere (Kampfer et al., 1998; Bandyopadhyay et al., 1999). DNA was transiently transfected into MM14 cells by a calcium phosphate-DNA precipitate method as described previously (Kudla et al., 1995). Primary satellite cells and myofibers were transfected either by calcium phosphate-DNA precipitate method or using Lipofectamine Plus (Invitrogen, Carlsbad, CA) according to the manufacturers protocol.
Muscle-Specific Promoter Assay

A differentiation-sensitive muscle-specific reporter activity assay was used as described previously with MSP denoting either a human α-cardiac actin promoter or troponin-I promoter (Kudla et al., 1995).

MyoD Activity Assay

A Gal4-reporter, pFR-Luc vector (2.5 µg), and MyoD-Gal4 (500ng) activator construct were co-transfected along with a CMV-LacZ (1 µg) vector into either MM14 cells or primary satellite cells as described above and extracts assayed as described previously (Fedorov et al., 1998).

Western Blotting

Whole cells lysates (10-20 µg) were resolved on polyacrylamide-SDS gels and transferred to Immobilon-P membrane as previously described (Fedorov et al., 2002). Proteins were detected using enhanced chemiluminescence (ECL), using antibodies directed against PKCλ and PKCζ (1:1000 dilution) purchased from Santa Cruz Biologicals. The blocking peptide was also purchased from Santa Cruz Biologicals and used at a 10-fold excess by weight according to the manufacturers’ instructions.
Chapter 4: The Par Complex

Regulates Activation Through The

Asymmetric Phosphorylation Of

p38α/β MAPK
**Introduction**

The asymmetric divisions of stem cells produce one committed daughter cell and one daughter cell that retains the stem cell identity (Knoblich, 2008). The Par complex establishes asymmetry in dividing stem cells and differentially regulates signaling between the two subsequent daughter cells. In many stem cells, the Par complex establishes asymmetric Notch signaling by restricting the localization of the Notch antagonist, Numb, to the opposite daughter cell. Numb then prevents the activation of Notch in one daughter cell while Notch signaling is uninhibited in the other daughter cell (Knoblich, 2008).

Satellite-SP cells are a subpopulation of satellite cells that divide asymmetrically in response to injury. During this asymmetric division, the Par complex is asymmetrically distributed and promotes commitment in one daughter cell, resulting in a proliferative, MyoD+ daughter cell and a quiescent, MyoD- daughter cell (see chapter 3). However, it is not clear how the Par complex manipulates satellite cell signaling to regulate activation and quiescence.

The transition of a quiescent MyoD- satellite cell to a MyoD+ myoblast involves multiple signaling pathways. The earliest detectable marker of satellite cell activation is the phosphorylation of p38α/β MAPK, which happens only minutes after muscle injury (Jones et al., 2005). Blocking the kinase activity of p38α/β MAPK prevents MyoD expression, proliferation and differentiation maintaining satellite cells in a quiescent like state (Jones et al., 2005). Therefore, it is likely that any daughter cell withdrawing from the cell cycle would have to down regulate p38α/β MAPK activity.

Pax7 is a transcription factor that is expressed in quiescent and proliferating satellite cells (Olguin et al., 2007). In active satellite cells, Pax7 negatively regulates differentiation by
promoting the degradation of MyoD (Olguin et al., 2007). Additionally, ectopic over-expression of Pax7 results in a complete loss of MyoD expression and induces satellite cell quiescence (Olguin et al., 2007). The ability of high levels of Pax7 to promote quiescence makes the regulation of Pax7 expression a potential mechanism to asymmetrically regulate MyoD after asymmetric division.

Notch signaling is another likely target of the Par complex as Notch is a demonstrated target of the Par complex and has an established role in myogenesis (Knoblich, 2008). In satellite cells, Notch promotes activation by negatively regulating the transcription of CDK inhibitors (Conboy et al., 2003; Carlson et al., 2008) and prevents differentiation by negatively regulating MyoD transcription and transcriptional activity (Buas et. al., 2010). Thus, Notch signaling appears to promote an active, proliferative state in satellite cells and its asymmetric regulation by the Par complex could potentially yield one proliferative and one mitotically quiescent daughter cell.

To understand how the asymmetric inheritance of the Par complex can regulate quiescence, I examined the roles of Pax7, Notch and p38α/β MAPK in the asymmetric division of satellite cells. While I found that high Pax7 expression is not associated with the asymmetric down regulation of MyoD, both p38α/β MAPK and Numb appear to be asymmetrically regulated in satellite cells. The Par complex binds p38α/β MAPK in dividing satellite cells and promotes its phosphorylation. Numb is expressed exclusively in satellite-SP cells and p38α/β MAPK is not active in Numb+ cells. These results lead me to propose that the Par complex directs Numb to one daughter cell and activates p38α/β MAPK in the other. The daughter cell that inherits Numb has neither Notch or p38α/β MAPK activity and withdraws from the cell cycle. In the other
daughter cell, Notch and p38α/β MAPK activity promote activation, proliferation and myogenic commitment, producing a committed myoblast.

Results

MyoD Is Not Asymmetrically Regulated By Increased Pax7 Expression

Satellite cells divide asymmetrically during the first division, generating a MyoD+ daughter cell and a daughter cell that loses MyoD expression. Pax7 promotes the degradation of MyoD and over-expressing Pax7 in satellite cells results in loss of MyoD and subsequent mitotic quiescence (Olguin et al., 2007). Thus, up regulating levels of Pax7 protein is a potential way for a satellite cell to down regulate MyoD and exit the cell cycle. If Pax7 is responsible for the asymmetric MyoD expressed between daughter cells, I would expect to find doublets containing one Pax7\textsuperscript{high}/MyoD- daughter cell and one Pax7+/MyoD+ daughter cell.

I examined doublets for asymmetric Pax7 distribution after the first division in response to injury. No doublets are asymmetric for Pax7 (Figure 4.1A, B) if I score using the same criteria I used to score asymmetric MyoD. However, Pax7 is expressed in both quiescent and active satellite cells and I would not expect Pax7 to be lost from either daughter cell after an asymmetric division. I instead examined doublets where one daughter cell displayed the high Pax7 expression (1 standard deviation above average) that could potentially result in the degradation of MyoD. Contrary to my prediction, the majority of doublets with one Pax7\textsuperscript{high} cell displayed above average Pax7 levels in the other daughter cell (Figure 4.1B, red). Similarly, cells with no detectable Pax7 are in doublets with Pax7\textsuperscript{low} cells (Figure 4.1B, blue). I then specifically scored doublets with asymmetric MyoD expression. In asymmetric doublets (Figure 4.1D,
**Figure 4.1: Pax7 is not asymmetrically expressed in doublets.** No satellite cell doublets asymmetrically express Pax7 on myofibers culture for 48 hours (A, B). In doublets where one cell is negative for Pax7 expression (B, blue circles), the other cell has low Pax7. In doublets where one daughter cell expresses Pax7 at one standard deviation above average or higher (B, red squares) the other daughter also displays high Pax7. Looking specifically in doublets with asymmetric MyoD (D, colored circles), high Pax7 expression does not correlate with absent MyoD (C, D). Instead, satellite cells maintain a positive correlation between Pax7 and MyoD levels (D).
asymmetric doublets represented by same color), Pax7 is either equivalently between the daughter cells or has increased expression in the MyoD+ daughter cell (Figure 4.1C, D). While these results do not disprove a role for Pax7 in the down regulation of MyoD after division, high Pax7 expression does not correlate with asymmetrically down regulated MyoD.

Rather than the expected negative correlation between Pax7 and MyoD levels, satellite cells display a positive relationship between Pax7 and MyoD (Figure 4.1D). The correlation between Pax7 and MyoD is statistically significant and maintained by satellite cells in mass culture after both 2 and 4 days in growth conditions (Figure 4.2A, B; growth conditions are FGF + 15% horse serum; p-value: day 2 <0.0001, day 4 <0.001). However, the balance between Pax7 and MyoD is lost when cells are grown under differentiating conditions (Figure 4.2 A; no FGF, 10% serum) as cells display a similar range of Pax7 levels (Figure 4.2A, D) but MyoD levels are increased an average of 9 fold irrespective of Pax7 expression at day 2 (Figure 4.2A, E). The MyoD levels in proliferative cells reach similar levels at day 4, but by then, cells in differentiation conditions have already down regulated Pax7 and MyoD and begun to differentiate (Figure 4.2B, D, E). Importantly, despite the differences in both MyoD and Pax7 expression between proliferating and differentiating cells at day 4, a similar Pax7+/MyoDlow/-, AraC-resistant population is still present in either condition (Figure 4.2C, D, E). Under differentiation conditions, an additional, differentiating Pax7-/MyoD+ population is detected as well (Figure 4.2C, D, E) (Olguin et al., 2007).
Figure 4.2: A subset of satellite cells resist AraC under both growth and differentiation conditions. Under growth conditions, satellite cells maintain a positive correlation between Pax7 and MyoD levels at day 2 (A) or 4 (B) in culture. However, this correlation is lost in cells grown in differentiating conditions (A, B). However, an AraC resistant, Pax7+/MyoD\textsuperscript{low/-} populations survives under both conditions (C) while differentiation conditions produce a second Pax7-/MyoD+ AraC-resistant population committed to differentiation (C). Both Pax7 (D) and MyoD (E) levels increase in cells grown in growth conditions from day 2 to 4 as cells remain proliferative. Pax7 and MyoD are down regulated by day 4 under differentiation conditions as cell commit to differentiation (D, E).
Satellite-SP Cells Display Asymmetric Numb

Canonically, the Par complex regulates Notch signaling in asymmetric division by directing the localization of Numb to the opposite daughter cell (Knoblich, 2008). As Notch has a documented roll in myogenesis (Luo et al., 2005), I asked if Numb was asymmetric in satellite-SP cells on myofibers. Numb is absent all in non-SP satellite cells (Figure 4.3A, B) but a subset of Sca1+, satellite-SP cells display Numb localized asymmetrically to one side of the cell (Figure 4.3C, D). Numb expression is up regulated during asymmetric division by the mitotic kinases, Aurora A and Polo (Knoblich, 2008) and it is possible that cell cycle specific regulation of Numb reduces the number of Numb positive cells I observe. Indeed, I observe brighter and more frequent Numb expression in satellite-SP cells paused in cytokinesis by Blebbistatin. The exclusive expression of Numb in Sca1+ cells implies that Numb regulates the return to quiescence of satellite-SP cells.

Notch Is Expressed And Regulated In Satellite Cell Clusters

In satellite cells, Notch activity promotes both activation and inhibits differentiation (Kondoh et al., 2007; Conboy et al., 2003; Carlson et al., 2008). I found that the majority of satellite cells express the Notch-1 at 36 hours (Figure 4.4A-D) as previously reported (Conboy and Rando, 2002). While Notch-1 localization was uniform in individual satellite cells (Figure 4.4A, B) heterogeneous Notch-1 expression was frequently observed in the clusters of cells containing satellite-SP cells (Figure 4.4C, D). Notch is activated by membrane bound ligands through cell contact with adjacent cells (Buas and Kadesch, 2010) and it is possible the cell interactions between these clustered cells regulate Notch activation.
Figure 4.3: Numb is asymmetric in satellite-SP cells. I do not observe Numb expressed in Sca1- satellite cells on myofibers cultured for 36 hours (A, B). However, Numb is expressed asymmetric in Sca1+ cells, indicating that its expression is exclusive to satellite-SP cells (C, D). Pausing cells in cytokinesis with Blebbistatin increases the frequency with which I observe Numb in satellite-SP cells suggesting that its regulation might be cell cycle specific (E).
Figure 4.4: Notch is expressed in both individual cells and Sca1+ cell containing clusters. Notch can be detected in both individual satellite cells (A, B) and in satellite cell clusters (C, D) on myofibers cultured for 36 hours.
Visualization of Notch protein does not necessarily indicate its activation, as the antibody I used labeled both active and inactive Notch. To understand the role of cell contact in Notch activation and to see if I could detect asymmetric Notch activity between daughter cells, I isolated muscle fibers from a Notch EGFP transcriptional reporter mouse (Duncan et al., 2005). At day 1 or day 2, I could detect no EGFP expression above background in satellite cells (Figure 4.5A-F). These data are contrary to previous reports that Notch is activated by 48 hours in culture (Conboy and Rando, 2002). It is possible that due to the high fluorescent background of the myofiber, I could not detect low levels of Notch activity. At days 3 and 4, while there is still no detectable Notch activity in individual satellite cells, EGFP is expressed in a subset of the cells in clusters (Figure 4.5G-L). I had previously noted that these satellite cell clusters display heterogeneous expression of Sca1 (Figure 4.4C, D; Figure 3.2H), Pax7 (Figure 4.6), MyoD (Figure 4.6) and Myogenin (Figure 2.4C) so I looked to see if Notch activity was restricted to a subset of these cells. In the majority of cases, EGFP fluorescence is limited to the proliferative Pax7+/MyoD+ cells in the cluster (Figure 4.6A-C) or the differentiating Pax7-/MyoD+ cells (Figure 4.6B). EGFP was low or absent in Pax7+/MyoD- cells and thus, it is likely that Notch signaling primarily promotes myogenesis in these clusters. Notch activity in differentiating Pax7-/MyoD+ cells contradicts previous reports that Notch signaling blocks differentiation (Conboy and Rando, 2002). However, due to the long half-life of EGFP (>24 hours), EGFP expression may not reflect current Notch activity but rather in cells from previous Notch activity. Additionally, there are multiple isoforms of Notch and, combined with the variety of ligands, binding partners and covalent modification that control Notch activation, this could allow Notch to regulate multiple pathways.
Figure 4.5: Notch is transcriptionally active in satellite cell clusters. EGFP expressed by a Notch transcriptional reporter is not detectable at 1 day (A-C) or 2 days (D-F) after injury. However, heterogenous EGFP is detectable in some satellite cell clusters at day 3 (G-I) and day 4 (J-L) suggesting cell-cell interaction is important for Notch activation.
Figure 4.6: Notch activity correlates with MyoD expression. A Notch transcriptional reporter expresses EGFP preferentially in MyoD positive cells in satellite cell clusters at day 4 (A-C).
Phosphorylation of p38α/β MAPK is necessary for satellite cell activation and blocking p38α/β MAPK kinase activity prevents MyoD expression, proliferation and differentiation (Jones et al., 2005). If satellite-SP cells become truly quiescent after an asymmetric division, I would expect to see asymmetric p38α/β MAPK activity in doublets. The expression and distribution of p38α/β MAPK is uniform in both non-dividing (Figure 4.7A) and mitotic cells (Figure 4.7A-C). However, the active, phosphorylated form of p38α/β MAPK is often asymmetric in Sca1+ (Figure 4.8A-D) and Sca1- cells (Figure 4.8E-H). Activation of p38α/β MAPK is involved in migration and cytoskeletal organization (Huang et al., 2004) and asymmetric phospho-p38α/β MAPK in non-mitotic cells could indicate either of these processes. Indeed, phospho-p38α/β MAPK is not asymmetric in dividing non-SP satellite cells (Figure 4.9A, B) or in non-SP satellite cell doublets (Figure 4.9C, D). However, in satellite-SP cell doublets, I often observed asymmetric distribution of phospho-p38α/β MAPK (Figure 4.9E-H) supporting the conclusion that one daughter cell is activated while the other is quiescent.

Regulating the phosphorylation of p38α/β MAPK to one daughter cell would be an effective way to regulate quiescence and activation. However, the Par complex has not been reported to regulate p38α/β MAPK activity. To see if p38α/β MAPK is asymmetrically regulated during division or if it is differentially activated in daughter cells after division, I examined phospho-p38α/β MAPK during the first division. While the majority of satellite cells phosphorylate p38α/β MAPK evenly during division (Figure 4.10A) p38α/β MAPK is
Figure 4.7: \(p38\alpha/\beta\) MAPK is symmetrically distributed in satellite cells. \(p38\alpha/\beta\) MAPK is distributed uniformly in mitotic (A-C) and non-dividing (A) satellite cells on myofibers cultured for 36 hours.
Figure 4.8: Phosphorylated p38α/β MAPK is asymmetric in non-mitotic satellite cells. Phosphorylated p38α/β MAPK (phospho-p38α/β MAPK) is asymmetric in both Sca1+ (A-D) and Sca1- (E-H) satellite cells on myofibers cultured for 36 hours.
Figure 4.9: Phosphorylated p38α/β MAPK is asymmetric in Sca1+ doublets. Phospho-p38α/β MAPK is symmetric in dividing Sca1- cells (A, B) and in Sca1- doublets (C, D). However, phospho-p38α/β MAPK is asymmetrically distributed in Sca1+ doublets on myofiber cultured for 36 hours (E-H).
Figure 4.10: Phosphorylated p38α/β MAPK is asymmetric in a subset of dividing cells. p38α/β MAPK is uniformly phosphorylated in the majority of dividing cells (A) but asymmetric in a subset (B). Asymmetric phospho-p38α/β MAPK colocalizes with Par complex members Par-3 (C, D) and PKCλ (E, F).
asymmetrically active in a subset of dividing satellite cells (Figure 4.10B). Additionally, asymmetric phospho-p38α/β MAPK colocalizes with Syndecan-4 (Figure 4.10B-F), Par-3 (Figure 4.10C, D) and PKCλ (Figure 4.10E, F) indicating that signaling from the Par complex may directly regulate p38α/β MAPK activation in asymmetrically dividing cells.

Par-3 Forms An Asymmetric Complex With Phospho-p38α/β MAPK

To determine if the colocalization of Par-3 and phospho-p38α/β MAPK truly indicates that the Par complex regulates p38α/β MAPK, we tested whether Par-3 and PKCλ form a complex with p38α/β MAPK. p38α MAPK and an HA-tagged PKCλ do interact when both are ectopically over-expressed in 10T1/2 cells. Immunoprecipitation with an HA-tag antibody isolates p38α MAPK as shown by Western blot (Figure 4.11A). However, we were unable to demonstrate that PKCλ interacts with endogenous p38α/β MAPK in 10T1/2 cells (Figure 4.11A), C2C12 cells (data not shown, Tyner, unpublished data) or MM14 cells (data not shown, Tyner, unpublished data). To determine if endogenous Par-3 and p38α/β MAPK complex in dividing satellite cells, I utilized a novel proximity ligation assay (PLA, DuoLink) (Pisconti et al., 2010). In the presence of the anti-phospho-p38α/β antibody and the anti-Par-3 antibody, I observed abundant complexes in mitotic satellite cell explants (Figure 4.11B, C) that were asymmetric and localized to the cell membrane adhering to the substratum (Figure 4.11D-G). Although asymmetric, these complexes may differ from those observed on myofibers as these cells were dispersed in tissue culture plates (Figure 4.11B-G). Complexes were not observed if the anti-Par-3 antibody was omitted (Figure 4.11B, H). These observations and data demonstrating that PKCλ is required for MyoD-dependent gene transcription support the idea that the Par complex
Figure 4.11: Par-3 and PKCλ complex with p38α/β MAPK. Coimmunoprecipitation shows that p38α/β MAPK and PKCλ bind when p38α MAPK and an HA-tagged PKCλ are over-expressed in 10T1/2 cells (A). Endogenous phospho-p38α/β MAPK and Par-3 form multiple complexes in a subset of dividing satellite cells as demonstrated by proximity ligation assay where green dots represent protein interactions (B, C). Phosphorylated histone 3 (pH3) labels mitotic cells and βgal indicates Pax7 expression in Pax7 LacZ/+ cells. Slices through a 3D reconstruction of a cell show that the majority of the complexes asymmetrically localize to the substratum and edges of the cell (D-G). Incubation with only the phospho-p38α/β MAPK antibody results in no dots (B, H). PKCλ is necessary of p38α/β MAPK activity as measured by a Chop reporter (I). Control, sense PKCλ (SE) or antisense PKCλ (AS) plasmids were cotransfected with Chop reporter assay which measures p38α/β MAPK kinase activity.
is involved in p38α/β MAPK signaling. To provide better support for this hypothesis, we utilized a CHOP reporter that provides a direct readout of p38α/β MAPK signaling (Aguirre-Ghiso et al., 2003; Jones et al., 2005). In the presence of ectopically expressed antisense PKCλ but not a sense control, CHOP reporter activity was reduced by 3-fold, indicating that PKCλ and the Par complex regulate p38α/β MAPK signaling (Figure 4.11I). The colocalization of phospho-p38α/β MAPK with asymmetric members of the Par complex during division, the demonstration that endogenous Par-3 and p38α/β MAPK complex in satellite cells and the requirement of PKCλ for p38α/β MAPK activity in myoblasts suggests that the Par complex can regulate p38α/β MAPK activation during the asymmetric division of satellite-SP cells.

*p38α/β MAPK Activity Regulates Satellite-SP Cell Identity*

If the Par complex controls satellite-SP cell self-renewal through p38α/β MAPK activation, it is possible that inhibiting p38α/β MAPK activity after the first division would result in both daughters becoming quiescent and an increased number of satellite-SP cells. Treating myofibers from a Sca1:GFP+/- mouse with the p38 inhibitor, SB203580, from 48 to 60 hours after injury increased the percentage of GFP+ cells by 3 fold when compared to the DMSO control (Figure 4.12A). The increase in Sca1+ cells was primarily due to increased numbers of GFP+ cells in satellite cell clusters and had little impact on the number of individual satellite-SP cells (Figure 4.12B, C). Interestingly, treatments from 60 to 72 hours after injury, during later rounds of proliferation, had no detectable effect. This result suggests that regulation of p38α/β MAPK plays a role in regulating the satellite-SP identity immediately following the first division after injury.
Figure 4.12: A p38α/β MAPK inhibitor after the first division increases the percentage of Sca1+ cells. An increase in the percentage of Sca1+ satellite cells is seen on myofibers treated with the p38α/β MAPK inhibitor, SB203580, from 48 to 60 hours (A). Increase in Sca1% is primarily due to expansion of Sca1+ cells in clusters (B, C).
The colocalization of p38α/β MAPK and Par-3 suggests that a daughter cell receiving the Par complex also activates p38α/β MAPK while the other daughter would not. In other stem cells, Par complex asymmetrically regulates Notch by directing Numb to the opposite daughter cell. Thus, I would predict that any daughter cell receiving Numb would not activate p38α/β MAPK. Indeed, I observe no phospho-p38α/β MAPK in Numb+ satellite cells (Figure 4.13A-C) supporting a model where Numb is inherited by one daughter cell and p38α/β MAPK activated in the other.

Par-3 Forms An Asymmetric Complex With Tiam1 In Mitotic Satellite Cells

The regulation of p38α/β MAPK by the Par complex has not been previously demonstrated. To understand what genes could be involved in p38α/β MAPK activation during asymmetric division, I assembled a putative signaling network linking the Par complex and p38α MAPK using Ingenuity Pathway analysis and literature searches (Table 4.1). To find genes specifically involved in the asymmetric phosphorylation of p38α/β MAPK in satellite-SP cells, I looked for differential gene expression between non-SP satellite cells and satellite-SP cells. Cells from uninjured muscle were sorted for Syndecan-4 or Syndecan-3 (Cornelison et al., 2001) expression to isolate satellite cells (Figure 4.14A, Kathleen Tanaka). These cells were then sorted into satellite-SP cells (Figure 4.14B, Kathleen Tanaka, ABCG2+/Sca1+) and non-SP satellite cells (Figure 4.14B, Kathleen Tanaka, ABCG2-/Sca1-). RNA was purified from each population of cells and analyzed by Affymetrix array. To visualize satellite cell gene expression, I overlaid the non-SP satellite cell expression data onto the putative signaling network (Figure 4.14C) where darker coloring indicates higher expression.
Figure 4.13: p38α/β MAPK is not phosphorylated in Numb+ cells. Phospho-p38α/β MAPK is absent in Numb+ satellite cells on myofibers cultured for 36 hours (A-C).
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**Table 4.1: A putative Par complex/p38α MAPK signaling network.** A putative signaling network linking the Par complex to p38α MAPK activation was assembled by Ingenuity Pathway Analysis and a literature search. Interactions between proteins and references are shown in the table.
Figure 4.14: Tiam1 and Ccm2 are preferentially expressed in satellite-SP cells. Satellite-SP cells (Syndecan-4+ or Syndecan-3+, ABCG2+/Sca1+) and non-SP satellite cells (Syndecan-4+ or Syndecan-3+, ABCG2-/Sca1-) were isolated via FACS (A, B). Gene expression values for non-SP satellite cells were overlaid on a putative signaling network linking the Par complex to p38α MAPK activation where darker coloring indicates higher expression (C, log10), demonstrating the high expression of the small GTPases, Rac1 and Cdc42, p38α MAPK and kinases upstream of p38α MAPK. Differential expression between non-SP and satellite-SP cells overlaid on the putative Par/p38α MAPK network, where intensity of green coloring reflects increased expression in satellite-SP cells and red coloring reflects increased expression in non-SP satellite cells, shows that Tiam1 and Ccm2 are preferentially expressed in satellite-SP cells (D).
As expected, transcripts of the small GTPases, Rac1 and Cdc42, which promote myoblast proliferation and differentiation, as well as p38α MAPK (Mapk14) and its upstream kinases, are all expressed highly in non-SP satellite cells (Figure 4.14, C; Table 4.2). To understand which of these genes could be involved in the regulation of p38α/β MAPK by SP satellite cells, I overlaid the differential expression between non-SP satellite cells and satellite-SP cells onto the signaling network where the intensity of green coloring indicates increased expression in satellite-SP cells and red indicates increased expression in non-SP satellite cells (Figure 4.14D; Table 4.2). As expected, p38α MAPK (Mapk14) and its kinases MKK4 (Map2k4) and MKK6 (Map2k6) are upregulated in non-SP satellite cells (Figure 4.14D, Table 4.2). In satellite-SP cells, the Rac1 GEF, T-cell lymphoma invasion and metastasis 1 (Tiam1) (van Leeuwen et al., 1995), and scaffold protein, Cerebral Cavernous Malformation 2 (Ccm2) (Uhlik et al., 2003) display increased expression (Figure 4.14D, Table 4.2), implicating these genes in the asymmetric activation of p38α/β MAPK in satellite-SP cells.

Tiam1 binds the Par complex and regulates Rac1 signaling and cellular polarity (Mertens et al., 2006). To confirm that Tiam1 binds Par-3 in satellite cells and, thus, could regulate the asymmetric activation of p38α/β MAPK, I used the PLA assay. In the presence of antibodies to phospho-p38α/β MAPK and Par-3, I detect numerous complexes in a subset of cells (Figure 4.15A, B) that asymmetrically localize to the substratum and the edges of the dividing cells (Figure 4.15C-F) and do not appear in the control containing the anti-Par-3 antibody alone (Figure 4.15G). Therefore, Tiam1 could regulate the Par-3 complex’s local activation of p38α MAPK. In an asymmetrically dividing satellite cell, the Par complex would asymmetrically activate p38α MAPK through Tiam1-mediated regulation of Rac1 signaling. A daughter cell
Figure 4.15: Tiam1 and Par-3 form a complex in dividing satellite cells. Endogenous Tiam1 and Par-3 form multiple complexes in a subset of dividing satellite cells as demonstrated by proximity ligation assay where green dots represent protein interactions (A, B). Phosphorylated histone 3 (pH3) marks mitotic cells and β-gal indicates Pax7 expression in Pax7^{LacZ/+} cells. Slices through a 3D reconstruction of a cell show that the majority of the complexes asymmetrically localize to the substratum and edges of the cell (C-F). Incubation with only the phospho-p38α/β MAPK antibody results in no detected interactions (A, G).
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**Table 4.2: Differential gene expression between satellite-SP cells and non-SP satellite cells.**

Affymetrix array data taken from satellite-SP cells and non-SP satellite cells was cross referenced with putative Par complex/p38α MAPK signaling network. Average expression (log\(_{10}\)) levels for each gene in the pathway are shown for non-SP satellite cells and the fold increase in satellite-SP cells compared to non-SP satellite cells and p-values are shown.
receiving the Par complex would sequester and activate p38α MAPK inducing MyoD expression and commit to the muscle lineage as a proliferating myoblast. The sister cell would reacquire quiescence, and lack MyoD protein, generating a satellite cell and maintaining the satellite SP cell population (Figure 4.16).

**Discussion**

The regulation of Notch signaling by the Par complex has been reported in many cell types (Knoblich, 2008) and, as Notch promotes proliferation in satellite cells (Conboy et al., 2003), it was not surprising to find asymmetric Numb in satellite-SP cells. Directing Numb to a daughter cell would block Notch signaling and promote cell cycle exit (Carlson et al., 2008). Notch appears to be additionally regulated by cell contact between satellite cells. The activation of Notch in only the MyoD+ cells suggests that Notch signaling might facilitate communication between cells to pattern the proliferation and differentiation in a cluster of satellite cells. Thus, satellite cells could ensure the optimal balance between the proliferation and differentiation of myoblasts.

The Par complex has not been reported to regulate p38α/β MAPK. As p38α/β MAPK is necessary for MyoD expression and proliferation, I anticipated the asymmetric activation of p38α/β MAPK in satellite-SP cell doublets. I was surprised to find, however, that p38α/β MAPK is directly regulated by the Par complex during asymmetric division. Tiam1 has been shown to be recruited to cell-junctions by Par-3 where Tiam1 activates Rac1 signaling and regulates migration (Pegtel et al., 2007) and cytoskeletal organization (Nishimura et al., 2005). p38 is a downstream target of Tiam1/Rac1 signaling (Buchsbaum et al., 2002), and recruitment of Tiam1,
Figure 4.16: A model for the asymmetric division of satellite-SP cells. A model where the Par complex is asymmetric in dividing satellite-SP cells. The Par complex promotes p38α/β MAPK activation in one daughter cell and regulates Numb to the other. Notch and p38α/β MAPK are active in one daughter cell which commits to myogenesis. The other daughter cell receives Numb and does not receive active p38α/β MAPK, resulting in quiescence and retention of the satellite-SP cell identity.
and possibly the Tiam1/p38 signaling scaffold, Ccm2, the Par complex utilizes preexisting pathways to regulate satellite cell activation. Thus, linking the Par complex to p38α/β MAPK phosphorylation is an evolutionarily efficient way to regulate activation and quiescence in asymmetrically dividing satellite cells.

By inhibiting Notch signaling in one daughter cell and promoting p38α/β MAPK activation in the other, the Par complex regulates satellite cell fate through two separate signaling pathways. Both p38α/β MAPK and Notch promote satellite cell activation and proliferation (Conboy et al., 2003; Jones et al., 2005; Carlson et al., 2008) and positively regulating both of these pathways in one daughter cell would generate a proliferative myoblast (Figure 4.16). The other daughter cell would receive Numb and not activate p38α/β MAPK and would retain the satellite-SP cell identity and withdraw from the cell cycle.

**Experimental procedures**

*Mice*

Mice were housed in a pathogen-free facility and all procedures and protocols were approved by the Institutional Animal Care and Use Committee at the University of Colorado. Mice were female B6D2F1 (The Jackson Laboratory) between 3 and 5 months of age. Sca1:GFP (B6.Cg-Tg(Ly6a-EGFP)G5Dzk/J) (Ma et al., 2002) and Notch transcriptional reporter (Tg(Cp-EGFP)25Gaia/J) (Duncan et al., 2005) mice were obtained from Jackson Laboratory.
Cell Culture

Primary satellite cells and myofibers were prepared as previously described (Cornelison et al., 2004). Primary satellite cells and MM14 cells were cultured on gelatin coated plates and myofibers were cultured unattached to the plate. Cells were cultured under growth conditions (primary cells: F12-C + 15% horse serum + 20 ng/ml bFGF; MM14 cells: F12-C + 15% horse serum + 0.3 to 2.5 nM bFGF depending on concentration every 12h) or differentiation conditions (primary cells: F12-C + 10% horse serum) at 37°C under 5% CO₂ and 6% O₂. Media was changed daily and unattached primary cells were centrifuged and added back to the culture dish. Unless stated otherwise, all pictures are of myofiber-associated cells 36 hours after injury. The p38 inhibitor (SB203580) was suspended in DMSO and added a final concentration of 50 µM.

Immunofluorescence And Proximity Ligation Assay

Immunofluorescence was performed as previously described (Tanaka et al., 2009). Tissue was sectioned as previously described (Hall et al., 2010). Primary antibodies and dilutions: mouse monoclonal Pax7 (Developmental Hybridoma Bank at Iowa University) at 1:5, rabbit polyclonal MyoD (C-20, Santa Cruz Biotechnology) at 1:800, chicken anti-Syndecan-4 at 1:1500, rat monoclonal anti-pH3 (Sigma Aldrich) at 1:500, rabbit polyclonal anti-pH3 (Millipore) at 1:250, rabbit polyclonal anti-PKCλ (Santa Cruz Biotechnology) at 1:50, rabbit polyclonal anti Par-3 (Upstate) at 1:250, rabbit polyclonal p38 (C-20, Santa Cruz Biotechnology) at 1:50, mouse monoclonal anti-phospho-p38 (Cell Signaling) at 1:50, mouse monoclonal Sca1 (BD Pharmingen) at 1:50, hamster monoclonal Notch-1 (Upstate Biotechnology) at 1:50, rat monoclonal Laminin at 1:250, goat polyclonal Numb (Santa Cruz Biotechnology) at 1:50 and
rabbit polyclonal anti-Tiam1 (Santa Cruz Biotechnology) at 1:100. Secondary antibodies (Invitrogen) conjugated with Alexa Fluor 488, Alexa Fluor 546, Alexa Fluor 555, Alexa Fluor 594 and Alexa Fluor 647 were used at 1:1000 dilution. For proximity ligation assay (Olink), samples were incubated with antibody as described above. Duolink proximity ligation assay was performed according to manufacturer’s protocol. Numbers of primary satellite cells were normalized to untreated day 2 numbers. Unless stated otherwise, pictures are of myofiber-associated cells 36 hours after injury. Nuclear levels of Pax7 and MyoD fluorescence were measured in SlideBook 4.0 by subtracting the average cytoplasmic intensity (determined by Syndecan-4) from the average nuclear intensity (determined by DAPI). Doublets containing one daughter cell with negative nuclear staining and one daughter cell with staining one standard deviation greater than the negative cell were scored as asymmetric. Background fluorescence was set to the secondary only control or fluorescence of the myofiber. Confocal images were displayed as 3D reconstructions or slices through a 3D reconstruction generated in Volocity. Blue represents DAPI staining unless stated otherwise.

**Arrays**

For SP and non-SP profiles, primary skeletal muscle cells were isolated from 3-6 month old B6D2F1 female mouse hindlimb as previously described (Tanaka et al., 2009). Cells were directly collected into 100 µl XB reagent from the Arcturus Pico Pure RNA isolation kit. Following the Pico Pure isolation (Arcturus), RNA was amplified using WT-Ovation FFPE RNA Amplification System V2 (NuGEN) and labeled with FL-Ovation cDNA Biotin Module V2 (NGEN). 5 µg of fragmented Biotin labeled cDNA was hybridized to Affymetrix 430 v.2 mouse
microarrays at the University of Colorado core facilities and scanned on a GeneChip Scanner 3000 (Affymetrix) with intensity data recovery by GCOS (Affymetrix). Three replicate gene chip CEL files were imported into Spotfire (TIBCO) and normalized by GCRMA.

Expression map was generated using Ingenuity Pathway Analysis combined with literature search. To display expression levels on maps, the shape representing each gene was colored black and the opacity determined by the average log_{10} expression of the most highly represented probe set where the highest expressed gene was set to 100 and the lowest to 0. To display differential expression between satellite cells and satellite SP cells, genes with a probe set showing at least a 2 fold change in expression and a p-value less than 0.02 were colored green (increase in satellite-SP cells) or red (increase in non-SP satellite cells). Intensity of coloring reflects the difference in expression between the two cell populations.

Transfection

Expression vectors encoding sense and antisense PKC\(\lambda\) fragments are described elsewhere (Kampfer et al., 1998; Bandyopadhyay et al., 1999). DNA was transiently transfected into MM14 cells by a calcium phosphate-DNA precipitate method as described previously (Kudla et al., 1995).

p38 MAP Kinase Activity Assay

Pathdetect CHOP reporting system (Stratagene) was used to determine p38 kinase activity (Aguirre-Ghiso et al., 2003). For this assay, MM14 cells were plated on 6 well plates at a density of 1x10^4 cells/well and co-transfected with 2.5 µg pFR-Luc reporter vector, 500 ng pFA-
CHOP vector and 1 µg CMV-LacZ vector per well. The cells were harvested and assayed for luciferase and β-galactosidase activities as described previously (Fedorov et al., 1998).

**Immunoprecipitation**

10T1/2 cells were harvested 40 hours after transfection, sonicated and centrifuged for 15 minutes. Protein quantified using a Pierce micro BCA kit. For each immunoprecipitation, 320µg of protein was pre-cleared with 30µl of protein A sepharose (upstate) for 1 hour at 4C. Proteins were incubated with 2.5µl of either monoclonal anti-p38α/β (cell signaling) or rabbit polyclonal anti HA (HA.11) overnight at 4C and then for 1 hour at 4C with 30µl of proteinA sepharose beads. The proteins were washed, and then released from the beads by boiling in SDS-PAGE sample buffer. Proteins were then run on a 10% SDS-PAGE gel.
Chapter 5: Jam2 Generates Asymmetry And Regulates Quiescence Through Cell-Cell Interactions
**Introduction**

The formation of cell-cell junctions between a stem cell and its niche serves to regulate the identity and behavior of the stem cells (Fuchs et al., 2004). Additionally, the sites of these cell-cell interactions serve to sequester PDZ domain containing transmembrane proteins, creating asymmetry in the stem cell. The Par complex then binds to the PDZ domains of these proteins and orients the mitotic spindle and signaling molecules to produce an asymmetric division (Suzuki and Ohno, 2006). It is not known what proteins polarize the Par complex in satellite-SP cells or what role they play in satellite-SP cell identity and self-renewal.

Junction Adhesion Molecule 2 (Jam2) is a membrane protein that localizes to adherens junctions and recruits the Par complex through its intracellular PDZ domain. Affymetrix array data from satellite cells during the first division show that Jam2 is highly expressed in satellite cells and rapidly down regulated upon activation. I show that Jam2 protein is expressed in quiescent satellite-SP cells and colocalizes with Par-3. Furthermore, disrupting the interaction of Jam2 and Par-3 prevents the generation of quiescent cells after the first division, suggesting that Jam2 is instrumental in the asymmetric divisions of satellite-SP cells. Surprisingly, I find that the extracellular domain of Jam2 promotes quiescence in adjacent cells. I propose that Jam2 recruits Par-3 to cell-fiber or cell-cell junctions, creating asymmetry in dividing satellite-SP cells. During the asymmetric division, Jam2 is inherited with the Par complex by the committed daughter cell. Jam2 expressed in the committed daughter can then signal to the adjacent daughter cell, promoting quiescence and self-renewal.
Results

*Jam2 Expression Is Regulated In Response To Injury*

To determine what proteins the Par complex binds to establish asymmetry in satellite cells, I searched Affymetrix array data for PDZ domain containing transmembrane proteins that are highly regulated during the first division. Jam2 mRNA is highly expressed in quiescent satellite cells before rapid down regulation upon injury (Figure 5.1A). Quantitative PCR confirmed the high level of Jam2 transcript in quiescent cells and its rapid down regulation showing as well that Jam2 is re-expressed 2 days after injury (Figure 5.1B, Tiffany Antwine). Expression of Jam2 protein has a different pattern than its RNA as it is expressed in 33% of satellite cells at day 1 (Figure 5.1C, E-G), 70% at day 2 (Figure 5.1C, H-J) and nearly all myofiber-associated satellite cells by day 4 (Figure 5.1C, K-M). Moreover, the cells expressing Jam2 display a range of Pax7 and MyoD expression (Figure 5.1D), indicating that, while Jam2 expression may be limited to a subpopulation of satellite cells at early time points, it is expressed in the majority of cells 2 days after injury.

*Jam2 Colocalizes With Par-3 In Satellite Cells.*

If Jam2 is responsible for binding and polarizing Par-3, I expect to see Jam2 colocalize with members of the Par complex. When detected, Par-3 is only localized to regions of satellite cells expressing Jam2 (Figure 5.2A-C) on myofibers 36 hours after injury. I then used proximity ligation assays to determine if Jam2 and Par-3 form a complex in dividing satellite cells. The assay detected complexes in dividing cells when incubated with antibodies to Jam-2 and Par-3 (Figure 5.2D-G, with Adam Cadwallader) but not with the Par-3 antibody only (Figure 5.2D, H,
Figure 5.1: Jam2 expression is regulated during the first division. Affymetrix probe sets for Jam2 show high levels of transcript in quiescent satellite cells but low levels at all other time points (A). Quantitative PCR confirms this expression pattern showing additionally that Jam2 is expressed again at day 2 (B). Examination of Jam2 expression by immunofluorescence shows that increasing numbers of cells express Jam2 from day 1 (C, E-F) through day 2 (C, H-J) and day 4 (C, K-L). Jam2 is not preferentially expressed in Pax7+/MyoD-, Pax7+/MyoD+ or Pax7-/MyoD+ cells (D-M).
Figure 5.2: Jam2 forms a complex with Par-3. Labeling cultured myofibers with antibodies for Jam2 and Par-3 shows that Par-3 localization is restricted to portions of the cell expressing Jam2 at 36 hours (A-C). Proximity ligation assays show that Jam2 and Par-3 form a complex as indicated by the increase in the number of bright green foci when Pax7LacZ/+ cells are incubated with both Jam2 and Par-3 antibodies (D-G) rather than Par-3 antibody alone (H).
With Adam Cadwallader) indicating that Jam2 and Par-3 do interact in dividing satellite cells.

Jam2 colocalizes with the Par complex member, PKCλ, where PKCλ is in the cytoplasm cytoplasmic in dividing (Figure 5.3A) and non-dividing (Figure 5.3B-E) satellite cells at 36 hours. Interestingly, in non-dividing satellite cells with Jam2 expression, I frequently noted that PKCλ was localized to the nucleus. Phosphorylation of PKCλ on tyrosine 256 results in the exposure of a nuclear localization signal and import to the nucleus (White et al., 2002). While no kinase has been implicated in the phosphorylation of PKCλ at this site, it is possible that Jam2 facilitates interaction between PKCλ and its kinase, promoting the transport of PKCλ to the nucleus. Our lab has previously shown that PKCλ is localized in the nucleus during satellite cell proliferation and that the transfection of a Y256F PKCλ mutant that cannot localize to the nucleus blocks satellite cell differentiation (Tyner, unpublished data). The localization of PKCλ to the nucleus in Jam2 expressing cells indicates that Jam2 may mark cells committed to myogenesis. Recently divide cells with asymmetric Jam2 expression are also asymmetric in their localization of PKCλ (Figure 5.3E) supporting a role for Jam2 in asymmetric division.

Syndecan-4 Expression Can Used To Isolate Quiescent Satellite Cells

If Jam2 regulates asymmetric division in satellite cells, it should be present in satellite-SP cells before the first division. While quantitative PCR confirmed high levels of Jam2 RNA in quiescent satellite cells, I wanted to confirm that Jam2 protein is present in quiescent satellite-SP cells. I used fluorescence activated cell sorting (FACS) on cells from uninjured muscle to examine Jam2 expression and to isolate the cells that express Jam2. Preparation of satellite cells for FACS requires the mincing of the muscles, resulting in a solution of both cells
Figure 5.3: Jam2 colocalizes with cytoplasmic PKCλ. Jam2 and cytoplasmic PKCλ colocalize in both dividing (A) and non-mitotic satellite cells (B-E) after 36 hours in culture. PKCλ is localized to the nucleus in Jam2+ satellite cells (B-E). Doublets with asymmetric Jam2 staining display asymmetry in the nuclear localization of PKCλ as well (E).
and pieces of muscle fiber. Treating cells with Vybrant DyeCycle (Live Dye), which marks the nuclei of both living and dead cells, and Propidium Iodide (PI), which is retained only by dead cells (Figure 5.4A) shows that only a small percentage of events are live cells (~4%). When gating live cells onto a forward scatter (FS) versus side scatter (SS) plot, they overlap significantly with events that likely represent debris and not a cell, resulting in a high level of background (Figure 5.4B). I frequently noted that cells are still bound to pieces of fiber after isolation by flow cytometry (data not shown) which is the likely cause of the highly variable forward and side scatters. To eliminate as much background as possible, I use Vybrant DyeCycle and Propidium Iodide to set the forward/side scatter gate (Figure 5.4A, B). I then eliminate dead cells by negatively selecting for DAPI retention (Figure 5.4C). The resulting population contains a variable percentage of live cells (10-40%).

Labeling satellite cells with a Syndecan-4 antibody labels only a small number of cells above the secondary and isotype controls (Figure 5.4D). However, 80% of adherent colonies formed from events above the isotype control (Figure 5.4E) and 47% of the adherent colonies from plating events above the secondary only control (Figure 5.4F) are myogenic indicating that Syndecan-4 antibody enriches for satellite cells. I had previously noted that Syndecan-4 expression is low in satellite cells shortly after injury (data not shown). To determine if this was the cause of low Syndecan-4 signal, I compared Syndecan-4 staining in freshly isolated cells and cells cultured for 5 hours. A 5 hour incubation in culture results a Syndecan-4 population with greater separation above the background (Figure 5.5A, B). The increased purity of this population is illustrated by labeling with the blood lineage marker, CD45 (Johnson et al., 1989). In freshly prepared satellite cells, a large portion Syndecan-4+ events are CD45+ (Figure 5.5C,
Figure 5.4: Sorting for Syndecan-4 expression enriches for satellite cells. Live cells marked by Vybrant DyeCycle (Live Dye) and no Propidium Iodide (PI) staining comprise only a small percentage of events isolated from uninjured muscle (A). A gate on the forward scatter (FS) versus side scatter (SS) plot is drawn to include all live cells (B) and the DAPI negative cells in this population are analyzed (C). Comparing Syndecan-4+ fluorescence to the secondary only and isotype controls shows very few events above background (D), but plating all events above the isotype (E) and secondary only controls (F) enriches for satellite cells.
Figure 5.5: **Syndecan-4 expression is increased 5 hours after injury.** Syndecan-4 expression in cells freshly isolated from an uninjured muscle (A) is near background levels but in cells cultured for 5 hours (B) Syndecan-4 marks a clear population. Plotting Syndecan-4 against CD45 shows reduced contamination of the Syndecan-4 population by blood cells at 5 hours (E, F) compared to freshly isolated cells (C, D).
D), indicating that they are not likely satellite cells. However, after 5 hours in culture, the majority of the Syndecan-4+ events are CD45- (Figure 5.5E, F), supporting the conclusion that low levels of Syndecan-4 expression in uninjured muscle are responsible for the high background to signal ratio.

I examined whether two other satellite cell markers, CD34 and α-7 integrin (Ieronimakis et al., 2010), could be used as a marker of myogenic Jam2+ cells. For each marker, I set the forward/side scatter gate based on the localization of live cells (Figure 5.6A, B), negatively gated for DAPI, which marks dead cells (Figure 5.6C), and CD45, which is expressed in blood cells (Figure 5.6D). I isolated Jam2+ and Jam2- events in cells positive for either CD34, α-7 integrin or Syndecan 4. I then scored the number of myogenic colonies formed per event plated (Figure 5.6E-J). Neither α-7 integrin (Figure 5.6E, F, K) nor CD34 (Figure 5.6G, H, K) enrich for myogenic cells in either Jam2+ or Jam2- populations. However, Jam2-/Syndecan-4+ and Jam2+/Syndecan-4+ cells display a 5- and 17-fold increase in myogenic colony formation over the negative population (Figure 5.6I-K). Thus Syndecan-4 is the most effective marker of quiescent, Jam2+ satellite cells of the three antibodies tested.

Quiescent Satellite-SP Cells Express Jam2

To determine if Jam2 protein is expressed in quiescent satellite cells and after activation, I analyzed Syndecan-4 and Jam2 expression in cells from uninjured muscle and 18 hours after injury. In both freshly isolated satellite cells (Figure 5.7A-E) and satellite cells 18 hours after injury (Figure 5.7F-J), Jam2 is present in a subset of Syndecan-4+ cells, indicating that a subset of satellite cells retain Jam2 protein after activation, despite the reduced expression of Jam2
Figure 5.6: Syndecan-4 is a better satellite cell marker than α-7 integrin or CD34. A forward/side scatter gate (B) was set to include all live cells (A) and this population was negatively gated for DAPI (C) and CD45 expression (D). Jam2 positive and negative events expressing α-7 integrin (E, F), CD34 (G, H) and Syndecan-4 (I, J) were plated and scored for their ability to form myogenic clones and normalized to double negative events (K).
Figure 5.7: Jam2 is expressed in a subset of quiescent and activated cells. A forward/side scatter gate (B, G) was set to include all live cells (A, F) and this population was negatively gated for DAPI (C, H). A subset of Syndecan-4+ cells express Jam2 in uninjured muscle (D, E) and 18 hours after injury (I, J).
mRNA. Flow cytometry gates were set as described previously in this chapter (Figure 5.7A-D, F-I).

Jam2 expression appears to be restricted to a subset of quiescent cells. Simultaneously labeling for Jam2 and the satellite-SP marker, ABCG2, reveals satellite-SP cells express Jam2 (Figure 5.8A-C), suggesting Jam2 as an additional marker of the satellite-SP population. While Jam2 appears to be universally expressed in ABCG2+/Syndecan-4+ cells, Jam2 also labels a small population of Syndecan-4+/ABCG2- cells (Figure 5.8C). To determine if the ABCG2-/Jam2+/Syndecan-4+ display similar behavior in culture to the satellite-SP cells, I plated ABCG2+/Jam2+ (Figure 5.8C-H) and ABCG2-/Jam2+ satellite cells (Figure 5.8C, I-M) in growth media. After 2 days, I switched the cells to differentiation media. After 7 days in culture, the ABCG2+/Jam2+ clones retain numerous Pax7+ cells (Figure 5.8C-H) while Pax7 expression is rare in colonies derived from ABCG2-/Jam2+ cells (Figure 5.8I-M). Moreover, ABCG2+/Jam2+ colonies displayed less differentiation and more evidence of recent fusion (Figure 5.8F, G, MyoD expression in myotubes) than ABCG2-/Jam2+ colonies (Figure 5.8I-M) suggesting that even at 7 days under differentiating conditions, ABCG2+ colonies were still proliferative or slow to differentiate.

The increased self-renewal of ABCG2+/Jam2+ cells compared to ABCG2-/Jam2+ cells implies that Jam2 is not exclusively expressed in satellite-SP cells in uninjured muscle. I confirmed that Jam2 is expressed by the ABCG2+ satellite cells (Figure 5.8N-V) and, as Jam2 clearly enriches for the satellite-SP population, I continued to study the behavior of Jam2 cells in culture.
Figure 5.8: Satellite-SP cells express Jam2. Satellite-SP cells, marked by Syndecan-4 (A) and ABCG2 (B, C) express Jam2. Colonies from ABCG2+/Jam2+ cells (D-H) display higher Pax7 expression at 7d than colonies derived from ABCG2-/Jam2+ cells (I-M) indicating that ABCG2 is a better marker for satellite-SP cells. Attempts to repeat the isolation of ABCG2+ satellite cells were unsuccessful as no ABCG2 signal was detected above background (N-S). Gating events on Syndecan-4 did not reduce background enough to reveal specific ABCG2 staining (T-V).
**Clones From Jam2+ Cells Display Reduced Myogenic Commitment**

If Jam2 enriches for the satellite-SP population, I would expect to see different behavior between progeny of Jam2+ and Jam2- cells. I isolated Jam2+/Syndecan-4+ and Jam2-/Syndecan-4+ cells by FACS (Figure 5.9A, B) and plated each population under growth conditions for 7 days. Clones derived from Jam2+ cells display increased colony size (Figure 5.9D, G), an increased number of Pax7+ cells (Figure 5.9C, D, H), reduced differentiation (Figure 5.8C, D, I, percentage of nuclei in myotubes) and increased self-renewal (Figure 5.9C, D, J, number of Pax7+/MyoD- cells) when compared to clones derived from Jam2- cells (Figure 5.9E-J). This implies that progeny from Jam2+ cells have an increased proliferative capacity and reduced myogenic commitment. This is potentially a result of the increased self-renewal capacity of Jam2+ cells, as there is a direct correlation between the number of Pax7+/MyoD- satellite cells and the size of the colony in clones derived from Jam2+ cells (Figure 5.9K) but not in clones derived from Jam2- cells (Figure 5.9L). If Pax7+/MyoD- cells were only generated from the asymmetric division of satellite-SP cells, I would expect to find only one Pax7+/MyoD- cell per clone. However, the presence of multiple Pax7+/MyoD- cells and their contribution to colony size in Jam2+ clones suggests that Jam2+, SP-satellite cells either can expand in culture or that there are two populations of Pax7+/MyoD- cells; one that is truly quiescent and one that is uncommitted to differentiation but continues to provide proliferative myoblasts.

I noted clusters of satellite cells in Jam2 derived clones that did not appear to be fusing into myotubes (Figure 5.10A-C, E-G). These clusters display similar organization of Pax7 and MyoD expression to the satellite cell clusters on cultured myofibers (Figure 5.10A-C; Figure 4.8). In the rare cases where I found a cell cluster in a Jam2- clone, the cells in the cluster did not
**Figure 5.9: Clones from Jam2+ cells display reduced myogenic commitment.** Clones derived from Jam2+/Syndecan-4+ cells (A-D) display reduced myogenesis when compared to clones from Jam2-/Syndecan-4+ cells (A, B, E, F). Jam2+ clones are larger (G), have increased Pax7 expression (H), reduced myotube formation (I) and an increased number of Pax7+/MyoD-satellite cells (J). Jam2+ clones display a correlation between size and number of Pax7+/MyoD-cells (K) while Jam2- colonies do not (L).
Figure 5.10: Jam2+ clones contain satellite cell clusters. Jam2+ cells form cell clusters in culture (A-C) that display organized Pax7 and MyoD expression. Jam2- cells rarely produce clusters and when they do, they do not display myogenic markers (D). The Jam2 expression correlates with cells expressing either MyoD or Pax7 (E-G).
express MyoD or Pax7 (Figure 5.10D), suggesting that Jam2- clusters have completely
differentiated or are composed of non-myogenic cells. The frequent observation of Sca1 cells in
clusters on myofibers and the restriction of satellite cell clusters to clones derived from Jam2+
cells indicates that these clusters are a property of satellite-SP cells. Examining Jam2 expression
in these clusters reveals that Jam2 appears to localize to cells expressing Pax7 or MyoD and not
the presumably differentiated cells that express neither (Figure 5.10E-G).

Disrupting Jam2 Alters Self-Renewal

To determine the function of Jam2 in satellite cells, I transfected cells on the second day
of mass culture with a plasmid expressing Jam2 and mCherry. Pax7+ cells transfected with Jam2
show a minor increase in number (Figure 5.11A, C) compared to cells transfected with an empty
vector (Figure 5.11A, D). Interestingly, in fields surrounding transfected cells, I noted an increase
in the total number of cells as well as Pax7+ cells (Figure 5.11B, C) when compared to the
control (Figure 5.11B, D).

To study the involvement of Jam2 in the asymmetric division of satellite cells, I
transfected satellite cells on freshly isolated myofibers (before the first division) with an empty
control plasmid (Figure 5.13B-D) or a plasmid expressing wild type Jam2 (Figure 5.13E-G,
Figure 5.12A, WT Jam2). Over-expressing Jam2 decreases the number of Pax7+ cells (Figure
5.13A, E-G) after 4 days compared to the control (Figure 5.13A-D) suggesting that over-
expressing Jam2 before the first division reduces proliferation or increases differentiation. To
disrupt Jam2 function, I over-expressed a Jam2 mutant without its intracellular domain (Figure
5.12B, Figure 5.13H-J, Jam2ΔIC), which would presumably compete with endogenous, wildtype
Figure 5.11: Over-expression of Jam2 increases proliferation in surrounding cells. Transfection of mass cultured satellite cells at day 2 with a plasmid expressing Jam2 and mCherry results in a minor increase transfected cells per field (A, C) when compared to transfection with an empty vector (A, D). When scoring the untransfected cells in the same field, transfection with Jam2 results in increased Pax7 expression and cell number.
Figure 5.12: Jam2 mutant constructs. Wild type Jam2 interacts with cell junction complexes through its extracellular IG domains and binds Par-3 through its intracellular PDZ domain (A). Jam2ΔIC mutant is missing its intracellular domain (B) and Jam2ΔEC is missing its extracellular domain (C).
Figure 5.13: Disruption of Jam2 eliminates AraC-resistance. Satellite cells transfected with a plasmid expressing mCherry and either Wild type Jam2 (A, E-G, WT Jam2), a mutant Jam2 without its intracellular domain (A, H-J, Jam2ΔIC) or a mutant Jam2 missing the extracellular domain (A, K-L, Jam2ΔEC) express less Pax7 than cells transfected with an empty vector (A-D). Untransfected cells in contact with cells transfected with WT Jam2 or Jam2ΔIC are Pax7+. Transfection with WT Jam2 (N, R-T) or Jam2ΔEC (N, X-Z) reduces or eliminates Pax7+ cells surviving an AraC treatment from day 2 to 4 relative to cells transfected with an empty vector (N-Q). However, treatment with Jam2ΔIC increases AraC resistance 6 fold.
Jam2 for ligand and disrupt polarity, or a Jam2 mutant without its extracellular domain (Figure 5.12C, Figure 5.13K-M, Jam2ΔEC), which has been previously shown to disrupt the localization of Par-3 to cell junctions (Ebnet et al., 2001). Expression of both Jam2 mutants reduced the number of Pax7+ cells and the total cell count after 4 days. I noted that over-expression of Jam2ΔIC and to a lesser extent, Jam2, increased the clustering of cells (Figure 5.13E-J) which may have indirectly promoted differentiation.

If Jam2 regulates the asymmetric division and self-renewal of satellite-SP cells, I predict that disrupting Jam2 function before the first division would result in reduced AraC-resistance. I transfected plasmids expressing mCherry and Jam2, Jam2ΔIC or Jam2ΔEC into satellite cells on freshly isolated myofibers and treated with AraC from day 2 to 4. At day 4, I scored the number of Pax7+, AraC-resistant cells and plotted the results relative to an empty vector control (Figure 5.13N-Q). While satellite cells transfected with WT Jam2 showed a 2-fold reduction in AraC-resistance (Figure 5.13N, R-T), over-expression of Jam2ΔIC resulted in a 6-fold increase in the survival of Pax7 cells (Figure 5.13N, U-W) and Jam2ΔEC eliminated the AraC-resistant population (Figure 5.13N, X-Z). I had previously noted that, in untreated cultures, while the cells transfected with Jam2 or Jam2ΔIC had lost Pax7 expression, adjacent untransfected cells were Pax7+ (Figure 5.13E-J). This indicates that the extracellular domain of Jam2 may be sufficient to induce quiescence or repress differentiation in a neighboring cell and could explain the increased AraC-resistance in cells transfected with Jam2ΔIC. If an increased number of cells express the Jam2 extracellular domain after the first division, this could allow daughter cells formed during the first division to mutually promote quiescence in each other. The extracellular domain of Jam2 can form homodimers between cells and over-expression in cells on untreated fibers might
promote a high degree of clustering, due to the increased number of cells, and induce
differentiation. Conversely, over-expression of the Jam2ΔEC mutant may disrupt the binding
between endogenous Par-3 and Jam2, disrupting polarity and eliminating self-renewal. This
supports a role for the interaction between Jam2 and Par-3 in the asymmetric division of satellite-
SP cells.

Transplanting Jam2+ Cells Promotes The Self-Renewal Of Endogenous Satellite Cells

Transplantation of the satellite-SP population results in increased engraftment to the host
satellite cell position compared to satellite cells (Tanaka et al., 2009). Jam2 labels ABCG2+ cells
and thus, I asked if Jam2 cells display increased engraftment. Selecting for live cells by forward
and side scatter and negatively for DAPI staining (Figure 5.14A-C), I sorted Jam2+/Syndecan-4+
and Jam2-/Syndecan-4+ cells from a LacZ expressing mouse (Figure 5.14D, E). I then cultured
the cells for 3 hours in FGF and injected the cells, coincident with a BaCl₂ induced injury, into
the Tibialis Anterior muscle of a host mouse. However, transfer to culture and then to BaCl₂
resulted in the loss of most of the cells and I only transplanted 600 cells of each type. There was
no engraftment of either the Jam2+ or Jam2- cells, possibly as a result of the low number of cells
injected (Figure 5.14G-L). However, the endogenous population of Pax7+ cells in the repaired
muscle increased 6-fold in the muscle injected with Jam2+ cells (Figure 5.14F-I) while injection
with Jam2- cells resulted in similar numbers of Pax7+ cells as the uninjured contralateral control
(Figure 5.14F, Figure 5.14F, M-O).

This result combined with the ability of the Jam2 extracellular domain to induce the
quiescence of surrounding cells implies that Jam2 functions to promote renewal in adjacent cells.
Figure 5.14: Transplant of Jam2+ cells increases self-renewal of host satellite cells. A forward/side scatter gate (B) was set to include all live cells (A) and this population was negatively gated for DAPI (C). Injection of 600 Jam2+/Syndecan-4+ cells (D-I) from a Rosa26 mouse yielded no detectable engraftment as measured by βGal staining. However, the number of endogenous Pax7+ cells increased 6-fold in the muscle injected with Jam2+/Syndecan-4+ cells (F-I) when compared to Jam2-/Syndecan-4+ cells (D-F, J-L) and the uninjured contralateral control (F, M-O).
If Jam2 establishes polarity in asymmetrically dividing cells by binding to Par-3, Jam2 would presumably sort with the Par complex during an asymmetric division. Jam2 would then promote quiescence in the other daughter cell and enforce the renewal of the quiescent satellite cell population (Figure 5.15).

**FGF-2 Promotes Pax7 Expression**

Fibroblast growth factor 2 (FGF-2) is a satellite cell mitogen (Olwin and Hauschka, 1986) that promotes satellite cell activation (Yablonka-Reuveni and Rivera, 1994) and inhibits satellite cell differentiation (Allen and Boxhorn, 1989; Jones et al., 2005). To understand the role of FGF-2 in self-renewal, I analyzed the effects of FGF on Pax7 and MyoD levels. Culturing cells in the presence of FGF-2 increases the number of satellite cells with high levels of Pax7 at day 2 (Figure 5.16A, C) while having little impact on MyoD expression. Interestingly, the greatest difference in Pax7 expression is displayed by the cells with the highest MyoD expression (Figure 5.16A, D). By day 4, cells cultured without FGF have down regulated Pax7 expression as they begin to differentiate (Figure 5.16B, C) while treatment with FGF maintains a Pax7+ population of cells (Figure 5.16B, C) and reduces MyoD levels (Figure 5.16B, D).

FGF-2 appears to effect Pax7 levels more than MyoD, perhaps hinting at the mechanism by which FGF-2 regulates satellite cell behavior. High levels of Pax7 promote MyoD degradation, and maintaining a high ratio of Pax7 to MyoD may delay the onset of myogenesis. I had previously noted that culturing cells under differentiation conditions (No FGF, 10% horse serum) did not eliminate the AraC-resistant population (Figure 4.2E), suggesting that FGF-2 is not necessary for self-renewal. However, satellite cells express low levels of endogenous FGF,
Figure 5.15: Model of satellite-SP cell asymmetric division. A model where Jam2 creates asymmetry in satellite-SP cells through its association with cell-cell junctions. The Par complex binds Jam2 and regulates the distribution of Numb and activation of p38α/β MAPK. The daughter cell that inherits the Jam2/Par complex commits to myogenesis. Jam2 then signals to the adjacent daughter cell to promote quiescence.
Figure 5.16: FGF increases Pax7 expression. Treatment with FGF increases the number of satellite cells expressing high levels of Pax7 at day 2 (A, C) with little effect on MyoD expression (A, D). At day 4, FGF treatment maintains Pax7 expression (B, C) and reduces MyoD levels (B, D) when compared to cells grown without FGF.
possibly providing an alternate source in culture. If FGF truly is not necessary for satellite-SP cells to resist commitment and enter quiescence, I would expect to satellite cells without Fibroblast growth factor receptor 1 (FGFR1) to maintain a Pax7+ population.

To analyze the ability of satellite cells null for FGFR1 to self-renew, we induced recombination in mice homozygous or heterozygous for a floxed FGFR1 allele. We then isolated satellite cells and cultured them in growth conditions for 6 days, scoring Pax7 expression and differentiation. While cells from both mice had similar numbers of Myogenin+ cells and fused nuclei (Figure 5.17 A-G), FGFR1−/− satellite cells retained no Pax7 expressing cells (Figure 5.17 A-D) suggesting that FGF signaling is necessary to resist differentiation, both to maintain a proliferative population of cells and to self-renew. This result indicates satellite-SP cells may not be able to self-renew without proper signaling from the surrounding tissue or cells.

Discussion

In order for Par-3 to establish asymmetry, it must bind to a transmembrane protein. Jam2 localizes to cell-cell junctions and binds Par-3 through intracellular PDZ domain and could potentially drive Par-3 asymmetry by sequestering it to the satellite cell-muscle fiber interface or to cell-cell junctions. I observed that the asymmetrically dividing satellite-SP cells express Jam2 and that Jam2 colocalizes with members of the Par complex. Over-expressing the Par-3 binding, intracellular region of Jam2 eliminates the generation of quiescent cells by presumably disrupting the interaction between Par-3 and Jam2. This would disrupt asymmetry, possibly resulting in both daughter cells receiving the Par complex and activating p38α/β MAPK (see chapter 4). These data support a role for Jam2 as the initial source of asymmetry for satellite-SP
Figure 5.17: FGFR1 is necessary to resist differentiation. A subset of FGFR1\textsuperscript{+/−} satellite cells resist differentiation after six days in culture as measured by Pax7 expression (A, C). No FGFR1\textsuperscript{−/−} cells express Pax7 after 6 days in culture (A, B) indicating that FGF signaling is necessary for self-renewal.
Over-expression of the extracellular and transmembrane domains appears sufficient to induce quiescence in neighboring cells. While surprising, this result supports our model as the daughter cell receiving the Jam2/Par complex would commit to myogenesis while inducing its sister cell to remain quiescent. Lateral cell-cell signaling is another mechanism to ensure retention of quiescent satellite cells during muscle injury.

Paradoxically, over-expression of the Jam2ΔIC mutant reduces the number of Pax7+ cells at 4 days on untreated fibers but increases the number of Pax7+ cells following AraC treatment. Perhaps this indicates the importance of the number or identity of surrounding satellite cells in the function of Jam2 as AraC treatment eliminates all proliferative myoblasts. Alternatively, the satellite cells ectopically expressing Jam2ΔIC may lose Pax7 expression via an indirect effect of the Jam2 extracellular domain. Expression of Jam2ΔIC appears to induce clustering in satellite cells, likely through the ability of Jam2 to form homophilic dimers (Cunningham et al., 2000), which may in turn promote differentiation. While transfected cells formed clusters on AraC treated fibers as well, the reduced size of the cluster or elimination of proliferative myoblasts may have limited any differentiation induced by satellite cell clustering.

Jam2 is expressed almost universally 4 days after injury. Clearly endogenous Jam2 expression alone is not sufficient to induce quiescence in neighboring cells as satellite cells are rapidly dividing and beginning to differentiate at this time. Jam2 interaction with Jam3 prevents the interaction of Jam3 with αMβ2 integrin in leukocytes (Lamagna et al., 2005). It is possible similar competitive binding occurs with Jam2 and that either the formation of homophilic dimers (Keiper et al., 2005) with other cells expressing Jam2 or interaction with another binding partner cells.
blocks the ability of Jam2 to promote quiescence in the adjacent cell. The If satellite-SP cells do not express Jam2, they might be more receptive to the quiescence signal of Jam2 in neighboring cells. In this case, a high local concentration of myoblasts would prevent satellite-SP cells from needlessly activating and dividing to provide more satellite cells.

Jam2 transcript is highly expressed in quiescent satellite cells while only a small percentage of cells express Jam2 protein likely indicating that the majority of satellite cells transcribe Jam2 but only a subset express the protein. It is possible that Jam2 protein is only stabilized in the presence of specific interactions or by environmental cues. The rapid down regulation of Jam2 gene expression after injury would ensure that only satellite cells in contact with a specific niche would retain the protein and be able to divide asymmetrically. If Jam2 is sufficient to promote an asymmetric division in satellite cells, this would suggest that the satellite-SP identity could be stochastically designated to any satellite cell through environmental interactions.

**Experimental Procedures**

*Mice*

Mice were housed in a pathogen-free facility and all procedures and protocols were approved by the Institutional Animal Care and Use Committee at the University of Colorado. Mice were female B6D2F1 (The Jackson Laboratory) between 3 and 5 months of age. Rosa26 (B6;129S-Gt(ROSA)26Sor) (Zambrowicz et al., 1997) mice were obtained from Jackson Laboratory. For floxed FGFR1 mice, we established a tamoxifen-inducible Cre-ER transgenic mouse line where ubiquitous FGFR-1 deletion is directed by floxed loxP inserts and
recombination efficiency is determined via β-galactosidase (β-gal) expression following excision of a floxed stop codon for Rosa26 (John Hall, thesis). Recombination was induced by administering 3x 2mg/dose tamoxifen IP (Sigma; 20mg/ml in 10% ethanol and corn oil) over 96 hours where mice were given a 48hr recovery following the initial dosage.

Cell Culture

Primary satellite cells and myofibers were prepared as previously described (Cornelison et al., 2004). Primary satellite cells were cultured on gelatin coated plates and myofibers were cultured unattached to the plate. Cells were cultured under growth conditions (F12-C + 15% horse serum + 20 ng/ml FGF-2) or differentiation conditions (F12-C + 10% horse serum) at 37°C under 5% CO₂ and 6% O₂. Media was changed daily and unattached primary cells were centrifuged and added back to the culture dish except in clonal assays. Unless stated otherwise, all pictures are of myofiber-associated cells 36 hours after injury. AraC (Sigma Aldrich) was added a final concentration of either 13 µM.

Immunofluorescence And Proximity Ligation Assay

Immunofluorescence was performed as previously described (Tanaka et al., 2009). Tissue was sectioned as previously described (Hall et al., 2010). Primary antibodies and dilutions: mouse monoclonal Pax7 (Developmental Hybridoma Bank at Iowa University) at 1:5, rabbit polyclonal MyoD (C-20, Santa Cruz Biotechnology) at 1:800, chicken anti-Syndecan-4 at 1:1500, rabbit polyclonal anti-pH3 (Millipore) at 1:250, rabbit polyclonal anti-PKCl (Santa Cruz Biotechnology) at 1:50, rabbit polyclonal anti Par-3 (Upstate) at 1:250, chicken polyclonal anti-
β-galactosidase antibody (Abcam) at 1:500, rat monoclonal Laminin at 1:250 and mouse monoclonal Jam2 (Acris) at 1:50. Secondary antibodies (Invitrogen) conjugated with Alexa Fluor 488, Alexa Fluor 546, Alexa Fluor 555, Alexa Fluor 594 and Alexa Fluor 647 were used at 1:1000 dilution. For proximity ligation assay (Olink), samples were incubated with antibody as described above. Duolink proximity ligation assay was performed according to manufacturer’s protocol. Numbers of cells were normalized to untransfected cells or uninjured contralateral control. Nuclear levels of Pax7 and MyoD fluorescence were measured in SlideBook 4.0 by subtracting the average cytoplasmic intensity (determined by Syndecan-4) from the average nuclear intensity (determined by DAPI). Fusion was scored by number of nuclei in myotubes. Background fluorescence was set to the secondary only control or fluorescence of the myofiber. Confocal images were displayed as 3D reconstructions or slices through a 3D reconstruction generated in Volocity. Blue represents DAPI staining unless stated otherwise.

Arrays

For injury time course, arrays were generated as previously described (Pisconti et al., 2010).

Transfection

Jam2 mutants were created by overlap extension PCR as previously described (Senanayake and Brian, 1995). Plasmid containing wild type and mutant Jam2 genes expressed Jam2 genes and mCherry from a single, IRES containing RNA. Primary satellite cells and
myofibers were transfected using Lipofectamine Plus (Invitrogen, Carlsbad, CA) according to the manufacturers protocol.

Flow Cytometry

Flow cytometry was performed as previously described (Tanaka et al., 2009). For cells 5 hours after injury, primary cells were cultured on gelatin coated plates in growth conditions for 5 hours before profiling. For cells 18 hours after injury, muscle was removed, dissected and cultured, unattached in growth conditions. Muscle was then minced and preparation resumed as normal. Vybrant DyeCycle (invitrogen) was added 30 minutes before profiling or sorting at 1:1000 at 37°C and Propidium Iodide (Invitrogen) was added at 1:1000 right before profiling or sorting. Primary antibodies were used at the following concentrations: 1:100 mouse anti-ABCG2 (BCRP1) 5D3 (BD PharMingen), 1:100 mouse anti-ABCG2-PE (BCRP1) 5D3 (Chemicon), 1:400 rat anti-CD45-FITC 30-F11 (BD PharMingen), 1:250 mouse monoclonal Jam2 (Acris), 1:100 rat anti-Scal-FITC E13-161.7 (BD PharMingen), 1:1600 rat anti-α-7 Integrin, 1:200 rat anti-CD34-647 1:100 rabbit anti-Syndecan-3 (Cornelison et al., 2001) and 1:2000 chicken anti-Syndecan-4 (Cornelison et al., 2004). Alexa conjugated secondary antibodies were used at 1:2000. Secondary antibodies were (at 1:2000 unless otherwise noted) as follows: anti-chicken-Alexa488 (Molecular Probes) 1:500, anti-chicken-PE (Open Biosystems), anti-mouseIgG-PE-Cy5 (Santa Cruz), and anti-ratIgG-PE-Cy7 (Santa Cruz). Data was analyzed in Summit 5.0, FlowJo 7.6.3 or FlowJo 8.
Cells were cultured on uncoated plates for 3 hours in growth conditions and resuspended in 70 µl BaCl₂. 600 cells were injected into tibialis anterior muscle and mice were allowed to recover for 30 days.
Chapter 6: Discussion
Muscle Stem Cells

The Maintenance And Regeneration Of Adult Tissue

Many adult tissues, like the skin, intestinal epithelium, blood and germ line are in a constant state of regeneration and must constantly turnover cells to maintain proper function (Blanpain and Fuchs, 2009; van der Flier and Clevers, 2009; Giebel and Bruns, 2008; Yuan and Yamashita, 2010). The cells comprising these tissues are terminally differentiated and cannot divide as a source of new cells. Instead, these tissues contain an undifferentiated population of adult stem cells that are capable of the proliferation required to regenerate these tissues (Blanpain and Fuchs, 2009; van der Flier and Clevers, 2009; Giebel and Bruns, 2008; Yuan and Yamashita, 2010). However, the high metabolic activity and repeated DNA synthesis necessary for a high degree of proliferation throughout the life time of the animal would lead to the accumulation of DNA damage and eventual cellular senescence (Hayflick, 2000; Finkel and Holbrook, 2000). Thus, there is a strong evolutionary pressure on organisms to ensure that the adult stem cells of each are protected from the stresses of prolonged proliferation while acting as a constant source differentiated cells for the life span of the animal.

Adult stem cells are capable of supplying countless differentiated cells to maintain, repair and regenerate tissues while dividing infrequently themselves. Stem cells accomplish this by dividing rarely to yield transient amplifying cells. Transient amplifying cells are committed cells that are capable of a period of proliferation, allowing them to generate a large number of progeny. By shifting the proliferative stress to their offspring, stem cells can produce massive numbers of progeny over the lifetime of the animal, while dividing infrequently themselves, allowing them to avoid senescence for the lifespan of the animal. However, stem cells cannot
exclusively produce transient amplifying cells as daughter cells, as this would result in the loss of
the stem cell population. For stem cells to persist, they must ‘self-renew’ by ensuring that some
of their offspring retain the stem cell identity. These different fates among the daughters of stem
cells are promoted through asymmetric divisions and interactions with varying niches.

*Satellite Cells As Muscle Stem Cells*

Like other tissues, the terminally differentiated myonuclei of skeletal muscle are
continuously replaced to maintain functional muscle (Dhawan and Rando, 2005). However,
skeletal muscle is unique in that it accumulates variable degrees of damage depending on use and
injury (Smith et al., 2001; Ambrosio et al., 2009). Additionally, skeletal muscle is a very plastic
tissue, capable of undergoing hypertrophy, to produce greater force, or hypotrophy, to reduce the
animals metabolic needs, as needed by the organism (Booth, 1982; Favier et al., 2008). Both the
repair and hypertrophy of skeletal muscle require the generation of new myonuclei (Oustanina et
al., 2004). Similar to other tissues, skeletal muscle maintains a population of uncommitted cells
that proliferate to provide differentiated myonuclei as needed. These ‘satellite cells’ maintain the
mass and function of uninjured muscle, repair both minor and severe damage from exercise of
injury and are responsible for muscle hypertrophy (Oustanina et al., 2004), filling a role
analogous to the stem cells of other adult tissues.

In healthy muscle, the satellite cells are generally quiescent (Mauro, 1961; Schultz et al.,
1978) and divide infrequently to replace or generate myonuclei (Smith et al., 2001). Following
injury or exercise, satellite cells generate a committed population of myoblasts, which are similar
in function to transient amplifying cells and are capable of considerable proliferative expansion
(Biressi and Rando, 2010) while also self-renewing to ensuring that the quiescent satellite cell population is not lost (Olguin and Olwin, 2004; Zammit et al., 2004; Sacco et al., 2008; Hall et al., 2010). Therefore, in behavior and function satellite cells are reminiscent of stem cells and the satellite cell population appears to contain or comprise a population of adult muscle stem cells.

Satellite cells are not uniform in their commitment to myogenesis, their proliferative response to injury and their ability to self-renew (Rantanen et al., 1995; Beauchamp et al., 2000; Olguin and Olwin, 2004; Zammit et al., 2004; Shinin et al., 2006; Kuang et al., 2007; Conboy et al., 2007; Tanaka et al., 2009). This suggests that satellite cells are not a pure population of muscle stem cells, but may instead be composed of a hierarchical progression from muscle stem cell to transient amplifying cell to differentiating muscle cell. Only subsets of satellite cells sort in the side population, express the stem cell markers, Sca1 and Abcg2 (Tanaka et al., 2009), have never expressed the myogenic marker, Myf5 (Kuang et al., 2007), divide asymmetrically (Kuang et al., 2007), display long term retention of DNA labels incorporated perinatally (Shinin et al., 2006) and are capable of significant self-renewal (Kuang et al., 2007; Tanaka et al., 2009), all characteristics of uncommitted, slowly cycling stem cells.

To understand the variation in satellite cell behavior, I examined the response of satellite cells to muscle injury. Hypothesizing that a subpopulation of satellite cells act as muscle stem cells, where they divide infrequently, and that the remaining satellite cells are responsible for the proliferation necessary to repair the muscle, I expected to find that not all satellite cells respond to muscle injury. Surprisingly, I found the opposite; that all satellite cells activate and divide following muscle injury. However, this division in response to injury is not equivalent among satellite cells. The satellite-SP cells divide asymmetrically to generate a proliferating myoblasts
and a quiescent, Pax7+/MyoD- satellite cell. This observation suggests that the satellite-SP cell population, the asymmetrically dividing Myf5- satellite cell population and the Pax7+/MyoD-reserve cell population overlap and likely consist of uncommitted, self-renewing, muscle stem cells.

**Muscle Stem Cell Self-Renewal**

*The Par Complex Regulates Satellite Cell Behavior*

Muscle stem cells divide asymmetrically to yield a committed Myf5+ satellite cell and an uncommitted, Myf5-, muscle stem cell (Kuang et al., 2007). However, the timing and mechanism of this asymmetric division is unclear. In other stem cells, asymmetric divisions are directed by the Par complex, which orients the plane of division and differentially directs the inheritance of signaling molecules between the daughter cells. To determine if the Par complex directs the asymmetric division of muscle stem cells, I analyzed the distribution of members of the Par complex during the first division following muscle injury. A subset of mitotic satellite cells localizes the Par complex to one side of the cell, supporting a role for the Par complex in the asymmetric division of satellite cells.

The observation that muscle stem cells divide to generate a single, AraC-resistant cell immediately following muscle injury suggests that the initial division of muscle stem cells is asymmetric and that the resulting in one proliferative myoblast daughter and one quiescent stem cell. If this is true, I would expect that daughter cells would vary in their phosphorylation of p38α/β MAPK, as that is the earliest reported marker of satellite cell activation (Jones et al., 2005). I found that p38α/β MAPK is not only differentially phosphorylated between the daughter
cells of muscle stem cells, but is asymmetrically activated in a subset of mitotic satellite cells during the first division after injury. Additionally, phosphorylated p38α/β MAPK is found in complex with and requires the activity of the Par complex supporting a role for the Par complex in the asymmetric regulation of the phosphorylation of p38α/β MAPK in dividing muscle stem cells. As a role for the Par complex in p38α/β MAPK has not been previously reported, I assembled a putative signaling network linking the Par complex to p38α/β MAPK and analyzed the gene expression of the members. The Rac1 GEF, Tiam1, and the Rac1/p38 signaling scaffold protein are more highly expressed in muscle stem cells, relative to non-stem cell satellite cells, and the detection of an asymmetric complex containing Tiam1 and Par-3 in mitotic satellite cells suggests that the Par complex regulates p38α/β MAPK activity through Rac1 signaling. The phosphorylation of p38α/β MAPK acts as a molecular switch for satellite cell activation (Jones et al., 2005) and the asymmetric regulation of p38α/β MAPK by the Par complex is a very effective way to differentially promote activation and quiescence between daughter cells (Figure 6.1).

I frequently observed that Syndecan-4 was localized to the daughter cell inheriting phosphorylated p38α/β MAPK in asymmetrically dividing cells. Syndecan-4 positively regulates FGF signaling by promoting the binding of FGF to its receptor (Steinfeld et al., 1996). In satellite cells, Syndecan-4 appears to facilitate the role of FGF signaling in activation (Jones et al., 2001) as Syndecan-4 null satellite cells are deficient in activation and slow to divide in response to injury (Cornelison et al., 2004). Sequestering Syndecan-4 from one daughter cell could be an additional pathway to inhibit activation by reducing that daughter cell’s responsiveness to FGF. While it is unclear how Syndecan-4 achieves this asymmetric distribution, in other cells Syndecan-4 localizes to cell junctions (Bass and Humphries, 2002)
Figure 6.1: Muscle stem cells divide asymmetrically. A model depicting the asymmetric division of muscle stem cells during the first division after injury. The Par complex is asymmetrically distributed in satellite cells where it negatively regulates Numb and promotes the activation of p38α/β MAPK. As Syndecan-4 segregates with the Par complex, one daughter cell inherits active p38α/β MAPK, active Notch and Syndecan-4 while the other daughter cell inherits Numb, which negatively regulates Notch signaling.
where it regulates Rac1 signaling (Tkachenko et al., 2004). Therefore, Syndecan-4 could function in the regulation of p38α/β MAPK through Rac1 signaling in asymmetrically dividing satellite cells, and subsequently differentially regulate the response to FGF-2 in the resulting daughter cells (Figure 6.1).

The Par complex regulates cell fate during the asymmetric divisions of other stem cells by directing the localization of the Notch inhibitor, Numb to the opposite daughter cell (Knoblich, 2008). Array data performed the asymmetrically dividing Myf5- satellite cells from uninjured muscle reveals that Myf5- cells preferentially express Notch3 compared to Myf5+ cells (Kuang et al., 2007). Additionally, a muscle null for Notch3 has enhanced muscle growth, but only after repeated injuries (Kitamoto and Hanaoka, 2010). This phenotype suggests that self-renewal is enhanced in these mice and that there is a greater population of muscle stem cells to contribute myoblasts for each subsequent injury. It is possible that the lack of Notch3 results in symmetric divisions generating two muscle stem cells, increasing the sized of the muscle stem cell pool. Thus, it seems likely that the Par complex influences cell fate through the regulation of Notch3 signaling (Figure 6.1).

To determine if the Par complex regulated Notch signaling by directing the localization of Numb, as in other stem cells, I analyzed the distribution of Numb during the first division. I found that, during the first division, Numb is exclusively expressed in muscle stem cells and its expression is mutually exclusive with the phosphorylation of p38α/β MAPK. Notch signaling has been shown to promote the activation of satellite cells (Conboy et al., 2003; Carlson et al., 2008) and the localization of Numb to the daughter cell destined to retain the stem cell identity is
consistent with this association between Notch3 and activation, suggesting that Numb blocks Notch3 activity in the stem cell daughter, resulting in its quiescence (Figure 6.1).

Thus, in satellite cells, Par complex appears to promote activation in only one daughter cell by manipulating at least three signaling pathways. By regulating the localization of Syndecan-4, phosphorylated p38α/β MAPK and Numb during division, the Par complex ensures differential FGF, p38α/β MAPK and Notch signaling between the two daughter cells in a manner that results in one quiescent muscle stem cell and one active myoblast (Figure 6.1).

**Jam2 Orients The Asymmetric Divisions Of Muscle Stem Cells**

To achieve asymmetry during division, the Par complex must be anchored to the membrane by a PDZ domain containing transmembrane protein (Knoblich, 2008). Then, through the polar interaction between the satellite cell and its niche, this transmembrane could establish the asymmetry of the Par complex during division (Fuchs et al., 2004).

Jam2 is a PDZ domain containing transmembrane protein that associates with cell-cell junctions and recruits the Par complex (Ebnet et al., 2003). The pattern of Jam2 expression during satellite cell activation suggests that asymmetry may be generated in dividing muscle stem cells by the recruitment Par-3 to sites of cell-cell or cell-fiber interaction by Jam2 (Figure 6.2A). This model is supported by the expression of Jam2 in satellite-SP cells, the enhanced self-renewal of clones derived from Jam2 expressing cells and the loss of the AraC-resistant cells when the interaction between Par-3 and Jam2 is disrupted.

Interestingly, the extracellular domain of Jam2 appears to be sufficient to induce quiescence in neighboring cells. Satellite cells over-expressing a Jam2 mutant without an
Figure 6.2: Jam2 orients the asymmetric divisions of muscle stem cells and promotes quiescence in adjacent satellite cells. A model depicting the asymmetric division of muscle stem cells during the first division after muscle injury. Through interaction with the muscle fiber and recruitment of the Par complex, Jam2 orients the asymmetric division of muscle stem cells (A). The Jam2 inherited by the myoblast daughter cell then reinforces the quiescence of the muscle stem cell daughter (B).
intracellular domain (Jam2ΔIC) cluster and are Pax7-, while untransfected satellite cells bind these clusters and become quiescent. While Jam2+ cells transplanted into an injured muscle display little engraftment, a 6 fold increase was seen in the number of endogenous Pax7+ cells in the muscle. These results indicate that the Jam2 extracellular domain promotes renewal in other cells. This suggests a function of Jam2 in asymmetric division separate from orienting the localization of the Par complex. As the myoblast daughter inherits Jam2, it could then signal to the through the extracellular domain of Jam2 to the quiescent daughter cell to reinforce quiescence (Figure 6.2B).

The Muscle Stem Cell Niche

The niche of the satellite cell is, by definition, between the plasma membrane of the muscle fiber (Mauro, 1961). The importance of muscle stem cell interaction with its niche is illustrated by the enhanced self-renewal of satellite cells transplanted in association with the muscle fiber compared to satellite cells transplanted in isolation (Montarras et al., 2005; Sacco et al., 2008; Tanaka et al., 2009). The stem cell niche typically serves to orient the asymmetric divisions of stem cells (Knoblich, 2008) and it is likely that the niche performs a similar function with muscle stem cells, possibly explaining the need of the fiber for satellite cell self-renewal.

The asymmetric division of muscle stem cells occurs perpendicular to the muscle fiber, in a manner that generates the Myf5- muscle stem in contact with the basal lamina and the Myf5+ myoblasts in contact with the fiber (Kuang et al., 2007). Typically, the stem cell remains in contact with the stem cell niche during an asymmetric division, which serves to reinforce the stem cell identity in that cell. The observation that the muscle stem cell daughter does not remain
in contact with the fiber could suggest that the basal lamina comprises a more important component of the muscle stem cell niche than the fiber. Alternatively, directing the division so that the myoblast daughter cell is in contact with the muscle fiber could be a mechanism to facilitate the future differentiation and fusion of that cell. Keeping the muscle stem cell isolated from the fiber might prevent the muscle stem cell from being mistakenly incorporated into the fiber during the fusion of other cells. The expression of Jam2 by the myoblast daughter cells could act as a temporary replacement for the muscle stem cell niche, maintaining the quiescence and identity of the muscle stem cell (Figure 6.2A, B).

The self-renewal of muscle stem when cultured in the absence of the myofiber was unexpected as interaction with a specific niche is typically necessary to establish the asymmetry necessary for an asymmetric division. The asymmetric division of non-adherent cells in culture, nucleated by interactions between satellite cells, suggests a role for cell-cell contact in directing the behavior of muscle stem cells. I frequently detected Sca1+ satellite cells located in clusters of satellite cells on the muscle fiber. The early appearance of these clusters and the motility of myofiber-associated satellite cells before the first division suggest that these clusters are not necessarily derived from clonal expansion. The over-expression of Sca1 leads to the clumping of lymphocytes suggesting that Sca1 promotes cell-cell adhesion (English et al., 2000) and hinting at a possible function of Sca1 in muscle stem cells where it promotes satellite cell interaction.

While I rarely observe the clustering of satellite cells in explanted culture, Jam2+ cells frequently form organized clusters of satellite cells, similar to the ones that appear on muscle fibers. Additionally, over-expressing Jam2 appears to promote the grouping of satellite cells, suggesting that Jam2 may play a role the formation or regulation of satellite cell clusters. Besides
promoting the formation of these clusters, Jam2 could orient the asymmetric divisions of cells in these clusters and could further regulate the quiescence of muscle stem cells through the extracellular domain of Jam2. The gathering of satellite cells into clusters could be directed to sites of injury and be a mechanism to organize the injury response. By grouping together, satellite cells would be able to assess how many muscle stem cells and myoblasts are available to repair the muscle and coordinate their behavior appropriately.

The Environment Regulation Of Muscle Stem Cells

It is unclear whether the muscle stem cell population truly reflects an uncommitted progenitor lineage or whether all satellite cells can adopt the muscle stem cell identity. Sophisticated lineage-tracking experiments on intestinal (Snippert et al., 2010), mouse ear epidermis (Doupé et al., 2010) and mouse germ line (Klein et al., 2010) stem cells have revealed that the stem cell identity is retained by a subset of equivalent cells through chance interactions. This demonstrates that stem cells do not necessarily have to divide asymmetrically to generate both transient amplifying cells and stem cells, but, instead, self-renewal can be ensured by the stochastic interactions with the stem cell progeny with specific niches. A similar process could provide an alternate explanation for the self-renewal of muscle satellite cells. If contact with a specific and rare niche designates satellite cells to adopt or retain stem cell characteristics, muscle stem cells would still comprise only a subset of the total satellite cell population (Figure 6.3).

Jam2 and Wnt7a are potential mechanisms by which the regeneration environment can regulate muscle stem cell behavior. Over-expression of Wnt7a in injured muscle results in an
Figure 6.3: Muscle stem cells as a lineage versus the stochastic determination of muscle stem cells. Models depicting possible mechanisms for the maintenance or generation of muscle stem cells. In the lineage model, satellite cells consist of two cell types, muscle stem cells (green) and myoblasts (yellow). In response to injury, each cell type exhibits a distinct behavior (A). In the stochastic model, all satellite cells are equivalent initially, but through variable interactions with the environment, they commit to different fates (B).
increased number of muscle stem cells during regeneration, increased quiescent satellite cells in
the repaired muscle as well as induces muscle hypertrophy (Le Grand et al., 2009). Wnt7a
accomplishes this by promoting the expansion muscle stem cell number through symmetric
divisions directed by the planar cell polarity pathway (Le Grand et al., 2009). I rarely detected
AraC-resistant or BrdU retaining satellite cells in pairs indicating that it is unlikely these cells are
products of symmetric divisions. However, this is not necessarily in disagreement with the
reports of symmetric division in muscle stem cells. Myf5 expression and AraC resistance
measure different aspects of muscle stem cell self-renewal, reflecting muscle stem cell identity
and quiescence respectively. Therefore, it is possible that muscle stem cells divide symmetrically,
but that one or both daughters divide again, either symmetrically or asymmetrically, so these
cells are not AraC-resistant, despite retention of the stem cell identity. I do often observe multiple
Sca1+ cells in satellite cell clusters, which could be due to symmetric divisions of the satellite-
SP cells. While it is unlikely that all the Sca1+ cells in clusters are quiescent, as Sca1+ cells are
generally found in isolation after AraC treatment, the additional Sca1+ cells could be undergoing
subsequent symmetric or asymmetric divisions. Alternatively, Sca1+ satellite cells have been
demonstrated to be capable of inducing Sca1 expression in neighboring satellite cells and this
could also explain the multiple Sca1+ satellite cells in clusters (Mitchell et al., 2005).

It is also possible that symmetric divisions rarely occur in culture. Wnt7a is primarily
expressed by regenerating muscle fibers and it is possible that culturing cells in the absence of
the myofiber reduces the frequency of muscle stem cell symmetric divisions. Even culturing
satellite cells associated with live myofibers selects for the less damaged muscle fibers that
survive isolation from whole muscle and these fibers may produce less Wnt7a than more
severely injured fibers found in an in vivo environment. Additionally, Wnt7a appears to be most highly expressed by regenerating muscle fibers 4 to 6 days after injury. This could result in the symmetric expansion of muscle stem cells at later stages of muscle regeneration further reducing my ability to detect these symmetric divisions in culture.

The production of Wnt7a by regenerating fibers is likely a mechanism by which muscle stem cells can alter their behavior based on the severity of the muscle injury and coordinate symmetric and asymmetric divisions to regulate the numbers of myoblasts and muscle stem cells (Figure 6.3A, B). A severely damage muscle will release more Wnt7a, resulting in a greater expansion of muscle stem cells. The increased population of muscle stem cells will then be able to generate a larger population of myoblasts to repair the muscle, as evidenced by the muscle hypertrophy measured following Wnt7a over-expression (Le Grand et al., 2009). Therefore, Wnt7a release by injured muscle fibers could regulate the size of the muscle stem cell population to ensure the optimal number of stem cells for repair (Figure 6.4A, B).

Jam2 is expressed on the majority of myoblasts after the first division and its extracellular domain promotes quiescence in neighboring cells. Thus, the expression of Jam2 by myoblasts could allow a muscle stem cell to gauge the number of myoblasts present. If there is a high density of satellite cells, a greater number might retain or adopt the muscle stem cell identity, withdrawing from the cell cycle until needed. Conversely, if the number of Jam2 expressing myoblasts decreases, muscle stem cells might reactivate and divide to generate more myoblasts. Therefore, the clustering of satellite cells could provide immediate feedback as to the number of satellite cells available to repair the muscle. This would allow the regulation of the number and behavior of both muscle stem cells and myoblasts through subsequent symmetric and
Figure 6.4: Wnt7a promotes the symmetric division of muscle stem cells. A model depicting the response of muscle stem cells to Wnt7a. Low levels of Wnt7a are produced by muscle fibers following a minor injury and, as a result, muscle stem cells undergo fewer symmetric divisions resulting in a reduced injury response (A). Severely damaged muscle fibers release more Wnt7a, resulting in the expansion of the muscle stem cell population through asymmetric division and an increased myogenic response (B).
asymmetric divisions.

Satellite cells activate with in minutes after muscle injury (Jones et al., 2005) and are capable of rapid proliferation but do not initially divide for the first 1 to 2 days after injury. There is substantial evolutionary pressure on animals to quickly restore muscle function and any unnecessary pause before regeneration would presumably be selected against. The only explanations for this delay in proliferation are either quiescent satellite cells simply cannot enter their first division after injury any faster due to physical limitations or that this delay somehow increases the speed or efficiency of muscle regeneration. Allowing satellite cells to migrate to the site of injury and to assess the severity of damage and the number of available satellite cells would allow satellite cells to organize their numbers and behavior in manner that would allow the most effective muscle repair possible.

The Repair Of Skeletal Muscle

Satellite Cells As Transient Amplifying Cells

The non-stem cell satellite cells in uninjured muscle may be analogous to the transient amplifying populations generated by other stem cells. Satellite cells are heterogeneous in their commitment to myogenesis (Beauchamp et al., 2000) and their proliferative potential (Schultz and Jaryszak, 1985). This variation can be explained if satellite cells consist of both muscle stem cells and more committed transient amplifying cells at various stages of progression toward myogenesis. Even in uninjured muscle, myonuclei must be generated to maintain muscle function and it would expected that a myoblast population be present in the muscle to provide differentiated muscle cells. However, as the requirement for new myonuclei is much lower in
healthy muscle than during injury, a much lower level of proliferation would be required than during injury repair. Thus, transient amplifying cells may exit the cell cycle, comprising a committed fraction of the quiescent satellite cell population, reactivating as needed.

The number of satellite cells in skeletal muscle increases following exercise and decreases during atrophy (Schmalbruch and Lewis, 2000; Smith et al., 2001). This variation could be due the expansion and reduction of the transient amplifying population as needed. The proliferative capacity of satellite cells, measured by their ability to form large myogenic colonies, varies extensively among satellite cells (Schultz and Jaryszak, 1985). However, the average proliferative capacity of satellite cells purified from an injured muscle is greatly decreased, relative to those purified from uninjured muscle (Schultz and Jaryszak, 1985), indicating that a lower proliferative capacity could be due to a greater progression toward myogenesis. Thus, the heterogeneity of proliferative potential among satellite cells could reflect variability in myogenic commitment. Indeed, differentiating myoblasts are detected as early as 12 hours following muscle injury (Rantanen et al., 1995), likely indicative of their progression to the brink of differentiation prior to the injury. By maintaining a transient amplifying population of satellite cells along side muscle stem cells, myonuclei could continuously be generated as needed by the healthy muscle while the muscle stem cell population would be protected from the stress of frequent activation and division.

*The Balance Of Proliferating And Differentiating Myoblasts*

Myoblast behavior must be regulated during muscle regeneration to ensure fast and complete muscle repair. To restore muscle function quickly, myoblasts must begin to differentiate
and fuse with the damaged muscle shortly after injury. However, to ensure that enough myonuclei are generated to completely repair the muscle, a portion of myoblasts must remain proliferative. Therefore, designating proliferating and differentiating populations of myoblasts during muscle repair would be an effective strategy to balance a fast restoration of muscle function while ensure complete repair. However, as the population of differentiating myoblasts would constantly decline due to fusion with the fiber and the population of proliferating myoblasts would expand through cell division, constant communication between satellite cells would be necessary to regulate the number of proliferative myoblasts that commit to differentiation.

The heterogeneity and pattern of Sca1, Pax7, MyoD and Myogenin expression in clusters of satellite cells suggests that these clusters are organized into differentiating, proliferating and self-renewing compartments. Notch transcriptional activity is only detected in a subset of cells in these clusters and appears to pattern with MyoD+ satellite cells. The restriction of Notch activity to a subset of cells in these clusters is reminiscent of Notch’s role in lateral inhibition during development. In lateral inhibition, Notch activity inhibits expression of Notch ligand and the natural variation in Notch activation among cells is amplified. A cell with greater levels of Notch activity would express less Notch ligand a cell, leading to reduced Notch activity and greater Notch ligand expression in surrounding cells. This then generates a feedback loop that organizes the pattern of Notch expression and cell identity (Lewis, 1998).

In satellite cells, Notch-1 promotes proliferation and inhibits differentiation (Conboy and Rando, 2002; Conboy et al., 2003; Carlson et al., 2008). Additionally, expression of the Notch ligands, Jagged (Lindsell et al., 1995) and Delta-1 (Jarriault et al., 1998) in adjacent cells
prevents myoblast differentiation. Thus, the patterning of Notch activity could reflect the mechanism by which satellite cells designate myoblast populations to proliferation and differentiation (Figure 6.5).

Additionally, the asymmetric distribution of Numb in dividing myoblasts could result in asymmetric divisions that generate daughters committed to either proliferation or differentiation. Numb is frequently inherited asymmetrically along with the template DNA strands during later myoblast divisions (Conboy and Rando, 2002; Shinin et al., 2006; Conboy et al., 2007). As neither daughter cell generated by these divisions enters quiescence (Shinin et al., 2006; Conboy et al., 2007), it is unlikely that these asymmetric divisions reflect satellite self-renewal and may instead generate a proliferative and differentiating myoblasts daughter cell.

The frequency with which asymmetric inheritance of template DNA is observed in satellite cells and myoblasts throughout regeneration and the observation that neither resulting daughter cell resulting is quiescent suggests that it is likely to be a mechanism of self-renew (Shinin et al., 2006; Conboy et al., 2007). Instead, it is possible that the template DNA contains DNA methylation patterns, specific to a proliferating or differentiating myoblasts, and serves as an additional means to asymmetrically regulate myoblasts fate between the daughter cells. The ectopic expression of Notch1 in satellite cells inhibits differentiation while ectopic expression of Numb promotes satellite cell differentiation (Conboy and Rando, 2002) suggesting that the regulation of Notch signaling by asymmetric Numb could additionally regulate the transition from proliferation to differentiation, generating differentiating myoblasts while maintaining a population of proliferative myoblasts (Figure 6.5). Thus, similar mechanisms of asymmetric division could be used in both muscle stem cells and committed myoblasts.
Figure 6.5: Myoblast proliferation and differentiation is regulated by Notch signaling. A model depicting potential roles of Notch signaling in regulating myoblast behavior. Through lateral inhibition or asymmetric regulation during division, myoblasts can organize themselves into proliferating and differentiating groups and maintain this balance through regeneration, despite the loss of differentiating myoblasts through fusion and the increase in proliferating myoblasts through division.
Satellite cells must also be able to modulate the injury response based on the severity of muscle damage to effectively regenerate the muscle. If a large proliferative population is generated in response to a minor muscle injury, more myoblasts than necessary would be generated resulting in muscle hypertrophy. Conversely, the maintenance of a small proliferative population of myoblasts after a massive injury would result in slow or incomplete muscle repair. FGF-2 signaling provides a potential mechanism for the damaged muscle tissue to communicate the extent of injury to satellite cells.

FGF-2 promotes satellite cell activation (Yablonka-Reuveni and Rivera, 1994) and proliferation (Olwin and Hauschka, 1986) and inhibits differentiation (Allen and Boxhorn, 1989; Jones et al., 2005). The mitogenic effect of FGF-2 may be due to the manipulation of Pax7 levels. Satellite cells cultured in differentiating conditions rapidly elevate cellular levels of MyoD before differentiating. Satellite cells under proliferative conditions delay this increase in MyoD expression and maintain a balance between Pax7 and MyoD levels. High levels of MyoD promote myoblast differentiation (Lassar et al., 1989) and over-expression of Pax7 blocks negatively regulates MyoD and blocks differentiation (Zammit et al., 2006; Olguin et al., 2007; Collins et al., 2009) suggesting that the balance between these proteins determine the fate of satellite cells. Satellite cells cultured in the presence of FGF-2 have increased levels of Pax7, which may in turn lead to reduced MyoD levels and a prolonged proliferative state. Maintaining a greater number of myoblasts in proliferation would then lead to an increased total number of myoblasts and a greater injury response (Figure 6.6A, B).
Figure 6.6: FGF-2 regulates myoblast number. A model depicting the potential role of FGF-2 signaling in regulating myoblast proliferation. After a minor injury, little FGF-2 is released by the damaged fibers resulting in the proliferation of only a few myoblasts and a small injury response (A). After a severe injury, a higher level of FGF-2 is released, resulting in a greater number of proliferative myoblasts and a larger injury response (B).
Treating satellite cells with FGF-2 increases myoblast number and culturing donor cells with FGF-2 before transplantation into an injured host muscle greatly increases the contribution of donor satellite cells to the host muscle (John Hall, Thesis). FGFR1 satellite cells display an opposite phenotype as they fail to resist differentiation in culture and do not effectively repair muscle \textit{in vivo} (John Hall, Thesis). Thus, it appears that FGF-2 increases the myogenic response of satellite cells through the maintenance of a larger proliferative myoblast population. FGF-2 is expressed by injured muscle (Kästner et al., 2000) and FGF-2 levels likely reflect the severity of muscle damage. A severe injury would result high levels of FGF-2, inhibiting the myoblast transition from proliferation to differentiation, resulting in a larger number of proliferative myoblasts and thus, increased myoblast contribution to muscle repair (Figure 6.6A, B).

**Conclusion**

The impressive capacity of skeletal muscle to fully regenerate from an extensive range of injuries as well as increase or decrease in size and force output depends on a source of new myonuclei. Therefore, satellite cells must be flexible in their behavior, capable of assessing the needs of the muscle and responding appropriately. Through a multitude of signaling pathways, muscle stem take measure of their environment and react accordingly, balancing self-renewal with the generation and maintenance of proliferating myoblasts. Thus, satellite cells have evolved to address the needs of the organism and ensure the optimal function of skeletal muscle.
References


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