

Context Matters: In Vivo and In Vitro Influences on Muscle Satellite Cell Activity

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ABSTRACT

Skeletal muscle is formed during development by the progressive specification, proliferation, migration, and fusion of myoblasts to form terminally differentiated, contractile, highly patterned myofibers. Skeletal muscle is repaired or replaced postnatally by a similar process, involving a resident myogenic stem cell population referred to as satellite cells. In both cases, the activity of the myogenic precursor cells in question is regulated by local signals from the environment, frequently involving other, non-muscle cell types. However, while the majority of studies on muscle development were done in the context of the whole embryo, much of the current work on muscle satellite cells has been done in vitro, or on satellite cell-derived cell lines. While significant practical reasons for these approaches exist, it is almost certain that important influences from the context of the injured and regenerating muscle are lost, while potential tissue culture artifacts are introduced. This review will briefly address extracellular influences on satellite cells in vivo and in vitro that would be expected to impinge on their activity. *J. Cell. Biochem.* 105: 663–669, 2008. © 2008 Wiley-Liss, Inc.

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Satellite cells are believed to be the primary adult stem cell type responsible for repair and regeneration of injured skeletal muscle. They exist as single cells distributed fairly uniformly throughout the muscle, located at the periphery of differentiated myofibers, and are mitotically quiescent in the absence of injury or disease. During quiescence, they are isolated from most extracellular signals by their location between the sarcolemma and external lamina of their host myofiber. Upon injury, they will become “activated,” exit the myofiber, and proceed to proliferate extensively, migrate to the site of injury, and differentiate into new muscle, fusing with existing damaged muscle or other satellite cell-derived myocytes. Lacking the well-defined “niche” common to other adult stem cell types even during quiescence, their regulatory environment when activated is instead composed of the multiple cell types either initially present in the injured muscle or recruited post-injury.

While much of the focus of skeletal muscle research in the last 10 years has shifted from development to regeneration, many of the essential questions remain the same, whether the myogenic precursor cells in question are in the embryo (somatic myoblasts) or the mature organism (satellite cells): Where do these cells come from? How are they specified to the myogenic lineage? What drives their commitment to myogenic differentiation? How is patterning

established in the resulting muscle? What are the genetic hierarchies within the cell, and the signals from outside the cell, that control timely and appropriate progress through myogenesis?

In the case of development, significant advances towards answering these questions have been made in the mouse and chick embryo systems. Using knockout technology, cell labeling, grafting and ablation studies, factor “bead” experiments, and cell transplantation, models have been constructed and refined that include not only the embryonic myoblasts themselves, but also their interactions with surrounding cell types such as the primitive nervous system, the overlying ectoderm, and the lateral plate mesoderm [reviewed in Kablar and Rudnicki, 2000; Christ and Brand-Saberi, 2002; Pownall et al., 2002].

Unfortunately, most of these powerful approaches are not, or are not yet, feasible to apply to satellite cell biology. The majority of experiments aimed at dissecting the physiology of satellite cells have been done on immortalized myogenic cell lines, and even primary cells are mainly evaluated in tissue culture. There are strong practical reasons for these approaches: satellite cells are rare in the muscle tissue, and make up a very small fraction of the total muscle mass, making them difficult to analyze as a population in the tissue. While application of new methods such as fluorescence-activated cell sorting and expression of transgenic selectable markers have

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made mass satellite cell isolation more feasible than in the past, they are still difficult to isolate in sufficient numbers for biochemical analysis without expansion. Satellite cells are activated by the isolation process, and therefore it is problematic to catch cells in the quiescent state. Satellite cells are also particularly refractory to re-graftment after they have been isolated and cultured, hampering both potential in vivo experiments and avenues of cell-based therapies. Finally, the adult animal is much more difficult to manipulate and interpret on a molecular scale than the embryo. Thus, in vitro studies on expanded populations of primary satellite cells, or on myogenic cell lines, have been the most feasible methods to examine the biochemical and genetic pathways that direct muscle regeneration.

While it is by no means a new observation, it is worth considering, particularly for those new to the field, the caveats of drawing conclusions about cell activity in vivo based upon experiments done in vitro. In the case of muscle injury in particular, there are many local players: the muscle fiber and its associated extracellular matrix; blood vessels, connective tissue, and neurons (which are likely to have suffered damage as well, at least in cases of myotrauma); the fibroblasts that populate the connective tissue and the extracellular matrix they produce; and non-resident cells recruited to the injury would all be expected to influence satellite cells in vivo. Tissue culture, in addition to lacking these in vivo interactions, will also introduce extracellular conditions on the satellite cells that will affect their activity. There are many excellent reviews available that cover in detail the characteristics of muscle satellite and other stem cells [Hawke and Garry, 2001; Shi and Garry, 2006; Zammit et al., 2006; Peault et al., 2007]; as a complement, this review will attempt to briefly address some characteristics of the signaling environments satellite cells will experience both in vivo and in vitro, and their physiological differences from each other.

IN VIVO: THE MUSCLE FIBER

Satellite cells are intimately associated with the differentiated muscle around them. During quiescence, they are sandwiched between the myofiber sarcolemma and external lamina of their host myofiber. They are activated by local damage to the muscle fiber, and will coordinate their activities during regeneration with the physiological requirements particular to the current injury. Accordingly, extract of crushed muscle is one of the most potent mitogens for primary satellite cells [Bischoff, 1986b]. The primary component of crushed muscle extract is hepatocyte growth factor (HGF), a heparin-binding, multifunctional cytokine that is currently the most likely candidate for the initial satellite cell activating factor [Allen et al., 1995]. The active form of HGF is present in uninjured muscle bound to heparan sulfate proteoglycan in deposits localized on the fiber surface, and is released when muscle is damaged [Tatsumi et al., 2006]. HGF is bound by its high-affinity receptor c-met, which is expressed on all satellite cells during both the quiescent and activated stages [Cornelison and Wold, 1997]. HGF has multiple effects on satellite cells, and has been shown in vivo and in vitro to promote activation, proliferation, differentiation, and chemotaxis [Bischoff, 1997; Sheehan and Allen, 1999].

Several members of the FGF family of growth factors are also associated with muscle injury. FGF-2, which is a potent mitogenic and survival factor for satellite cells [Sheehan and Allen, 1999], is released from the myofiber upon injury [DiMario et al., 1989] and is later expressed by proliferating myoblasts as well [Anderson et al., 1991]. Blocking antibodies directed against FGF-2 injected in vivo [Lefaucheur and Sebillé, 1995] decrease both the number and diameter of regenerating myofibers, underscoring its importance in satellite cell proliferation and the requirement for coordinated proliferation and differentiation.

Insulin-like growth factor (IGF)-I and IGF-II each increase following muscle injury, and are produced by both myoblasts and de novo differentiating myofibers [Jennische et al., 1987]. Their actions are non-redundant and promote both myoblast proliferation and myofiber differentiation [Engert et al., 1996; Coolican et al., 1997] as well as enhancing muscle cell survival and hypertrophy both in vivo and in vitro [Stewart and Rotwein, 1996]. These actions have made them popular targets for therapies aimed at reducing atrophy and enhancing regeneration in diseases such as muscular dystrophy or in aging [reviewed in Machida and Booth, 2004; Glass, 2005]. While not as intensely studied as IGFs, vascular endothelial growth factor (VEGF) appears to have similar expression, effects and potential in regeneration [Germani et al., 2003].

In addition to secreted factors, it appears that contact-mediated signaling from the host myofiber also has a role in regulating satellite cell activity. Bischoff noted that in single-fiber culture, satellite cells adhering to viable myofibers proliferate at a slower rate than cells on a dead fiber or an empty lamina tube [Bischoff, 1990]. However, the nature of the interactions that cause this effect remains unknown.

IN VIVO: CONNECTIVE TISSUE, FIBROBLASTS, AND ECM

Muscle fibers are surrounded by an external lamina made up of type IV collagen, laminin, and heparan sulfate proteoglycans; the interstitial matrix that surrounds them contains collagen types I, III, and V, fibronectin, and perlecan [reviewed in Grounds, 1990]. These structures provide physical stability and orientation, sequester and present heparin-binding growth factors such as HGF and FGF, participate in signaling to the differentiated fibers through dystroglycan and sarcoglycan complexes, and transduce mechanical forces from the tendons. Specific components of the muscle matrix mediate satellite cell adhesion, motility, and proliferation; the matrix is also continually modified by the action of metalloproteases released by inflammatory cells, injured myofibers, and satellite cells themselves [Grounds et al., 2005]. Injury-specific matrix components such as osteopontin are also incorporated, further diversifying the adhesion possibilities during regeneration [Hirata et al., 2003].

The chemokine SDF-1/CXCL12 has also been shown to be secreted by muscle fibroblasts to signal to satellite cells [Ratajczak et al., 2003]. SDF-1, whose cellular receptor is CXCR4, is uniquely associated with chemotaxis and homing of many different types of adult stem cells [reviewed in Miller et al., 2008]. Both SDF-1 and

CXCR4 are required for appropriate muscle development, and SDF-1 signaling is both mitogenic and motogenic when added to the myogenic cell line C2C12 [Odemis et al., 2007]. Particularly intriguing in the context of satellite cell activity is the suggestion that SDF-1 can attract satellite cells or other mesenchymal stem cells to both a quiescent niche and a site of recent injury, a potentially bimodal effect that could mediate both early events in muscle regeneration and repopulation of the satellite cell compartment afterward [Miller et al., 2008].

IN VIVO: VESSELS AND VESSEL-ASSOCIATED CELLS

Blood vessels and microvasculature are critical for supplying oxygen to skeletal muscles; injury to the muscle tissue usually includes concomitant injury to the vessels. Vessel-associated cells such as pericytes have recently been proposed to be a source of myogenic stem cells distinct from satellite cells [Dellavalle et al., 2007]. Originally described by the Cossu lab as mesangioblasts [De Angelis et al., 1999], these cells may be competent to contribute not only to muscle regeneration but also to the production of new satellite cells, a suggestion that is partially supported by the finding that myoblasts and endothelial cells derive from a common somitic progenitor [reviewed in Buckingham et al., 2003]. If there is cooperation or joint participation in regeneration and its aftermath, an analysis of the crosstalk between satellite cells and non-satellite stem cells could yield important insights into maintenance or acquisition of the satellite “stem” cell state. While the field has not yet reached a consensus on their role in muscle repair, these cells present very intriguing possibilities for advances in gene and cell therapies for muscle disease and dysfunction as well as basic inquiry into satellite/stem cell biology.

With respect to other cells of the vasculature, there is significant evidence for a functional relationship if not a lineal one. It has recently been shown that quiescent satellite cells are closely associated with capillaries, potentially allowing them to interact rapidly upon activation with neighboring endothelial cells. After activation, satellite cell-derived myoblasts and endothelial cells participate in reciprocal signaling involving VEGF and possibly also FGFs and IGFs [reviewed in Christov et al., 2007]. This crosstalk would facilitate regeneration by coordinating myogenesis and neovascularization, to provide the growing muscle with adequate and appropriate blood supply according to the conditions present in the tissue.

IN VIVO: NERVES AND INNERVATION

While innervation is critical for muscle fiber survival, growth, and activity, and a class of neuromuscular diseases such as muscular dystrophy and ALS appear to involve lesions in both muscles and their associated motor neurons, very little work has been done to date on the potential influences of neuronal cells on satellite cells or the early events in muscle regeneration. This may be due to the prevailing opinion that the role of the neuron in myogenesis is limited to electrical stimulation of the myofiber. However, recent

experiments using conditioned medium from embryonic neurons suggest that neuronally derived soluble factors, possibly sonic hedgehog (Shh) or neurotrophin 3, can both promote satellite cell proliferation and prevent satellite cell apoptosis in culture [Pelletier et al., 2006]. The local effects of damaged and regrowing axons and glia on satellite cells represent an unexplored but potentially significant area for both basic and clinical research.

IN VIVO: INFLAMMATORY CELLS

The primary non-resident cell types satellite cells encounter and interact with are infiltrating immune cells; the most commonly studied in the context of satellite cell-mediated muscle regeneration are macrophages and monocytes. Pioneering work by Miranda Grounds demonstrated that depletion of macrophages and monocytes impairs subsequent muscle regeneration [Grounds, 1987] and that satellite cells and leukocytes mutually attract one another, via signaling through small chemokines [Robertson et al., 1993]. While the role of macrophages was originally thought to involve only the clearing of damaged cells through phagocytosis, these studies and others have revealed a critical role in both the initiation and progression of satellite cell-mediated regeneration and repair [reviewed in Tidball, 2005].

Several groups have investigated crosstalk between leukocytes and satellite cells, particularly with respect to chemoattraction. Satellite cells have been shown to secrete multiple pro-inflammatory cytokines including IL-1, IL-6, TNF- α , and MCP-1/CCL2 [reviewed in Chazaud et al., 2003]. Satellite cells *in vitro* express MDC/CCL22, MCP-1/CCL2, FKN/CXC3CL1, VEGF, and urokinase plasminogen activator and its receptor (uPA/uPAR), which together account for nearly 80% of their monocyte chemoattractant potential [Chazaud et al., 2003]. Proposed reasons for this attraction *in vivo* include aiding in satellite cell escape from the fiber external lamina, generation of proteolytic fragments of ECM that will act as signaling factors for satellite cell proliferation or chemotaxis, and secretion of pro-growth and anti-apoptotic signaling factors as detailed below.

Hematopoietic cells are the source of a wide variety of diffusible signaling factors, as well as matrix components and matrix-modifying factors. Macrophage-conditioned medium is mitogenic for satellite cells and increases the number of MyoD⁺ cells [reviewed in Tidball, 2005]; while the identity of the active factor(s) remains undefined as yet, macrophages secrete IGF-I and II, HGF, FGFs, PDGF-BB, EGF, and IL-6 [reviewed in Chazaud et al., 2003], all of which have mitogenic and/or pro-myogenic effects. Adhesive signals through fragments of ECM liberated by macrophage digestion have been proposed to have a chemoattractive or mitogenic effect on satellite cells *in vivo*; while it is a much newer concept, adhesion-based signaling between satellite cells and macrophages has also been shown to impinge on satellite cell and myotube survival [Sonnet et al., 2006].

IN VIVO: ENDOCRINE AND CIRCULATING FACTORS

While all of the factors discussed to this point have been active at the level of paracrine or cell-cell interactions, endocrine or systemic

influence from the whole organism can readily affect satellite cell activity. Muscles of young animals regenerate more efficiently than those of old ones, yet the experiments of Carlson and Faulkner [1989] showed that whole muscles transplanted from a young rat to an old one would regenerate according to the norm for the age of the animal, not the graft. This result was also found when treating satellite cells from young or old mice with crushed muscle extract from young or old mice [Mezzogiorno et al., 1993]. Most recently, this was elegantly demonstrated by the parabiosis of a young mouse with an aged one, followed by injury: the injured muscle from either mouse recovered to an intermediate degree, and the possibility of a contribution from young cells to the regenerating aged muscle was ruled out [Conboy et al., 2005]. This work, done in the Rando lab, proposes that circulating factors influence the activity of the Notch contact-mediated signaling pathway between satellite cells and their surrounding muscle, and that increased Notch signaling is required for successful regeneration. The identity of the circulating factor or factors has yet to be determined.

Other circulating factors that have been shown to affect satellite cell activity *in vivo* include insulin, androgens, IL-6, growth hormone, and HGF produced in sites other than the muscle [reviewed in Hawke and Garry, 2001; Shi and Garry, 2006]. However, many of the existing reports on these factors are conflicting, suggesting that additional focused research will be necessary to begin to define their physiological roles in muscle regeneration.

IN VIVO: PHYSICAL STRESSES

In addition to molecular factors, physical stress such as stretch has not only been suggested as a potential activating mechanism for satellite cells (via synthesis of NO and subsequent release of HGF) [Tatsumi et al., 2006] but has also been shown to affect satellite cell activity *in vitro*. Cyclic or static stretch of C2C12 or primary satellite cells on specially designed tissue culture plates such as Flexiwell produces wide-ranging effects on cell shape [McGrath et al., 2003], production and secretion of cytokines [Tatsumi et al., 2002], cell cycle [Kook et al., 2008], intracellular signaling [Zhang et al., 2007], and gene expression [Rauch and Loughna, 2005]. However, particularly in the absence of connective tissue attachments that would normally transduce contractile force *in vivo*, replicating (rather than simulating) these forces *in vitro* has not yet been accomplished. Thus, while the *in vivo* environment will certainly include the application of physical stresses to satellite cells and their differentiated progeny that will exert pleiotrophic effects on the regeneration response, we are only beginning to be able to experimentally dissect and quantify these influences using current technology.

IN VITRO: MYOGENIC CELL LINES

Due to the technical difficulties associated with isolating and maintaining cultures of primary satellite cells, immortalized cell lines are frequently used as satellite cell models. The most commonly

used cell line, C2C12, was isolated from clonal cultures derived from thigh muscles of 2-month-old C3H mice 70 h after crush injury [Yaffe and Saxel, 1977]. Although they are fibroblastic in appearance while proliferating, when they are cultured in low serum (2% instead of 20%) or at high density they robustly form multinucleate myotubes. Their transcriptional and cell-signaling responses remain sufficiently similar to those of primary embryonic and adult myoblasts that they have been used broadly and successfully to model both: much of the data cited in this review was produced in C2C12 cells. However, there are still many caveats associated with their use, such as their morphology (what changes in adhesion factors led to such a significant change?), immortal state (how has the cell cycle machinery been altered to allow escape from senescence?) and divergence from patterns of gene expression seen in recently harvested primary satellite cells (in one study, over 25% of regulatory genes surveyed were present in C2C12 but absent in satellite cells [Cornelison, 1998]).

MM14 cells, which are used less frequently, were derived from leg muscle of a 2-month-old Balb/C male mouse [Linkhart et al., 1980]. Their morphology and transcription profile are more similar to primary cells than are C2C12 [Cornelison, 1998] making them a potentially more appropriate model. However, they also have some known differences from primary satellite cells, including an absolute dependence on exogenous FGF stimulation during G1 to prevent terminal differentiation [Linkhart et al., 1980].

Both of these cell lines are frequently referred to in the literature as “satellite cells,” however due to their physical and biochemical differences from primary cells this is not strictly appropriate. Ideally, conclusions reached from experimentation on immortalized myoblast lines such as these should be tested in primary cells, *in vivo* if possible, instead of assuming that the same principles will necessarily convey.

IN VITRO: TISSUE CULTURE SUBSTRATES

Cells grown in a monolayer *in vitro* are adhered to treated plastic, frequently coated with one or more purified extracellular matrix proteins. While C2C12 cells are grown on uncoated dishes [Yaffe and Saxel, 1977], MM14 cells are cultured on gelatin-coated dishes [Linkhart et al., 1980], and primary cells are grown on these as well as various substrates including entactin-collagen-laminin (ECL) and Matrigel (BD Biosciences), a membrane preparation from a mouse sarcoma line containing undefined amounts of multiple ECM components, growth factors, and cytokines. While these substrates support adhesion, differentiation, and, in some cases, migration, it would be naïve to assume that satellite cell behavior under any of these conditions is entirely reflective of an *in vivo* situation.

A partial solution to this difficulty is to isolate and culture whole living myofibers, with their attached satellite cells [Bischoff, 1986a; Cornelison and Wold, 1997]. The satellite cells are activated by the isolation procedure, and will emerge from beneath the myofiber basal lamina, activate the myogenic program, proliferate, and eventually both populate the fiber surface and emigrate from the host fiber (particularly when the fibers are also cultured in Matrigel). However, while satellite cells *in vivo* would be presumed to contact

the matrix in three dimensions, culture on the surface of a fiber still leaves them exposed to media on all but one surface, which represents only one of the ECM environments that would be represented in vivo.

IN VITRO: TISSUE CULTURE MEDIA

Myoblasts in vitro are typically cultured in medium (DMEM or F-12) supplemented with either fetal bovine serum (C2C12 cells) or horse serum (MM14 cells or primary myoblasts), a source of growth factors such as chick embryo extract or purified growth factor, and occasionally additional factors such as insulin. It has been determined by several labs that exposure to culture renders adult myoblasts dramatically less viable for re-engraftment [Smythe and Grounds, 2000], a difficulty that must be overcome if cell-based therapies are to become successful [Peault et al., 2007].

In addition to exposure to potentially deleterious external factors or concentrations of factors, primary cells in culture are also to an unknown extent deprived of the paracrine and endocrine factors discussed above. Use of crushed muscle extract as a source of physiological stimuli would potentially provide a full complement of soluble factors, but this is rarely done, primarily because of the undefined makeup of CME, questions of appropriate concentration, and technical inconvenience. Single-fiber culture allows cells access to paracrine factors secreted by their host myofiber, but the extent to which this is physiologically relevant in a floating culture situation is unclear.

IN VITRO: OXYGEN TENSION

It has been established in several systems that stem cells, possibly due to their function in vivo in early tissues or in less-vascularized niches, maintain “stemness” better and are more proliferative under physiological oxygen tension (about 6% O₂) or even “hypoxia” (about 2% oxygen) [reviewed in Csete, 2005]. Adult stem cells, including satellite cells, display physiologically abnormal traits when cultured under standard lab conditions, which include ambient oxygen tension (about 20% at sea level). Compared to satellite cells cultured at 5% O₂, satellite cells in room air display deleterious phenotypes including delayed proliferation and expression of pro-myogenic genes, inefficient differentiation, increased apoptosis, and an increased tendency to transdifferentiate into adipose cells [Csete et al., 2001].

Redox conditions under high oxygen tension may account for some of these effects, particularly the increase in apoptosis. In addition, many of the secreted factors described above, including HGF, SDF-1, TNF- α , and VEGF are either directly or indirectly regulated by oxygen [Csete, 2005]. However, given the difficulties already stated above in analysis of satellite cells in vivo, it is difficult to make comparative observations. Even maintenance in O₂-controlled incubators is unlikely to successfully mimic physiological gas conditions. This is not only because cells will be exposed to room air when they are removed from the incubator and manipulated, but because the actual oxygen tension in the cell is

function not only of the O₂ in the ambient air, but of medium depth, cell density, cellular respiration, etc. [Csete, 2005].

CONCLUSIONS AND FUTURE DIRECTIONS

As inquiry into satellite cell biology has expanded in both quantity and scope, there has been an increasing emphasis on the satellite cell primarily as a stem cell, rather than as a muscle cell. This has led to conceptual and technical innovations including novel markers for satellite cells in different stages, insight into potential “niche” interactions, promising new isolation and engraftment techniques for cell-based therapies, and a greater understanding of satellite cell potential. In particular, comparisons with other adult stem cells including hematopoietic stem cells and other mesenchymal stem cells have suggested novel avenues of research into the role of local “niche” signals in muscle regeneration. Today, thanks to these advances in identification, isolation, and analysis, our knowledge of what a satellite cell is, does and can do has advanced dramatically since the cells were first identified almost 50 years ago. A major hurdle yet to be crossed, however, is finding ways to do so reliably, reproducibly, and quantitatively in vivo. It is to be hoped that, in conjunction with lessons learned from myogenesis in the embryo, this will finally lead to a more complete picture of the satellite cell, in context.

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