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## S1P lyase in skeletal muscle regeneration and satellite cell activation: Exposing the hidden lyase★

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### Abstract

Sphingosine-1-phosphate (S1P) is a bioactive sphingolipid whose actions are essential for many physiological processes including angiogenesis, lymphocyte trafficking and development. In addition, S1P serves as a muscle trophic factor that enables efficient muscle regeneration. This is due in part to S1P's ability to activate quiescent muscle stem cells called satellite cells (SCs) that are needed for muscle repair. However, the molecular mechanism by which S1P activates SCs has not been well understood. Further, strategies for harnessing S1P signaling to recruit SCs for therapeutic benefit have been lacking. S1P is irreversibly catabolized by S1P lyase (SPL), a highly conserved enzyme that catalyzes the cleavage of S1P at carbon bond C<sub>2-3</sub>, resulting in formation of hexadecenal and ethanolamine-phosphate. SPL enhances apoptosis through substrate- and product-dependent events, thereby regulating cellular responses to chemotherapy, radiation and ischemia. SPL is undetectable in resting murine skeletal muscle. However, we recently found that SPL is dynamically upregulated in skeletal muscle after injury. SPL upregulation occurred in the context of a tightly orchestrated genetic program that resulted in a transient S1P signal in response to muscle injury. S1P activated quiescent SCs via a sphingosine-1-phosphate receptor 2 (S1P2)/signal transducer and activator of transcription 3 (STAT3)-dependent pathway, thereby facilitating skeletal muscle regeneration. Mdx mice, which serve as a model for muscular dystrophy (MD), exhibited skeletal muscle SPL upregulation and S1P deficiency. Pharmacological SPL inhibition raised skeletal muscle S1P levels, enhanced SC recruitment and improved mdx skeletal muscle regeneration. These findings reveal how S1P can activate SCs and indicate that SPL suppression may provide a therapeutic strategy for myopathies. This article is part of a Special Issue entitled Advances in Lysophospholipid Research.

### Keywords

Satellite cell; Skeletal muscle; Sphingosine phosphate lyase; Sphingosine-1-phosphate; Sphingolipid; STAT3

### 1. Introduction

Sphingosine-1-phosphate (S1P) is a bioactive lipid that binds to a family of five G protein coupled receptors [1]. Through activation of S1P receptors and their G protein partners, S1P modulates the activities of adenylyl cyclase, the Ras-related C3 botulinum toxin substrate (Ras)/mitogen activated protein kinase cascade, AKT and STAT3 signaling, phospholipase

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C and small Ras-homologous (Rho) GTPases. Through these actions, S1P signaling affects cell survival, proliferation, migration and cell–cell interactions [2]. Recent studies have revealed the additional capability of S1P to modulate the activity of intracellular targets including NF $\kappa$ B, histone deacetylases and mitochondrial proteins through molecular mechanisms that appear to be independent of S1P receptors [3]. S1P signaling is essential for many physiological processes including angiogenesis [4,5], hematopoietic cell trafficking [6–8], embryonic development [9,10] and skeletal muscle homeostasis [11].

S1P is generated from sphingosine by a phosphorylation reaction catalyzed by the sphingosine kinases, SphK1 and SphK2 [12]. Sphingosine can be regenerated from S1P through the actions of S1P-specific phosphatases (Sgpp1 and Sgpp2) [13] and nonspecific lipid phosphatases (LPPs) [14]. However, only one enzyme, S1P lyase (SPL), is responsible for the irreversible catabolism of S1P (Fig. 1) [15,16]. SPL is an integral membrane protein located in the endoplasmic reticulum. SPL degrades S1P in the cytosol by catalyzing its cleavage at the C<sub>2-3</sub> carbon bond, resulting in the formation of ethanolamine phosphate and the long chain aldehyde, hexadecenal. S1P activity has a major impact on intracellular S1P stores as well as tissue and circulating S1P levels, illustrated by the marked accumulation of S1P observed in cells, model organisms and genetically modified mice in which the gene encoding SPL has been knocked down or knocked out [17–19]. By removing S1P in cellular, vascular and tissue compartments, SPL regulates the S1P pools available for interactions with intracellular targets and for autocrine and paracrine receptor-mediated signaling [20].

Based on the ability of SPL to generate S1P gradients required for lymphocyte trafficking, SPL inhibition via treatment with the drug LX2931 (Lexicon Pharmaceuticals) is now being tested as a strategy for immunomodulation in the treatment of autoimmune disease such as rheumatoid arthritis [21]. SPL induces apoptosis in response to stressful conditions including DNA damage and ischemia through mechanisms involving both substrate depletion and generation of reactive aldehydes [22,23]. Delivery of a soluble bacterial SPL has also been shown to reduce circulating S1P levels and block the proangiogenic effects of S1P receptor signaling [24]. These findings suggest that modulation of SPL may be useful in the prevention and treatment of a wide variety of human diseases including autoimmunity, cancer, ischemic tissue damage, fibrosis, inflammation and pathological angiogenesis.

The first SPL gene to be identified was the *Saccharomyces cerevisiae* dihydrosphingosine phosphate lyase 1 (*Dp11*) gene [25]. Over the past 15 years, the SPL genes of various bacterial, fungal, nematode, plant and mammalian genomes as well as murine and human *Sgpl1* genes encoding SPL have been identified and characterized. In many cases, knockout models have been generated, and the mutant phenotypes in these organisms have revealed critical roles for SPL in cellular function, development and physiology. Interestingly, *Drosophila Sply* mutants lacking SPL expression exhibit a myopathic phenotype affecting the muscles of the thorax that power the wings and enable flight [26]. Based on this observation, we hypothesized that SPL has an essential and conserved role in skeletal muscle homeostasis. Using a murine model of skeletal muscle injury, we have demonstrated that an S1P signal is generated in response to muscle injury and, through activation of S1P<sub>2</sub>, leads to downstream events that involve the transcription factor STAT3 and the recruitment of skeletal muscle stem cells called satellite cells (SCs) that are needed for efficient skeletal muscle regeneration [27]. Further, we found that dystrophic mdx mice, which serve as a model of Duchenne MD, are S1P-deficient due to chronic injury-induced upregulation of SPL. Pharmacological inhibition of SPL improved SC recruitment and muscle regeneration in a STAT3-dependent manner in mdx mice, thereby illustrating the potential utility of targeting SPL for therapeutic benefit in MD.

This review will highlight our recent findings on the role and mechanism of action of S1P signaling in SC recruitment and muscle regeneration. These observations will be related to the known functions of S1P as a skeletal muscle trophic factor and SC activator. We present some new findings regarding the potential cellular sources of SPL in injured muscle and demonstrate the presence of SPL expression in SC-derived myoblasts. We will discuss remaining questions and propose potential next steps toward further elucidating the biology and clinical potential of modulating S1P metabolism and signaling for therapeutic purposes in human diseases affecting skeletal muscle. We refer readers interested in learning more about SPL structure, function and regulation to numerous recent reviews describing the biochemical characterization of SPL, its subcellular localization, tissue distribution, regulation, role in development, function in apoptosis, development of SPL inhibitors, and structure/function relationships predicted by recent crystallization of a bacterial SPL [16,20,28–31].

## 2. The muscular dystrophies

MDs are a heterogeneous group of genetic diseases characterized by the progressive loss of skeletal muscle strength associated with pathological features including pseudohypertrophy, muscle necrosis and fiber splitting, regeneration and centralized nuclei, variation in fiber size, and eventual muscle replacement by adipose and fibrotic tissues [32]. Together, these effects compromise patient mobility and quality of life, and in the most severe cases lead to premature death. In 1987, Eric Hoffman identified mutations in the dystrophin gene as the cause of the most common and severe form of MD, Duchenne MD (DMD), which affects 1 in 4000 newborn males [33]. The dystrophin protein links the plasma membrane to the internal cytoskeleton through interactions with  $\gamma$ -actin and with a plasma membrane complex called the dystroglycan-associated protein complex (DGC). The DGC also interacts with the extracellular matrix (ECM). These connections anchor the plasma membrane internally and externally, thereby facilitating the distribution of forces generated upon muscle contraction. The lateral distribution of force stabilizes the myofiber and prevents membrane disruption with each contraction. Subsequent to the cloning of dystrophin, over 30 genes have been linked to hereditary MDs [32,34,35]. MD mutations are found in the components of the DGC, DGC-interacting proteins, and enzymes that regulate the expression, modification, and function of the DGC. In addition, mutations in the ECM proteins laminin and collagen VI also cause MD [35]. Disruption of the normal bridge between ECM, DGC and  $\gamma$ -actin in patients with MD leads to high stress on fragile membranes, resulting in membrane lesions that overwhelm the membrane repair and muscle regeneration systems, ending finally in calcium influx and muscle cell death [34,36,37]. In addition, there is evidence that modifying factors independent of the DGC may influence the severity of dystrophy in MD [32,38]. Novel treatments for MD focus on reducing muscle atrophy, inhibiting cell death pathways, and restoring membrane protein scaffolding and repair through the use of skeletal muscle stem cells [39]. Characterizing the signaling pathways that control these processes may reveal new targets for therapeutic intervention in MD and muscle wasting disease. While it remains controversial whether or not SC reserves and/or functions are reduced/impaired in MD, finding ways to expand endogenous stem cell reserves and/or enhance their myogenic potential may be of therapeutic benefit in MD.

## 3. S1P signaling, SCs and skeletal muscle regeneration

Adult skeletal muscle is a unique tissue that has the capacity to regenerate after injury [40]. Skeletal muscle regeneration involves the activation of SCs found between the basal lamina and sarcolemma of each myofiber (Fig. 2). After muscle injury, SCs are stimulated to leave their quiescent state and reenter the cell cycle. SCs proliferate, and their progeny (myoblasts) adhere to and fuse with the damaged muscle cell (multinucleated syncytia or

myofiber), stimulating a program of differentiation that leads to regeneration of healthy tissue [41–43].

S1P generated from catabolism of muscle membrane sphingolipids was recently identified as the signal that causes SCs to enter the cell cycle, whereas chemical inhibition of S1P formation prevented skeletal muscle regeneration [44–46]. This suggests a central role for S1P in skeletal muscle homeostasis. However, the mechanism responsible for this was not known. Rodent muscles express three of the five known S1P receptors, and S1P exerts a trophic action on denervated muscle fibers [46]. Sphingolipids including S1P have also been implicated in the regulation of muscle cell signaling, contractile properties, insulin resistance and muscle fiber trophism [47]. These findings suggest that S1P metabolism and signaling contribute to muscle homeostasis and repair, although the fundamental mechanisms responsible for the actions of S1P in muscle remain poorly understood. The chronic/degenerative phase of MD is caused by a failure of skeletal muscle regeneration to keep up with the ongoing injury and destruction of muscle fibers [48]. This may be accounted for in part by a reduction in SC reserves or SC myogenic potential. Elucidating the specific roles that S1P signaling plays in SC biology, muscle regeneration, aging and MD may help devise new approaches to maintaining muscle function and reducing loss of muscle mass in these diseases.

#### 4. The *Sply* mutant as an invertebrate model for muscular dystrophy

*Sply* mutants harboring a transposon insertion mutation in the gene encoding the *Drosophila* homolog of SPL cannot catalyze the degradation of S1P and accumulate sphingolipid intermediates. *Sply* mutants are flightless due to degeneration of the thoracic flight muscles that power the wings [26]. The thoraces of wild type fruit flies exhibit an invariant pattern of six pairs of dorsal longitudinal muscles (DLMs) as shown in Fig. 3A. In the *Sply* mutants, one or more fibers of the DLM are typically absent, and the severity of this myopathy was observed to increase with age (Fig. 3B). The *Sply* flightless phenotype can be recapitulated by knockdown of *Sply* in myoblast precursor cells using RNA interference, indicating that the defect is intrinsic to muscle (our unpublished observations). Interestingly, we have observed that RNAi-mediated knockdown of *Sply* expression in *Drosophila* S2 cells leads to reduction of dystrophin expression (our unpublished observations). These cumulative findings provide dramatic evidence that sphingolipid metabolism plays a crucial and intrinsic role in muscle stability and that tight control over sphingolipid metabolism is necessary to maintain muscle homeostasis. They also specifically implicate SPL in skeletal muscle homeostasis and suggest that the *Sply* mutant may potentially serve as an invertebrate model of DMD.

#### 5. Murine muscle injury elicits a metabolic response that produces an S1P signal

The genetic and molecular pathways that govern muscle development are largely conserved between *Drosophila* and humans [49,50]. To explore whether the *Sply* phenotype accurately predicts a role for S1P signaling in mammalian skeletal muscle homeostasis, we first examined the expression of the murine SPL gene *Sgpl1* in skeletal muscle at rest. Our findings indicate that SPL is expressed at low or undetectable levels in resting skeletal muscle [27]. However, we have observed that SPL expression is induced in response to various stressful conditions, such as radiation and ischemia [22,51]. This raised the possibility that expression of the lyase, while hidden at rest, may be “exposed” in response to injury. Therefore, we examined the effect of muscle injury on *Sgpl1* as well as other genes involved in S1P biosynthesis, catabolism and signaling. To accomplish this, we employed a commonly used system for analyzing murine skeletal muscle injury, the notexin

(NTX) injury model. NTX is a snake venom phospholipase A2 that when injected into muscle causes small lesions in the sarcolemma, resulting in muscle fiber degeneration. Due to the capacity of skeletal muscle for regeneration, the muscle recovers from this injury over the course of two to four weeks, [37,52,53]. Within 6 h of NTX injection into the gastrocnemius muscles of wild type mice, we observed induction of *Sphk1* gene expression 100-fold over baseline levels as determined by quantitative RT-PCR. The expression of *Sgpl1* was induced 20-fold over baseline several days after injury, concomitant with upregulation of the muscle transcription factor, *Myf5*. Other genes involved in S1P signaling including *Sphk2*, *Sgpp1* and genes encoding S1P receptors 1-3 were upregulated after injury, but the changes were more modest than those of *Sphk1* and *Sgpl1*. Using additional detection methods, we confirmed that SPL gene and protein expression are induced in muscle fibers after treatment with NTX. Not surprisingly, when we measured S1P plasma levels, we found that they are increased by 50% compared to baseline levels within the first 24 h after injury [27]. Thus, a dynamic and tightly orchestrated genetic program modulates S1P metabolism and receptor expression in response to muscle injury. These changes lead to the generation of a plasma S1P “signal.” Based on these findings, it became important to determine the significance of that signal for skeletal muscle regeneration.

## 6. SphK1 is essential for efficient skeletal muscle regeneration and SC functions

Two sphingosine kinases (SphK1 and SphK2) contribute to S1P biosynthesis in mammals. In mice, the disruption of one SphK gene is not lethal, provided the other is functional. Although SphK1 and SphK2 knockout mice appear healthy under routine conditions, some phenotypes have been revealed in response to specific stressful stimuli. For example, SphK1 knockout mice fail to achieve cardiac preconditioning and are more susceptible than control mice to anaphylactic shock, whereas SphK2 knockout mice are more susceptible to kidney ischemic injury [54–57]. These findings suggest that the developmental functions of SphKs are redundant, whereas individual SphKs may be responsible for specific functions in postnatal life.

Therefore, to address whether the profound upregulation of SphK1 and corresponding peak in plasma S1P levels we observed within hours of intramuscular NTX injection signified a specific function for SphK1 in the injured muscle, we compared the biochemical and functional consequences of NTX muscle injury in SphK1 global knockout mice and wild type controls. We found that the skeletal muscles of wild type mice were largely repaired by 10 days after injury. The point of maximal injury exhibited mostly newly regenerated fibers containing a centralized nucleus, the hallmark of a regenerated muscle fiber. In contrast, at the same time point the SphK1 knockout mouse muscles exhibited many residual necrotic fibers and only partial regeneration at the site of maximal injury. Importantly, when we measured SCs at the injury site, SphK1 knockout mice lacked a robust recruitment of SCs compared to wild type mice. In addition, plasma S1P levels in SphK1 knockout mice were approximately half the levels found in the circulation of wild type mice throughout the course of the experiment [27].

To study the requirement for S1P signaling in SC functions in more detail, we isolated SCs from the injured muscle fibers of wild type and SphK1 knockout mice and characterized them by measuring their capacity for proliferation and differentiation using in vitro assays. In comparison to wild type SCs incubated in media supplemented with wild type serum, we observed that SphK1 knockout SCs incubated in media supplemented with SphK1 knockout serum did not perform as well, exhibiting reduced proliferation and differentiation. The functions of the SphK1 knockout SCs were enhanced by incubation with wild type serum, although not to the level of wild type SCs. Conversely, wild type SCs incubated in medium

containing serum from SphK1 knockout mice were also functionally compromised, although not as severely as SphK1 knockout SCs [27]. These experiments revealed that SphK1 induction and S1P signaling contribute to efficient SC recruitment and muscle regeneration in response to acute injury. They also suggest that after muscle injury SCs may respond to S1P signals derived from multiple sources. S1P production by SphK1 within the SC itself may be required for autocrine stimulation. In addition, when the sequestered niche of the SC is compromised by injury, SCs are likely exposed to S1P derived from the injured fiber, the vasculature or from other source within the muscle fiber microenvironment. It seems likely that maximal SC activation requires S1P signals generated from both sources. However, it is also possible that SphK1 knockout mice fail to mobilize other circulating factors independent of or complementary to S1P that are needed for maximal SC activation and recruitment. Further investigation of the cytokine responses in SphK1 knockout mice may reveal more about the contribution of SphK1 and S1P signaling to the global inflammatory response to skeletal muscle injury.

## 7. Dystrophic mice exist in a state of S1P deficiency

Considering the dramatic changes in S1P metabolism and signaling that we observed in response to acute muscle injury, we predicted that chronic, global skeletal muscle injury that occurs in patients suffering from MD might also have an impact on S1P levels and metabolism. If so, this could have potential consequences for muscle regeneration and SC recruitment in these conditions and, conversely, could present opportunities for therapeutic intervention. Male C57BL/10ScSn-Dmd<sup>mdx</sup>/J (mdx) mice harbor a single point mutation in the mouse dystrophin gene located on the X chromosome [58]. This mutation is equivalent to the mutation that causes DMD in humans, making mdx mice a useful model of the disease, although the murine version is milder (due to the presence of revertant fibers and utrophin expression) and has greater capacity for regeneration due to other genetic factors [59]. When we compared the expression levels of the genes by RT-PCR involved in S1P metabolism in the muscles of mdx mice and control mice of the same background, we found no changes in *Sphk1* or *Sphk2* levels but a significant increase in *Sgpl1* expression. The upregulation of *Sgpl1* was confirmed by immunoblotting, which revealed a very high level of SPL expression in the dystrophic muscles of mdx mice compared to controls. Skeletal muscle comprises the largest single organ of the body making up approximately half of the body's mass. Thus, it was perhaps not too surprising that the upregulation of SPL in dystrophic skeletal muscle correlated with a significant reduction of plasma S1P in mdx mice compared to controls. The S1P levels in mdx mouse plasma were as low as those observed in SphK1 knockout mice [27]. This suggested that muscle regeneration and SC recruitment might be compromised by progressive S1P deficiency in dystrophic muscles. Further, it suggests that inhibiting SPL might provide a useful way to boost S1P levels and improve efficiency of skeletal muscle regeneration as therapeutic.

## 8. SPL expression in SCs, myoblasts, myofibers and other cells of skeletal muscle

The findings above point toward an important role for S1P in SC proliferation and a potential role in muscular dystrophy. However, the role in the muscle regeneration process of intracellular S1P and of SPL, the enzyme responsible for degrading S1P and controlling its intracellular levels, have not been fully explored. Specifically, our study did not investigate the cellular source of SPL responsible for increasing SPL levels in skeletal muscle in the aftermath of injury. In our laboratory, we have conducted a pilot study in which SC-derived myoblasts were isolated from mdx mouse skeletal muscles, cultured for 24 h, fixed and stained with specific antibodies for SPL and paired box protein-7 (Pax7), the latter which serves as a marker for SCs. Fig. 4 shows that majority of cells we isolated

express SPL. Further, some of these cells were positive for both Pax7 and SPL. This suggests that SPL expression is present in activated SCs and/or in SC progeny that have differentiated to myoblasts. Our observation is consistent with other results from our laboratory indicating that murine C2C12 cells express high levels of SPL and that knockdown of SPL in this cell line has profound effects on the ability of the cells to differentiate into myoblasts (our unpublished observations).

Skeletal muscle regeneration involves the complex interaction of many cell types, not only the injured fiber and SCs, but also vascular cells, vascular support cells and inflammatory cells that enter the skeletal muscle interstitium in response to injury. In other studies, we have observed that bone marrow-derived blood cells amply express SPL (Borowsky et al., submitted). Thus, inflammatory cells that infiltrate injured skeletal muscle likely contribute to the total amount of skeletal muscle SPL pool after injury. To directly address this possibility, as well as to further explore the presence of SPL in cells within the SC–myoblast–myofiber continuum, cryosections of wild type skeletal muscle (uninjured and 10 days post NTX injection) were stained for SPL and Pax7. We observed that quiescent SC and muscle fibers at rest do not express detectable amounts of SPL (Fig. 5). In response to injury, many mononucleated infiltrating cells were found to express SPL, confirming that some of the SPL “upregulation” we observed in injured muscle can be explained by inflammatory changes. However, we did not observe SPL expression in the regenerating muscle fibers or SCs in situ at 10 days after injury. Taken together with our finding of SPL expression in isolated SCs and SC-derived myoblasts in culture, our histological results suggest that SPL upregulation may largely represent an inflammatory response to muscle injury, but that SPL may also be transiently expressed in activated SCs and/or in the progeny of activated SCs (Fig. 5). Further imaging studies that follow SPL and Pax7 expression in situ throughout the time course of the first week after injury will be required to define whether SPL expression within SC-derived myoblasts may contribute to localized S1P gradients and affect the S1P levels of regenerating fibers in the aftermath of muscle injury. It is interesting to note that acute stress responses have been characterized by rapid and significant lymphopenia. Further, snake venoms, including notexin, have been shown to induce lymphopenia in rats [60,61]. The kinetics of induced lymphopenia after notexin injection, which occurs within 24 h, is similar to the kinetics of SphK1 upregulation and S1P increase that we have observed after notexin-induced injury in mice. Thus, there may be a causal connection between the increased S1P release and retention of cells in peripheral tissues as a mechanism leading to notexin-induced lymphopenia.

## 9. SPL inhibition improves the response to acute muscle injury in mdx mice

Tetrahydroxybutylimidazole (THI) is a byproduct of sugar caramelization that is found in many food substances containing caramel food coloring type III. THI was recently shown to serve as an immunomodulatory agent by virtue of its ability to inhibit SPL [62]. Chemical derivatives of THI have subsequently been developed and are undergoing clinical evaluation for therapeutic efficacy in patients with rheumatoid arthritis [21]. To test the effect of SPL inhibition on muscle regeneration in a dystrophic model, we compared the short-term effects of THI administration compared to vehicle in mdx mice after acute muscle injury with NTX. We observed a significant improvement in the number of regenerating fibers after injury when mice received THI. Quantification of SCs by both flow cytometry and immunostaining revealed that THI treatment improved SC activation as well [27]. These important findings demonstrate that SPL inhibition may have potential as a strategy to improve SC function and recruitment in the context of muscular dystrophy. Interestingly, T-cells have been shown to promote pathology of mdx mice [63]. Further, the immunosuppressant rapamycin ameliorates the dystrophic phenotype of mdx mice [64]. One of the main effects of THI and genetic knockout of SPL is retention of lymphocytes in

lymphoid organs and preventing them from reaching sites of injury and inflammation. Therefore, it is conceivable that such immunosuppressive activity may add to the potential therapeutic efficacy of SPL inhibition in disease states that involve muscle injury.

## 10. THI promotes muscle regeneration and SC recruitment through STAT3 signaling

Zammit and colleagues showed that S1P contributes to the activation of SCs [44], but the molecular mechanism underlying this effect was not well understood. The ability of THI to promote muscle regeneration and SC recruitment gave us a tool with which to investigate this question in vivo. Recent studies by the laboratory of Hua Yu demonstrated the existence of cross-talk between signaling pathways involving S1P and the transcriptional regulator STAT3 [65]. The interactions between S1P and STAT3 were found to be responsible for the chronic STAT3 activation in some types of cancer. Additional reports provided further evidence for S1P and STAT3 signaling interactions in the context of prostate cancer and cardiomyocyte function [66,67]. When we measured STAT3 activation in the skeletal muscles of mdx mice 5 days after NTX injury, THI treatment appeared to have a positive effect, as shown by increased phosphorylation on STAT3 Tyrosine 703 [27]. Importantly, inhibition of STAT3 signaling using the small molecule WP1066 reversed the ability of THI to promote muscle regeneration and increase the number of SCs several days after injury, indicating the importance of STAT3 activation in this process.

## 11. S1P activates SCs via S1P2/STAT3-dependent repression of cell cycle inhibitors

Since S1P can act via receptor-dependent as well as intracellular, receptor-independent mechanisms, we next measured the effect of inhibitors of the three major S1P receptors in muscle, S1P1-3, on STAT3 activation in isolated SCs. Since the isolation of SCs from injured muscle fibers readily activates them, we were not surprised to find a significant level of phosphorylated, active STAT3 at baseline. We found that treatment of SCs with S1P2 inhibitor prevented STAT3 activation in SC-derived myoblasts, whereas inhibitors of S1P1 and S1P3 did not alter STAT3 activation. We found that siRNA-mediated knockdown of S1P2 also reduced STAT3 activation in murine SC-derived myoblasts [27]. Importantly, the inhibition of S1P2 led to the upregulation of two cell cycle inhibitor proteins, p21 and p27. These proteins are known to be under repressive control by STAT3. We interpreted these findings as revealing a plausible mechanism by which S1P recruits SCs into the cell cycle, i.e. by activating STAT3, leading to repression of cell cycle inhibitors, thereby allowing the SC to re-enter the cell cycle and begin proliferating in response to injury. A schematic diagram of this mechanism is illustrated in Fig. 6. There remained one additional question we hoped to answer, i.e., by what mechanism did S1P ligation to S1P2 induce the activation of STAT3? We suspected that this process might involve members of the Rho family of small GTPases, including Ras-related C3 botulinum toxin substrate (Rac) 1, cell division control protein 42 homolog, and RhoA. These proteins function downstream of G protein coupled receptors including S1P2 and have been shown to promote tyrosine phosphorylation of STAT3 and its translocation to the nucleus in various cellular contexts including carcinogenesis. Our results using a combination of Rac1 and RhoA inhibitors and plasmid constructs with which we modulated Rac1 and RhoA expression and activity demonstrated that S1P2 activation inhibits Rac1 and activates RhoA, thereby promoting STAT3 phosphorylation on Tyrosine 703 in myoblasts (Fig. 6) [27].



## 12. Other recent findings regarding S1P, SCs and skeletal muscle regeneration

Extensive research has been conducted to better understand the role of S1P in the biology of SCs and the process of skeletal muscle regeneration. S1P has been shown to stimulate the proliferation of quiescent SCs in vivo after cardiotoxin-induced damage [44]. Further, in vitro studies have demonstrated that exogenous S1P increases the intake of labeled thymidine incorporation in a PI3K-dependent manner and that the proliferative action of the sphingolipid depends on S1P2 and S1P3 [68]. The involvement of S1P in the migration of SCs has been also explored. Employing S1P receptor agonists and antagonists as well as specific siRNAs, Calise et al. found that S1P1 and S1P4 are involved in mediating the pro-migratory effects in SCs [68].

The C2C12 murine SC line is widely used to study in vitro skeletal muscle growth and differentiation. Early studies by Bruni and colleagues demonstrated that addition of S1P to cultured C2C12 cells led to the activation of phospholipase D [69], monomeric GTPase Rho [70] and increased cytosolic calcium [71]. C2C12 cells do not express S1P5 but do have S1P1–S1P4. The most highly expressed S1P receptors in myoblasts and myotubes are S1P1 and S1P3, respectively. During myogenic differentiation, S1P2 is down-regulated and almost absent in myotubes [72]. The dynamic modulation and critical role of S1P2 in the myogenic process has been observed in other studies, as well. Danieli-Betto et al. [73] observed an increase in expression of S1P1 after muscle damage induced by bupivacaine. S1P2 was noted to be absent in quiescent cells but transiently expressed in the early phase of regeneration. The expression patterns of the S1P receptors during myogenic differentiation in vitro and in vivo suggest that they exert complex and possibly counteracting signals in this process. Future studies should elucidate how these receptor signals are synchronized to coordinately regulate the sequential phases of SC and skeletal muscle response to injury required for efficient muscle regeneration.

## 13. S1P and cell therapy for skeletal muscle regeneration

The main goal of cell therapy in patients with muscle injury, disease or sarcopenia (frailty of aging) is to repair tissue damage and replenish lost myofibers through systemic or local delivery of cells with myoregenerative properties and thereby restore skeletal muscle function. SCs, recognized as true muscle progenitor cells, have been extensively explored for this purpose in laboratory animals and in patients. However, one of the major problems encountered in these studies was the limited cell survival of the transplanted SCs [74,75]. In the last few years, in addition to SCs and SC-derived myoblasts, other somatic cell types exhibiting myogenic potential have been identified and characterized. Some of these cell types (most of them multipotent stem cells) have proven capable of contributing to myogenic regeneration (e.g. endothelial cells, mesoangioblasts) [76–78], and a few [e.g. pericytes, endothelial cells, mesenchymal stem cells (MSCs)] have demonstrated the ability to incorporate directly into the SC niche [79–82]. Pericytes and mesoangioblast are blood vessel-associated stem cells that originate from embryonic and postnatal tissue respectively. Both of these cell types hold promise for clinical application, because a significant proportion is capable of reaching skeletal muscles following systemic administration. In mesoangioblasts, S1P strongly stimulates proliferation and protects from apoptosis elicited by different stimuli [83]. Treatment of mesoangioblast ex vivo with S1P enhanced their survival after delivery intramuscular into mice [78]. This exciting finding supports the view that S1P-preconditioned allogeneic mesoangioblast cells might enhance their survival, overcoming a critical barrier to cell therapy.

In order to explore the potential impact of S1P/S1PR2 signaling on STAT3 activation, we applied exogenous S1P to selected cell types: human umbilical vein endothelial cells (HUVECs), the pericyte cell line C3H10T1/2 and M25.2 cells, a clonally-derived population of mesoangioblasts generated by Suzanne Berry-Miller (University of Illinois). Of all the cells we tested, SC-derived myoblasts responded most robustly to S1P with regard to the activation of STAT3 [27]. In contrast, HUVECs and M25.2 mesoangioblast cells exhibited minimal STAT3 phosphorylation in response to S1P, and STAT3 phosphorylation was undetectable in C3H10T1/2 cells under the conditions we employed. However, additional experiments will be required to establish whether endogenous or exogenous S1P signaling plays a role in the biology of these cell types or on their potential for myogenic differentiation and in vivo incorporation of these cells into muscle.

MSCs are characterized by their capacity for extensive proliferation *ex vivo*, relative ease of isolation and ability to differentiate into adipogenic, osteogenic, chondrogenic and myogenic lineages. S1P has been shown to stimulate MSCs isolated from adipose tissue to differentiate into smooth muscle in a dose-dependent manner [84]. S1P2 was shown to be a mediator of the differentiation toward smooth muscle, whereas S1P3 was shown to be less important in this process. Although these studies are intriguing, the potential of MSCs to be used for cell therapy in skeletal muscle disease, and the extent to which S1P signaling and metabolism may be used to enhance cell therapy using this source of stem cells remains to be further explored.

## 14. Summary

The involvement of sphingolipids in many aspects of skeletal muscle biology is becoming increasingly clear (see Donati and Bruni this issue). Further, the evidence supporting a central role for S1P signaling and metabolism in SC biology and the coordinated control of skeletal muscle regeneration is now irrefutable in light of results from investigations of S1P signaling in myoregeneration by many research groups. These cumulative findings have revealed at least the theoretical potential for harnessing S1P's actions to modulate the activation of SCs and enhance muscle repair for therapeutic purposes in a variety of clinical contexts, including muscle wasting diseases, trauma and sarcopenia, wherein reduction of SC numbers contributes to the frailty of aging. Considering the availability of small molecule S1P receptor agonists and antagonists and SPL inhibitors already in clinical trials for other diseases, it seems likely that these basic research findings will be translated into more clinically relevant large animal model systems in the near future.

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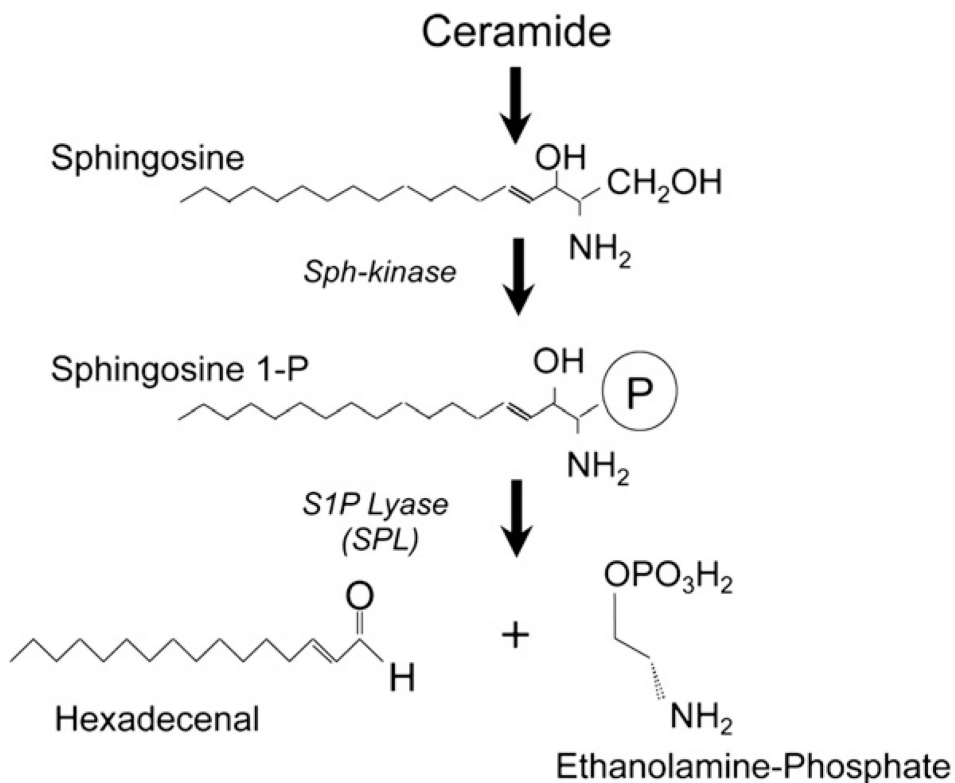
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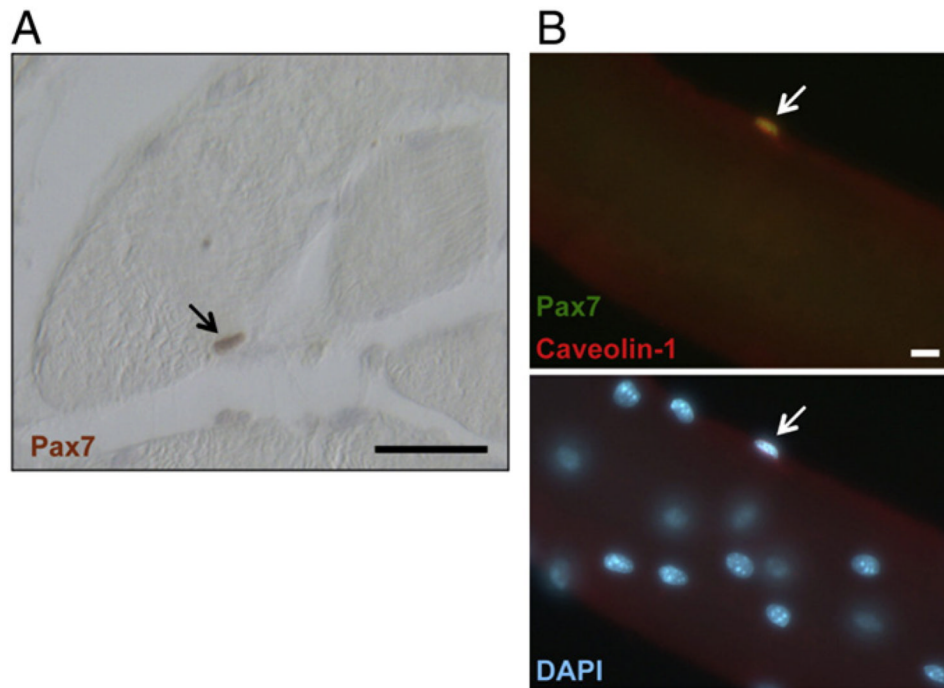
## Abbreviations

<b><i>Dp11</i></b>	dihydrosphingosine phosphate lyase 1
<b>DLMs</b>	dorsal longitudinal muscles
<b>DMD</b>	Duchenne muscular dystrophy
<b>DGC</b>	dystroglycan-associated protein complex
<b>ECM</b>	extracellular matrix
<b>HUVECs</b>	human umbilical vein endothelial cells
<b>LPPs</b>	lipid phosphatases
<b>MSCs</b>	mesenchymal stem cells
<b>MD</b>	muscular dystrophy
<b>NTX</b>	notexin
<b>Pax7</b>	paired box protein-7
<b>Rho</b>	Ras-homologous
<b>Rac</b>	Ras-related C3 botulinum toxin substrate
<b>SPL</b>	S1P lyase
<b>SCs</b>	satellite cells
<b>STAT3</b>	signal transducer and activator of transcription 3
<b>S1P</b>	sphingosine-1-phosphate
<b>S1P2</b>	sphingosine-1-phosphate receptor 2
<b>THI</b>	tetrahydroxybutylimidazole

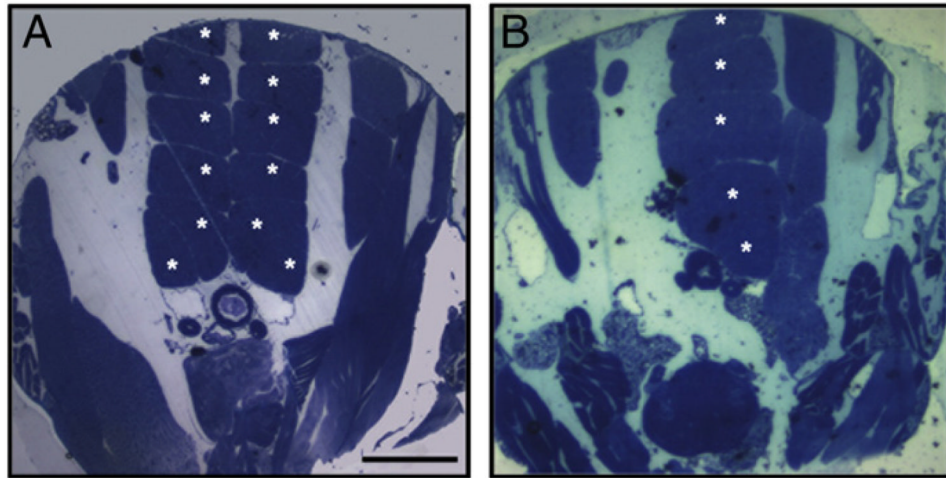


**Fig. 1.** Formation of sphingosine 1-phosphate. Diagram of the formation of sphingosine 1-phosphate starting from ceramide which is a central molecule of sphingolipid metabolism. Sphingosine kinases (Sph-kinase) catalyze the phosphorylation of sphingosine, generating S1P. S1P lyase (SPL) catalyzes the cleavage of S1P into the long chain aldehyde hexadecenal and ethanolamine-phosphate.

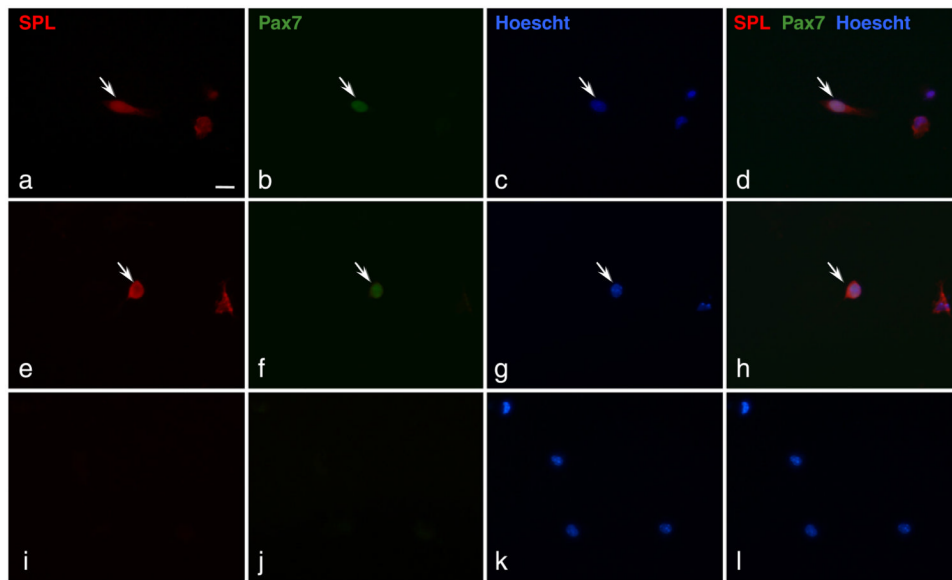




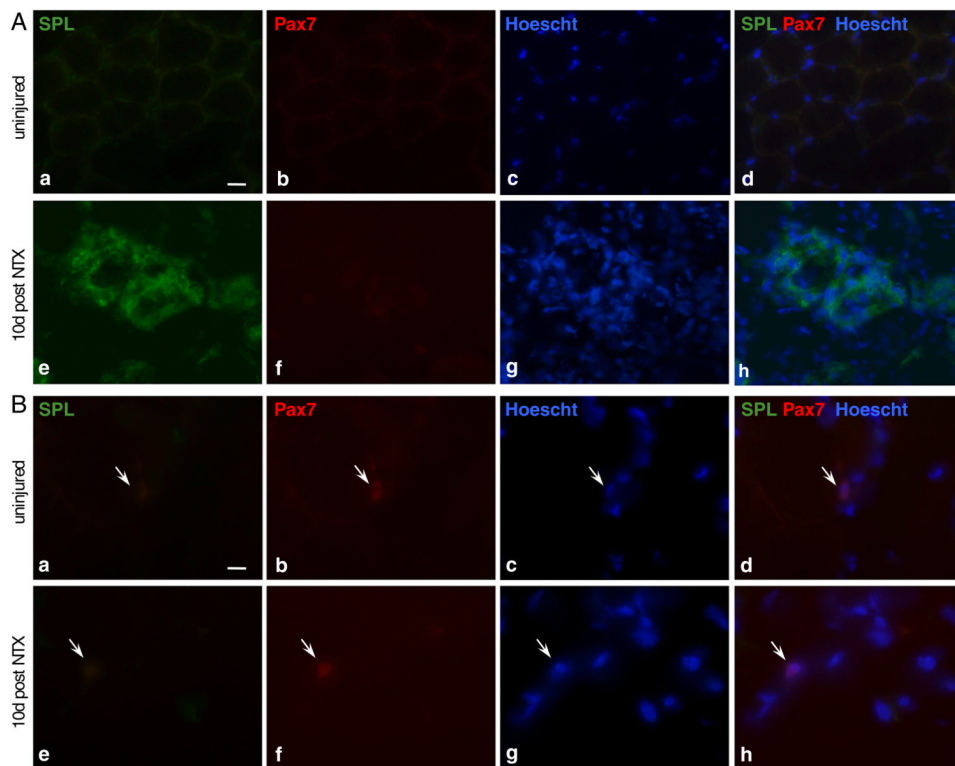
**Fig. 2.** SC localization. (A) SC stained with Pax7 antibody (red, marked by arrow) in a cross-section of human skeletal muscle. Scale bar 50  $\mu$ m. (B) Upper panel, co-immunostaining (merged yellow) of SCs with Pax7 (green) and Caveolin-1 (red) antibodies in isolated fibers of adult mouse muscle; lower panel, DAPI (blue) counterstained for nuclei. Scale bar 20  $\mu$ m arrow points to SC. Taken from A.S. de la Garza-Rodea [85].



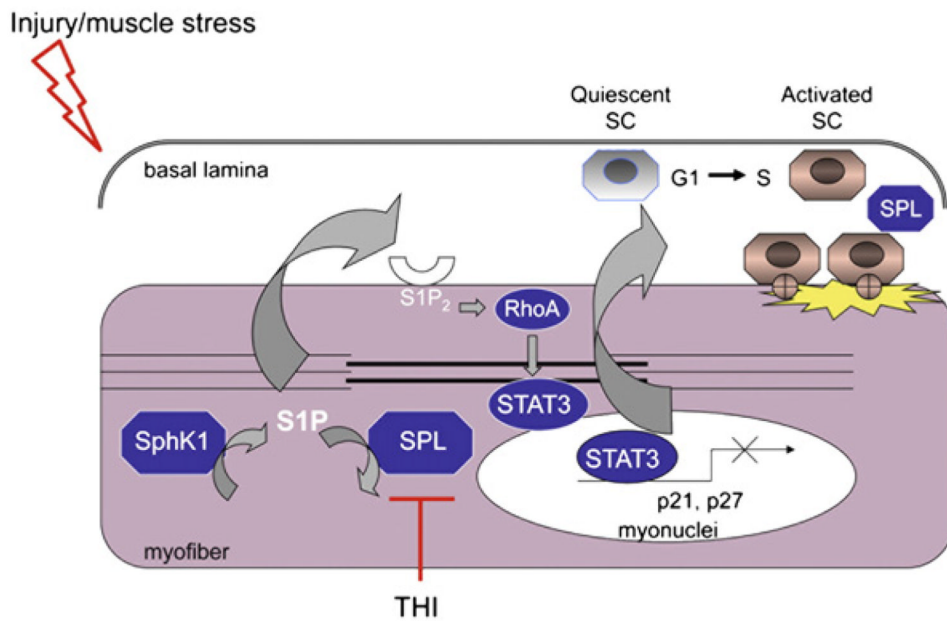
**Fig. 3.** The *Drosophila Sply* mutant myopathy. Methylene blue and Azure II stain of transversally sectioned thoraces of a normal fruit fly (A) and *Sply* mutant (B). The 6 pairs of dorsal longitudinal skeletal muscles (asterisks) present in wild type *Drosophila* (A). Degenerating muscles in a *Sply* mutant (B). Scale bar 100  $\mu\text{m}$ .



**Fig. 4.** Mdx SC-derived myoblasts express SPL. SC-derived myoblasts were isolated from skeletal muscle of mdx mice. Cells were in culture for 24 h before being fixed and stained with specific antibodies for SPL and Pax7. Nuclei were counterstained with Hoechst 33342. Two areas (a to d) and (e to h) show a Pax7 positive cell among several SPL positive cells. (i to l) cells stained with secondary antibodies (Alexa 488 and Alexa 546) and Hoechst only. Scale bar 100  $\mu$ m.



**Fig. 5.** SPL is expressed in inflammatory cells of degenerating/regenerating skeletal muscle. Cross-sections of cryopreserved skeletal muscle of an uninjured wild type (C57BL/6) mouse at rest (upper panels) and 10 days post-NTX-injury (bottom panels). Muscles were excised, sectioned, stained with antibodies specific for SPL and Pax7 and counterstained with Hoechst 33342. A: (a to d) resting myofibers do not express SPL; (e to h) strong stain is observed in degenerated/necrotic myofibers undergoing regeneration. Scale bar 100  $\mu\text{m}$ . B: SCs (arrows) that were detected in both injured and uninjured muscles did not co-stain for SPL. Scale bar 10  $\mu\text{m}$ .



**Fig. 6.** Role of S1P in response to skeletal muscle repair. After skeletal muscle injury, SphK1 generates S1P, which activates S1PR2, leading to RhoA and STAT3 activation, thereby repressing small cell cycle inhibitor proteins. SCs reenter the cell cycle and contribute to muscle regeneration. Inhibition of SPL using THI leads to a boost in S1P signaling, thereby promoting SC activation and efficient muscle regeneration. Note that the localization of SPL is assigned to the myofiber for convenience. However, SPL in SCs and inflammatory cells may also regulate S1P levels in and around the injured muscle.