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Review

The Rho family of small GTPases: crucial regulators of skeletal myogenesis

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Abstract. The Rho family of small GTPases is involved in a diverse array of cellular processes, including regulation of the actin cytoskeleton, cell polarity, microtubule dynamics, membrane transport pathways and transcription factor activity. Recent findings have implicated the Rho proteins as key regulators of the skeletal myogenic program; however, much controversy presently exists as to the precise role of these proteins in this process. This review examines the present controversial findings pertaining to the Rho family's regulation of skeletal myogenesis and extrapolates from both other differentiation systems and recently published data the possible mechanisms by which these proteins function in the myogenic cascade.

Key words. Rho; Rac; Cdc42; GTPases; myogenesis; skeletal myogenesis.

Introduction

The development of skeletal muscle is a multistep process involving pluripotent mesodermal cells which withdraw from the cell cycle, differentiate and undergo a cellular fusion process to become plurinucleated myotubes [1, 2]. Terminal differentiation is regulated by muscle-restricted regulatory factors (MRFs) belonging to the basic helix-loop-helix (bHLH) family of transcription factors, and E2A and myocyte enhancer factor 2 (MEF2) families [3–6]. E2A, MEF2 and MRF members collaborate to bind to sequence specific elements present in muscle regulatory and structural enhancers and control the irreversible withdrawal from the cell cycle by interacting with key cell cycle regulators. In addition to these regulators of myogenesis, other proteins and signaling pathways have been identified which control this process, including the Rho family of small GTPases.

Members of the Rho family of small GTPases are molecular switches that control a wide variety of signal transduction pathways relating to the regulation of the actin cytoskeleton, cell polarity, microtubule dynamics, membrane transport pathways and transcription factor activity [7–13]. The Rho proteins are members of the Ras superfamily of small GTPases, and are similar to Ras proteins in size and sequence [14–16]. The Rho family of small GTPases currently consists of well over 20 GTP-binding protein members divided into 6 subfamilies based on primary amino acid sequence, structural motifs and biological function. These include the RhoA-related subfamily, the Rac1-related subfamily, the Cdc42-related subfamily, the Rnd subfamily, the RhoBTB subfamily, and the Miro subfamily. The Rho-family members RhoD, Rif and TTF/RhoH do not belong to any of these subfamilies [17]. Rho GTPases oscillate between an inactive, guanosine diphosphate (GDP)-bound and an active guanosine triphosphate (GTP)-bound state, and switch between these states in a highly regulated manner [18] (fig. 1A). Signals received at the cell surface are transformed through a large family

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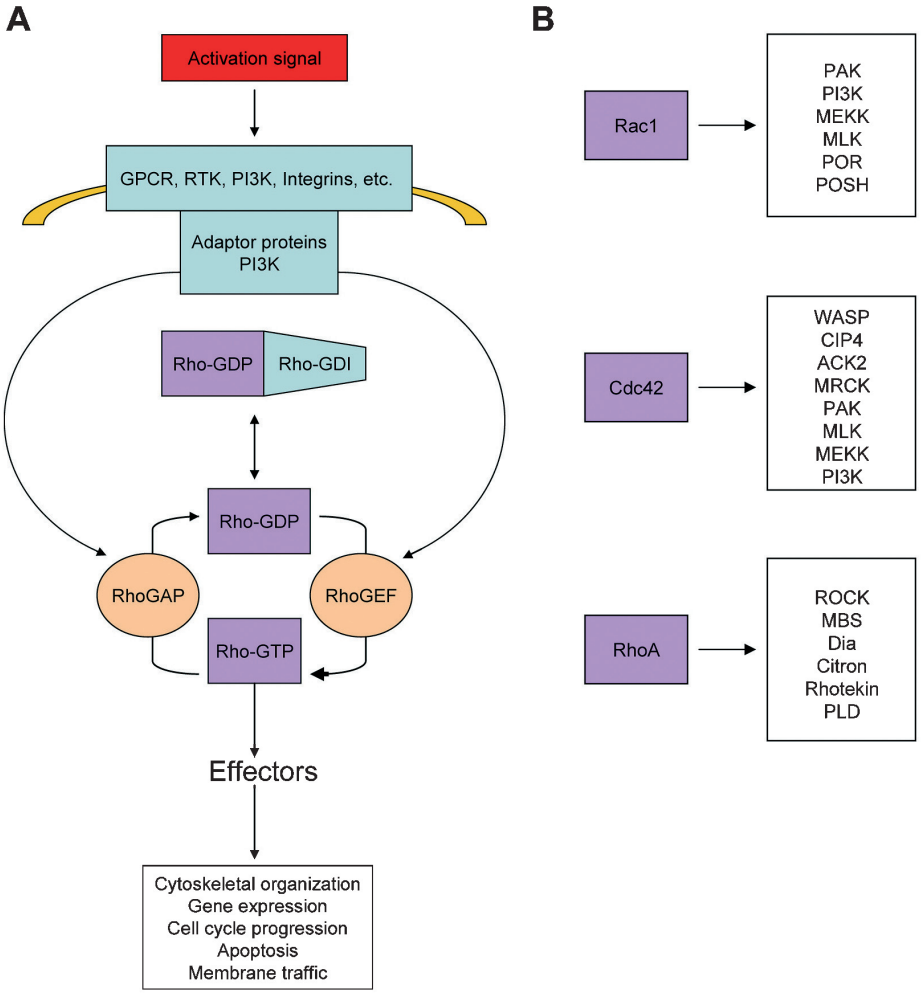

Figure 1. The biochemical model of Rho-GTPase regulation in the cell. (*A*) The cycle between the GTP-bound (active) and GDP-bound (inactive) Rho-GTPases are regulated by guanine nucleotide exchange factors (GEFs), GTPase activating proteins (GAPs) and Rho GDP-dissociation inhibitors (GDIs). Extracellular signals conveyed upon specific membrane receptors modulate the activities of GEFs and GAPs, which then regulate the GTP/GDP-bound states of the Rho-GTPases. Additionally, GDIs play an important role via binding to GDP-bound Rho proteins to inhibit the generation of active GTP-bound Rho proteins. Activated Rho-GTPases are capable of activating downstream effectors that lead to a diverse set of biological responses, primarily converging on the actin cytoskeleton. (GPCR, G-protein-coupled receptor; RTK, receptor tyrosine kinase; PI3K, phosphoinositol-3-kinase.) (*B*) Well-studied downstream effectors of the three prototypical Rho-family GTPases. Many of these effectors play direct roles in regulating the actin cytoskeleton.

of temporally and spatially regulated molecules called guanine nucleotide exchange factors (GEFs) and GTPase activating proteins (GAPs). GEFs function to activate the GTPases by catalyzing the exchange of bound GDP for GTP [19, 20]. The GTP bound forms of the Rho proteins are then transported from the cytoplasm to the membrane where they associate with their downstream targets [21–25]. Activated Rho family members function to trigger downstream signaling pathways, many of which are dominated by kinase cascades (fig. 1B). Highly regulated control of these signaling pathways is maintained by their intrinsic GTPase activity, which is induced through activation of GAPs [26–29], and the short half-life of many downstream targets. The cellular function of the Rho GTPases was defined when RhoA, Cdc42 and Rac1 were found to regulate actin assembly when microinjected into fibroblast cells ([30–34]). The Rho GTPases are key regulators of actin dynamics that lead to organized actin-based structures associated with the structure and motility of cells. RhoA is responsible for the activation of stress fibers, which are long bundles of actin that traverse the cell and are linked to integrins at sites of focal adhesion. Stress fiber formation results in more contracted cells that exhibit enlarged focal adhesions. Rac1 expression leads to polymerization of filamentous actin, which results in lamellipodium formation and membrane ruffling at the leading edge of migrating cells. Cdc42 causes a third type of actin structure, filopodia, which are long, fingerlike projections at the edges of lamellipodia.

Though the signaling mechanisms both upstream and downstream of the Rho GTPases are well understood, the role of the Rho GTPases during differentiation is both complex and controversial. Recent studies have attempted to characterize the function of RhoA, Rac1 and Cdc42 during skeletal muscle myogenesis. These studies have led to interesting conclusions involving the role that RhoA plays in this process; however, much is unknown, particularly with respect to the function of Rac1 and Cdc42 in this process. It is the purpose of this review to examine the present controversial findings pertaining to the Rho family's regulation of skeletal muscle myogenesis and extrapolate from both other differentiation systems and recently published data the possible mechanisms by which these proteins function.

RhoA as an activator of myogenesis

By the mid-1990s it was well established that RhoA was critically involved in the physiology of muscle contraction, but it was not until the late 1990s that the role of this GTPase in skeletal myogenesis began to be studied. Ramocki et al. first examined whether RhoA could regulate skeletal myogenesis using multipotent CH310T1/2 fibroblast cells [35]. CH310T1/2 fibroblast cells are an important model cell line for studying skeletal myogenesis in that they will only differentiate into skeletal muscle with exogenous expression of strong myogenic activating proteins such as MRF4 or MyoD. This study demonstrated that although activated H-Ras mutants could inhibit myogenesis, RhoA exhibited no effect on myotube formation or quail troponin I (TnI) luciferase activity. However, in order to induce myogenesis with this particular cell line, a strong inducer of muscle-specific gene transcription must be present, of which RhoA, in these studies, was apparently not.

However, it was known at the time that serum response factor (SRF), a DNA-binding protein containing a highly conserved DNA-binding/dimerization domain termed the MADS box, was a strong activator of myogenesis via activation of both the myogenic response and muscle differentiation, and was required for MyoD expression [36–39]. RhoA was known to mediate the activation of SRF [40], so the hypothesis that RhoA might regulate myogenesis was again tested by Carnac et al. using C2.7 myoblasts [41]. In this study, RhoA was found to be required for the expression of MyoD, and blocking the activity of RhoA using the general inhibitor lovastatin, or more specific antagonists of RhoA proteins such as C3-transferase or dominant negative RhoA constructs, resulted in a dramatic decrease in MyoD protein levels and promoter activity. In addition, the RhoA-dependent transcriptional activation of MyoD required functional SRF, suggesting that RhoA promotes expression of myogenic transcription factors via activation of SRF. Further work from another lab demonstrated that RhoA and its downstream SRF signaling pathway were essential for skeletal myogenesis [42]. These data confirmed a synergistic activation of skeletal α-actin expression with co-transfection of RhoA and SRF. Inactivation of SRF or RhoA with dominant negative mutants led to inhibition of myoblast fusion, a block in post-replicative myogenic differentiation and a loss of expression of SRF targets such as skeletal α-actin and indirect targets such as myogenin and α-myosin heavy chain (α-MHC). In addition, RhoA was shown to stimulate the auto-regulation of the murine SRF gene promoter. Recent studies have shown that RhoA controls SRF activity through several important mechanisms via redistribution of SRF cofactors from the cytoplasm into the nucleus. SRF activation has been shown to correlate directly to the ratio of F-actin (polymerized actin) to G-actin (unpolymerized actin). Indeed, two downstream effectors of RhoA, Rho kinase and Dia1, have been shown to promote F-actin stabilization and de novo assembly. A key cofactor of SRF, MAL, is sequestered in the cytoplasm when substantial G-actin is present; however, accumulation of F-actin via activation of the Rho-effector proteins leads to a release of MAL from G-actin, where it translocates to the nucleus and interacts with several SRF target promoters to mediate transcriptional activation of serum response element (SRE)-containing genes and regulate skeletal myogenesis [43–47]. In addition to these studies, several reports have confirmed the necessity of RhoA in the promotion of skeletal myogenesis, as evidenced by its regulation on myotube formation, expression of myogenic markers and accumulation of plurinucleated myotubes [48–52] (fig. 2).

Moreover, severe skeletal muscle deformity is observed in Trio-GEF deficient mice [53]. In Trio(–/–) embryos, primary skeletal myofibers exhibited highly unusual spherical myofibers at E18.5, and the authors suggest that a Trio deficiency may cause a defect in secondary myogenesis, as the appearance of the abnormal Trio(–/–) skeletal myofibers temporally coincided with the onset of secondary myogenesis, and smaller secondary myofibers located adjacent to the primary myofibers were absent. To date, Trio is the only mammalian Rho-family GEF implicated in regulating myogenesis, and other animal model studies need to be performed with Rho-family regulators in order to understand the physiological relevance of these proteins to skeletal myogenesis.

Upstream activators of RhoA during myogenesis

Extracellular ligand-mediated activation of myogenesis has been extensively studied, and both cell-cell adhesion and the insulin (INS)/insulin-like growth factor (IGF) signaling pathways have been shown to regulate RhoA

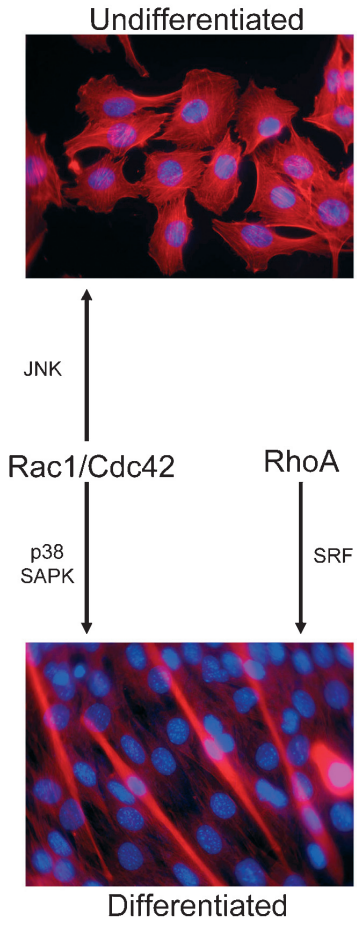

Figure 2. Rho-family regulation of myogenesis. Skeletal muscle differentiation is a multistep process involving permanent withdrawal from the cell cycle, activation of muscle-specific genes and fusion of differentiated myocytes to form multinucleated myotubes. The Rho family of small GTPases is essential for proper terminal differentiation of muscle cells; however, much controversy exists as to the precise role of RhoA, Rac1 and Cdc42 in this process. RhoA has been reported to positively regulate the serum response factor (SRF)-mediated activation of several muscle-specific gene promoters. Much confusion exists about the role of Rac1 and Cdc42 in skeletal myogenesis. These proteins activate the stress-activated protein linase (SAPK) and p38 pathways, which promote myogenesis; however, Rac1 and Cdc42 also promote the activation of C-jun N-terminal kinase (JNK), which is a strong inhibitor of myogenesis.

activity during skeletal myogenesis. Skeletal myogenesis has been demonstrated to be highly regulated by cell-extracellular matrix and cell-cell adhesion molecules [4, 54]. One specific group of adhesion molecules, the cadherins, consists of transmembrane glycoproteins that promote calcium-dependent homophilic cell-cell adhesion. Cadherin cytoplasmic domains provide F-actin cytoskeleton attachment points through association of catenins and other cytoskeletal-associated proteins [55]. Several studies suggest that N-cadherin-mediated cell-cell adhesion plays an important role in skeletal myogenesis. Perturbation of N-cadherin function inhibits myotube formation [56, 57] and decreases myosin heavy chain and MyoD accumulation [58]. In addition, N-cadherin-coated beads induce myogenesis in cultured myoblasts, suggesting a role of N-cadherin-mediated adhesion in myogenic induction [59]. Using C2C12 myoblast cells, Charrasse et al. demonstrated that N-cadherin-dependent cell-cell adhesion increases RhoA activity and induces the activity of skeletal α-actin, MyoD and myosin light chain 1A skeletal muscle-specific promoters [60, 61]. These data potentially link cell-cell adhesion with downstream signaling of RhoA, namely SRF, to induce muscle-specific gene expression of MyoD and other proteins required for skeletal myogenesis.

Mesenchymal cells can differentiate into muscle or fat cells, depending on exposure to growth factors. Sordella et al. have shed light on the regulation of myogenesis/adipogenesis by identifying IGF as an upstream mediator regulating RhoA and its negative regulator, p190 RhoGAP [62]. The authors used targeted disruption of p190 RhoGAP gene in mice, which resulted in increased Rho activity and subsequent activation of downstream Rho targets. The p190 RhoGAP knockout mouse exhibited a significant reduction in adipocyte formation. Cells derived from embryos lacking p190 RhoGAP strongly undergo myogenesis in response to IGF treatment, suggesting that RhoA is an essential modulator of IGF signals that directs myogenesis/adipogenesis cell fate decisions.

Rac1 and Cdc42: dual roles to create a fine balance

While the role of RhoA in skeletal myogenesis is well defined, much confusion and controversy exists regarding the roles of Rac1 and Cdc42 in myogenesis. The expression of Rac1 and Cdc42 was first found to be high in mesodermal cells of *Drosophila,* and knockouts of Rac1 and Cdc42 in *Drosophila* led to incomplete muscle formation via a failure to form myotubes [63]. Additionally, the *Drosophila* Rac-specific GEF, myoblast city (mbc), has been shown to regulate myoblast fusion [64–66]. These data demonstrated the necessity of these Rac1 and Cdc42 proteins in skeletal myogenesis, and several groups have since attempted to understand their roles in this process. Using C2C12 mouse myoblasts, Takano et al. demonstrated that dominant negative mutants of Rac1 and Cdc42 inhibited transcription of muscle-specific genes [48]. Conversely, expression of constitutively active mutants of Rac1 and Cdc42 increased expression of myogenic genes, suggesting that Rac1 and Cdc42 both promote myogenesis. However, another study using primary avian myoblasts demonstrated that expression of constitutively active Rac1 and Cdc42 mutants inhibited myogenin expression [50]. In addition, stable Rac1-expressing cell lines underwent myogenesis, but exhibited severely decreased levels of muscle specific proteins

and failed to assemble ordered sarcomeres. Conversely, dominant negative Rac1 expression increased sarcomere maturation. In an attempt to clarify these discrepancies, Meriane et al. utilized L6 rat myoblasts, C2.7 and C2C12 cell lines to examine the role of Rac1 and Cdc42 in skeletal myogenesis [51]. These authors discovered that overexpression of either constitutively active or dominant negative mutants of Rac1 or Cdc42 inhibited myogenin, troponin T and myosin heavy chain, and ultimately blocked myogenesis via activation of C-jun N-terminal kinase (JNK), a potent inhibitor of myogenesis, which resulted in the cytoplasmic accumulation of Myf5. It had previously been shown that Rac1 and Cdc42 activated the JNK, stress-activated protein kinase (SAPK) and p38 pathways [40, 67, 68], and that SAPK and p38 promoted and were absolutely essential for myogenesis [69]. This was the first study to demonstrate duel roles for Rac1 and Cdc42: activation of SAPK and p38, which were necessary for myogenesis, and activation of JNK, which inhibited myogenesis by redistribution of an essential myogenic regulatory transcription factor, leading to confusion as to the actual role of Rac1 and Cdc42 in myogenesis (fig. 2).

Other studies have supported the idea that Rac1 and Cdc42 could play a role in inhibition of myogenic differentiation. One such study by Heller et al. demonstrated that Rac1 inhibits myogenic differentiation by preventing the complete withdrawal of myoblasts from the cell cycle [70]. The authors additionally show that Rac1 activity is high in proliferating myoblasts, and its activity decreases upon initiation of myogenesis, suggesting perhaps that Rac1 activity must be precisely modulated in order for myogenesis to occur. Having previously demonstrated that Rac1 and Cdc42 inhibit skeletal myogenesis, Meriane et al. further examined whether expression of these two GTPases affected mitogenesis and proliferation of myoblasts [51, 71]. Using a combination of bromodeoxyuridine (BRDU) incorporation and cyclin D1 expression, they demonstrated that Rac1 and Cdc42 but not RhoA impair cell cycle exit of L6 myoblasts cultured in differentiation medium. Additionally, loss of cell contact inhibition and anchorage-dependent growth was observed upon exogenous expression of these two GTPases. This suggests that perhaps Rac1 and Cdc42 play a more important role during the proliferative stage of the myoblast, rather than in the differentiation process. While much of the data on Rac1 and Cdc42 apparently demonstrate that these GTPases inhibit myogenesis, none of these findings necessarily rules out the results presented by Luo et al. and Tanako et al. [48, 63], which demonstrated that inhibition of Rac1 and Cdc42 by exogenous expression of their dominant negative forms results in a block in myogenesis, indicating that at some level, these proteins are essential for this process.

Upstream activators of Rac1 and Cdc42 during myogenesis

Two important regulators of Rac1 and Cdc42 have been implicated in myogenic determination: N-cadherin and transforming growth factor beta (TGFβ). While N-cadherin was shown to increase activation of RhoA in response to cell-cell contact, Charasse et al., using antibodies that specifically recognize the extracellular domain of N-cadherin thus mimicking its activation, demonstrated a decrease in Rac1 and Cdc42 activity [60]. This downregulation of Rac1 and Cdc42 activity was accompanied by inactivation of JNK, a potent inhibitor of myogenesis. Additionally, the TGFβ pathway is an important regulator of myogenic differentiation. TGFβ has been shown to promote the proliferative state of cultured myoblasts [72, 73] and strongly inhibit their differentiation by repressing MEF2-dependent transcription in myogenic differentiation [74, 75] and activating the JNK pathway [76]. Data presented by Meriane et al. demonstrated that TGFβ addition to C2- and L6-cultured myoblasts activates Rac1 and Cdc42 GTPases and their downstream effector, JNK, in order to inhibit skeletal muscle differentiation [77]. However, this lab was unable to reverse TGFβ-induced myogenesis inhibition by the expression of dominant negative forms of Rac1 and Cdc42, since the expression of these mutants inhibits myogenesis through a decrease in p38 activity, which is essential for myogenesis [51, 69, 78]. Interestingly, in the developing embryo, precursor cells in a proliferative state migrate from the dermomyotome into the limbs. During this time, TGFβ family members regulate delamination of neural crest cells from the dorsal neural tube, and since Rac1 and Cdc42 GTPases have previously been implicated in migration in muscle and non-muscle cell types [33, 79], it is possible that the primary role of Rac1 and Cdc42 is during proliferative migration of myogenic precursors. Though TGFβ appears to promote Rac1 and Cdc42 in skeletal myocytes, it must be taken into account that TGFβ is a potent activator of RhoA during the epithelial-mesenchymal transition [80, 81] and during RhoA- and ROCK-induced cell cycle inhibition [82]. These data suggest the final tissue-specific response of TGFβ might depend on whether this ligand predominately activates Rac1/Cdc42 or RhoA in a particular cell type.

RhoA, Rac1 and Cdc42 as antagonists: it's never really that simple

When examining non-muscle cell types, it is observed that different Rho GTPase pathways antagonize rather than activate each other. Often, Rac1 and Cdc42 produce cellular responses that are reportedly antagonistic to that of RhoA. For example, in fibroblasts, Cdc42 and Rac1

promote membrane protrusions in the form of filopodia and lamellipodia, while RhoA promotes membrane retraction through contractile actin and myosin filaments [31, 32]. In neuronal cells, Cdc42 and Rac are positive regulators of neurite outgrowth, whereas RhoA promotes growth cone collapse [83, 84, 89]. However, several studies have demonstrated that the dynamic regulation of these GTPases is not black and white. For example, temporal activation/deactivation of Rho proteins has been reported during the clustering of acetylcholine receptors (Arc-R) in muscle cells [85]. This dynamic regulation occurs when a transient activation of Rac1 initially induces Ach-R localization, while RhoA is essential for the final clustering of the receptors. In another recent example, the E3-ubiquitin ligase Smurf1 has been shown to spatially regulate the stability of RhoA during microspike formation, thus allowing positive microspike formation to occur permissively via Rac1 and Cdc42 activity [86, 90]. Further verifying this idea using cardiomyocyte differentiation, Puceat et al. demonstrated that Rac1 effects depend on the stage of cell differentiation [87]. At early stages of cardiac differentiation, Rac1 delays differentiation by repressing expression of MEF2C, a major cardiac transcription factor. However, at later stages, Rac1 is activated by cardiogenic factors such as TGFβ and becomes essential in the process of cardioblast proliferation and myofibrillogenesis of cardiomyocytes. Thus, it is very possible, considering the impressive modulation these GTPases exhibit in a variety of systems, that the maintenance of a dynamic balance between Rho and Rac/Cdc42 activities is crucial for proper myogenic differentiation.

Indeed, one recent study has shed light on the dynamic relationship between the Rho-GTPases during skeletal myogenesis [88]. It is widely reported that the expression and level of activation of distinct members of the Rho-family starkly differs depending on the cell type and growth conditions. Furthermore, although many reports have examined the effects of activated or dominant negative forms of the Rho-GTPases on skeletal myogenesis, very little is actually known about the activation state and dynamics of RhoA, Rac1 and Cdc42 during the myogenic differentiation process. In this study, the authors demonstrated that the active form of RhoA was detectable in proliferating myoblasts; however, activation was virtually lost in the first 24 h of the myogenic differentiation cascade, but returned to the level observed in proliferating myoblasts after 72 h of differentiation. Rac1 was active in proliferating myoblast cells and until 24 h of differentiation. However, the level of active Rac1 dramatically decreased by 72 h of differentiation. Finally, activated Cdc42 was barely detectable in proliferating myoblasts, but was dramatically increased upon differentiation and remained high throughout terminal differentiation. Combined, these data suggest that the roles of the GTPases during myogenesis are not starkly antagonistic. There exists the necessity of an off switch for the RhoA protein during the initial steps of the differentiation process. In addition, Rac1 appears to have a role in the initial phases of myogenesis, but must be inactivated in order to achieve the terminal differentiation of myoblasts to myotubes. Though much of the previous findings suggest Cdc42 inhibits myogenesis, a high level of Cdc42 activation occurs upon myogenic differentiation, suggesting the necessity for this protein. The authors suggest that instead of a clearly antagonist role for the GTPases, that the Cdc42/Rho balance would control the initiation of the differentiation cascade, whereas the Rac/Rho balance plays a part in terminal differentiation.

Future studies

A great controversy still exists in an attempt to explain the role of the Rho family of small GTPases in skeletal muscle differentiation, and perhaps the discrepancy existing among findings from different authors is a reflection on the limitations of in vitro studies, namely the use of overexpression strategies widely used by the researchers involved in this field. For instance, a different level of Rho activation caused by transfection of varying amounts of Rho-GTPase complementary DNA (cDNA) can potentially lead to a completely different response. While very little is known about the precise developmental roles of the Rho family during skeletal myogenesis and the regulatory factors that control their expression and activation, the recent findings presented by Travaglione et al. have given a new and exciting view of the dynamic relationship governing the roles of the Rho family of small GTPases during skeletal myogenesis, allowing insightful opportunities for further investigations on this matter.

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