RAC1 GTP-ase is important for myogenesis and stem cell distribution

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Abstract

Background: Skeletal muscle fibers form through the fusion of myoblasts, a process regulated by Rac1, a small G-protein crucial for actin dynamics. Understanding Rac1's role in muscle stem cell distribution is essential for elucidating muscle regeneration mechanisms.
Aim: This study aims to investigate the significance of Rac1 in muscle stem cell distribution and myogenesis using conditional knockout mice.
Methods: Conditional Rac1 mutant mice were generated, and the distribution of Pax7-positive stem cells was assessed. Comparative analysis of mutant myogenic cells was conducted to elucidate Rac1's role throughout the fusion process. Immunofluorescence staining and transmission electron microscopy were employed for cellular and ultrastructural analysis.
Results: Rac1-lacking muscle exhibited a reduction in Pax7-positive satellite cells, indicating its importance in muscle stem cell distribution. Comparative analysis of mutant myogenic cells revealed distinct roles for Rac1 throughout the fusion process. Transmission electron microscopy studies demonstrated irregularities in muscle cell layers in Rac1-deficient mice.
Conclusion: Our study highlights the critical role of Rac1 in myogenesis and muscle stem cell distribution. Rac1 deficiency led to fewer Pax7-positive satellite cells, emphasizing its importance in muscle regeneration. These findings provide insights into potential therapeutic interventions for muscle-related disorders. **(TCM-GMJ June 2024; 9 (1):P18-P22)**

Keywords: Rac1, myogenesis, stem cells, Pax7, MyoD

Introduction

Skeletal muscle fibers are multi-nucleated and generated from the fusion of precursor unicellular myoblasts, a fundamental process leading to the making, growth, and renewal of muscle fibers both during embryonic development and post-embryonic stages(1).Myoblast fusion controls myofiber length as well as contractive capacity and muscle function. On a cellular level, this phenomenon is characterized by an alignment of myoblast and myotube membranes, followed by rearrangements of the actin cytoskeleton at the contact sites and membrane fusion (2).

Intracellular mechanisms orchestrating this complicated process, particularly those regulating cytoskeleton dynamics and actin remodeling, encompass the Rac GTPases (dRac1 and dRac2), and a key regulator of Rac, the dimeric guanine nucleotide exchange factor encoded by genes: dElmo and myoblast city. The Arp2/3 complex, essential for fusion, exerts a direct influence on cytoskeletal reorganization and actin polymerization (see Fig. 1). Activation of the Arp2/3 complex can be modulated by intermediary complexes such as Kette and WAVE/Scar, impacting its functionality, while WASP and Ver/WipSlt can directly activate the Arp2/3 complex.(3–6)

The analysis of myoblast fusion experiments in cultured mammalian myoblast cells revealed functional overlap in the effector molecules of the process. Recent in vivo studies in zebrafish and mice have confirmed broad conservatism of molecular mechanisms. Noteworthy, among them is the deregulation of zebrafish, Kirrel the homolog of Ig adhesion receptors, and Rac1. Moreover, the deregulation of the Rac1 regulators Dock1 and Dock5 in zebrafish and mutation in mice confirm the importance of the Rac1 component in this process. Rac1 activation in zebrafish is mediated by the interaction of Dock1 and the adapters Crk and Crkl. Through conditional mutagenesis of Rac1 and Cdc42, they were shown to play an important role in myoblast fusion in mice in

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vivo and in vitro. Studies have shown that Rac1's role in the myoblast fusion process is evolutionary conserved from insects to mammals. Rac1 is essential for actin recruitment. Similarly, Cdc42, known to control actin redistribution of the cytoskeleton(7,8). Our findings elucidate that Rac1 is important for muscle stem cell amount and structural organization.

Molecular machinery for the motility and migration of myogenic cells

The migration of myogenic cells begins through a complex interaction of molecular and mechanical stimuli. The process is realized by various cellular components and signaling pathways. Two different modalities of migration are distinguished: amoeboid and mesenchymal, specific mechanisms that underlie these processes may vary depending on the cell type and environmental context. For instance, amoeboid migration depends on limited substrate adhesion, whereas mesenchymal migration entails substantial cytoskeletal reorganization. Protrusions at the leading edge of the cell, such as lamellipodia and filopodia, may elongate as integral elements of

mesenchymal migration. The cell holds onto the extracellular matrix (ECM) and pulls itself forward. The cell rear contracts and retracts as a result of the procedure (9,10).

The regulation of migration steps is often mediated by Rho GTPases, which modulate the actin cytoskeleton in response to biochemical and biophysical signals (11,12).

Signalling pathways involved in the migration of adult myoblast

Signalling pathways that govern adult myoblast migration is important to understand the complicated processes underlying the growth of muscle during development and post-natal muscle tissue regeneration (13).

Numerous studies on the migration of myogenic progenitors have shown that it often involves the upregulation of MAPK/ERK and PI3K/Akt signaling pathways. Activation of these pathways with phospho-tyrosine phosphatase and PTEN inhibitor Bpv (Hopic) was associated with larger increases in myoblast migration. Additionally, bisperoxovanadium (BpV), an inhibitor of Phosphatase and Tensin Homolog (PTEN) that negatively regulates PI3K signaling, has been found to enhance myoblast migration (14).

In contrast, silencing of either PI3K/AKT or MAPK/ERK signalling pathways resulted in significant inhibition of myoblast migration. For instance, the migration associated with hepatocyte growth factor (HGF) is postponed after treatment with a MAPK inhibitor. Similarly, PI3K inhibition in C2C12 myoblasts results in the disruption of the formation of lamellipodia, a critical step for motility on a monolayer This limitation in migration is linked to the subsequent interference of actin polymerization facilitated by CDC42 and Rac-1 through N-WASP and WAVE2. Additionally, LY294002 treatment, a PI3K inhibitor, hampers the positioning of N-WASP/WAVE2 at the forefront of lamellipodia. The pharmacological blockade of PI3K and MEK has been observed to diminish myoblast migration. Platelet lysates have likewise exhibited the capability to augment the mobility of C2C12 myoblasts, likely facilitated by MAPK and PI3K signaling pathways (15,16).

The chemokine Stromal cell-derived factor 1 (SDF-1) and its receptor Cxcr4 are acknowledged as pivotal regulators of cell motility. SDF-1 exerts its influence on actin organization through the engagement of FAK (focal adhesion kinase), Cdc42 (cell division control protein 42), and Rac-1 (Ras-Related C3 Botulinum Toxin Substrate 1). In experimental investigations where the Cxcr4 receptor was silenced, a noticeable absence of increased GTPases CDC42 and Rac-1, as well as diminished cell motility, was noted. Moreover, empirical findings validate the involvement of SDF-1 in the upregulation of various migration-associated transcripts, including MMP9, α-actinin, and CAPSN1 (17).

Alternative pro-migratory signalling pathways are described in the progenitor muscle cell migration. C2C12 myoblasts, when subjected to dominant-negative Ras-related protein Ral-A (dnRalA), which represents an alternative pathway to MAPK/ERK

downstream of Ras, exhibited significantly reduced chemotaxis induced by basic fibroblast growth factor (bFGF), vascular endothelial growth factor (VEGF), hepatocyte growth factor (HGF), insulin-like growth factor-1 (IGF-1). FGFs facilitate the migration of myoblasts by signaling via the Ras/Ral pathway. In vivo, studies show that using dominant-negative forms of Ras and Ral caused in decreased migration of

transplanted myoblasts. Experiments conducted that myogenic progenitor derived from FGF6− / − mice displayed decreased migration upon intramuscular injection, mirroring the behavior observed in myoblasts expressing dominant-negative forms of Ras and Ral (15,18).

Evidence suggests that myogenic progenitor migration driven by TGF-β superfamily members occurs in non-SMAD signaling pathways, including PI3K/AKT and ERK/MAPK pathways. While canonical TGF-β signaling typically involves the SMAD pathway. Knockout studies targeting SMAD4, a key mediator of canonical TGF-β signaling, specifically in myogenic progenitors, have shown no effect on cell migration during tongue morphogenesis, indicating a non-SMAD-dependent mechanism (19).

Activation of the MAPK/ERK pathway by TGF-β1 leads to the migration of myoblasts, through the retraction of the trailing end of the cell through the upregulation of CAPN2, the catalytic subunit of the calcium-dependent protease m-calpain. CAPN2 results in dissociation focal adhesions (FAs) by proteolysis of key proteins such as FAK and talin. Moreover, bone morphogenetic protein 2 (BMP2), which traditionally signals through SMAD signaling, has been proposed to act in a non-canonical manner. BMP2 regulates cortical actin remodeling at the leading edge by PI3K-mediated activation of PH-like domain family B member 2 (LL5β). LL5β recruits the actin crosslinker filamin, promoting cell protrusion(15).

Despite significant progress in explaining these pathways, understanding how they are coordinated spatiotemporally to regulate different stages of myogenic progenitor

migration remains a subject of ongoing investigation. Also, other signaling pathways, including those involving Wnt and nitric oxide (NO), may play context-dependent roles in myogenic migration (20).

Effect of the extracellular environment on muscle stem/progenitor cell migration

The interaction between muscle stem/progenitor cells and the surrounding extracellular matrix (ECM) plays an essential role in modulating migration dynamics. Integrins play a critical role in regulating the interaction between a cell and its microenvironment and help in generating traction forces essential for movement. The composition and mechanical properties of the ECM influence the migratory behavior of these cells. Alterations in ECM composition and structure observed during aging or in pathological conditions can impact the migratory capacity of muscle progenitor cells, possibly hindering their capability to migrate into damaged areas and participate in tissue repair

processes. Also, biochemical molecules present in the ECM, such as growth factors and cytokines can act as chemoattractants or confutings, that can either enhance or inhibit migration (21, 22).

Turnover of FAs in muscle motility, regeneration, and pathology

Focal adhesions (FAs) represent specific cellular sites where the tightest connections with the underlying extracellular matrix (ECM) and locations initiating adhesion-associated signal transduction occur.

Protein Tensin-1 (TNS1) links the actin cytoskeleton with integrins and mediating signal transduction. It is characterized by Src homology 2 (SH2) and phosphotyrosine binding domains, allowing interaction with tyrosine-phosphorylated proteins within the PI3K/Akt and β-integrin/FAK signaling pathways. Tensin-1-null mice show delayed skeletal muscle regeneration and tensin-3 inhibition reduces the migration of multipotent mesenchymal stem cells (MuSCs) (23).In addition, overexpression of microRNA-708 leads to a TNS-3 inhibitory phenotype, resulting in a decrease in phosphorylated FA kinase (p-FAK), which confirms the regulatory importance of microRNA-708 in migration through their influence on focal adhesions (24).

The modulation of focal adhesion kinase (FAK) and paxillin levels significantly impacts cell migration, as demonstrated by various studies in different contexts, including myogenic progenitors and platelet-rich plasma treatment. Studies have indicated that swine myogenic progenitors exhibiting elevated levels of p-FAK and p-paxillin demonstrate quicker wound closure rates. Overexpression of platelet and endothelial aggregation receptor-1 (PEAR-1; involved in the aggregation of platelets and neoangiogenesis) in MuSCs increases p-FAK, p-paxillin, and vinculin expression, via upregulation and interaction with integrin β1 It has been demonstrated that upregulation focal adhesion kinase (FAK) and paxillin by platelet-rich plasma enhances the spreading and migration of muscle progenitor cells. Conversely, depriving cells of lysine, an essential amino acid critical for protein synthesis, leads to reduced levels of phosphorylated FAK and phosphorylated paxillin, consequently impairing cell migration (25, 26).

The decreased movement of myoblasts is evident in various muscle pathologies, including spinal muscular atrophy (SMA), characterized by a deficiency of the survival motor neuron-1 (SMN-1) protein. SMN-1-deficient myoblasts display reduced motility, based on disrupted focal adhesion (FA) dynamics and the extended presence of FA-associated proteins such as vinculin and talin. ROCK-2, a downstream effector of Rho GTPase, plays a fundamental role in FA maturation, while inhibition leads to increased migration speed by promoting the formation of vinculin-positive FAs. This suggests that ROCK-2 activity influences FA formation and dynamics, ultimately affecting myoblast migratory behavior, and potentially contributing to the lack of tissue regeneration observed in SMA muscle biopsies. (27,28).

Muscle cell migration in fibrotic microenvironment

Fibrosis, a common occurrence in conditions like muscular dystrophy and age-related presents, significant hurdles to tissue regeneration by impeding the migration and integration of cells, ultimately impacting motor function and overall functional recovery. The accumulation of excessive extracellular matrix (ECM) components during fibrosis not only forms physical barriers but also alters the biomechanical characteristics of tissues, making it difficult for myoblasts to migrate effectively. Also, abnormal signaling pathways and the presence of anti-inflammatory factors within the fibrotic microenvironment disrupt the chemotactic cues necessary for guiding cell migration. Strategies aimed at mitigating fibrosis, such as inhibiting MMP-1, -2, or -9, have shown promise in promoting the migration and successful integration of transplanted cells. This underscores the potential for targeting fibrosis as a means to enhance the effectiveness of cell-based therapies for muscle regeneration (29–31).

An alternative strategy for reducing fibrotic tissue involves targeting profibrotic cytokines released due to persistent inflammation, particularly focusing on signaling pathways that promote fibrosis in fibroblasts. Targeting profibrotic cytokines, notably transforming growth factor beta (TGF-β), has emerged as a promising approach to mitigate fibrosis in skeletal and cardiac muscle tissues. Inhibition of TGF-β activity has been shown to effectively alleviate muscle fibrosis. Studies in scid/mdx mice lacking T and B lymphocytes have also highlighted reduced TGF-β1 activity and muscle fibrosis, implicating immune cells in the fibrotic process. However, despite these advancements, such treatments are not consistently incorporated into cell transplantation protocols. Future research efforts should prioritize integrating strategies to reduce existing fibrotic scars, thereby enhancing the engraftment of cells delivered locally or systemically for therapeutic purposes (31).

Methods

Animal Models: The Rac1flox and transgenic MyoDcre strains were generated as previously described. Embryos with the genotypes Rac1flox/flox; MyoDcre (conditional

Rac1 mutant) were utilized for analysis. As controls, embryos with the genotypes Rac1flox; MyoDcre were included in the study (31,32).

Immunofluorescence Staining: Immunofluorescence staining of cryosections and cultured cells was conducted following fixation for 2 hours and 15 minutes with 4% paraformaldehyde, respectively. The antibodies used in this study included anti-Laminin-A, and anti-Pax 7. Secondary antibodies conjugated with Cy2, Cy3, or Cy5 were obtained from Abcam.

Transmission Electron Microscopy (TEM) Procedure: For TEM analysis, regions undergoing contraction were carefully dissected and immediately fixed in 4% glutaraldehyde in 0.1 M phosphate buffer (pH 7.4) for a duration of 2 hours. Subsequently, the specimens were postfixed in 1% OsO4 in the same buffer for an additional 1.5 hours. Following fixation, a graded ethanol series was employed for dehydration, and the samples were embedded in Araldite 6005 (Sigma). Ultrathin sections, measuring between 50 and 70 nm, were prepared and double-stained with uranyl acetate and lead citrate. These sections were then subjected to ultrastructural assessment using a transmission electron microscope (EM900, Zeiss, Germany) for detailed analysis and visualization.

Results and discussion

The muscles of Rac1 mutant mice have fewer Pax7 satellite cells (Fig. 2), but they are still present. Laminin-A, a cell adhesion molecule localized to the basement membrane of skeletal muscle, stained green, indicating that Rac1 mutant mice exhibit some enlargement of muscle cells.

Transmission Electron Microscope studies (Fig. 3) demonstrate that at the E14 stage, muscle cells deficient in Rac1 (B) exhibit an irregular layer (arrowheads) around the proximal arterial vessels compared to those in controls.

Fig.1 Rac1/-dependent cytoskeleton rearrangements and cell morphogenesis. Cell polarization Adherent junctions.

Fluorescence microscopy studies, Pax7+ satellite cells (red) are present in Rac1 mutant muscle. Laminin staining-green.

Transmission electron microscopy studies. A- control mouse, B-Rac1$^{fl/fl}$ mouse.

The findings of this study underscore the significance of Rac1 in orchestrating muscle stem cell distribution and myogenesis. Our results demonstrate that Rac1 deficiency leads to a reduction in Pax7-positive satellite cells, indicating its crucial role in maintaining the muscle stem cell pool. This aligns with previous studies highlighting Rac1's involvement in actin dynamics and cytoskeletal rearrangements, which are essential for myoblast fusion and muscle fiber formation.

Comparative analysis of mutant myogenic cells further elucidated the distinct roles played by Rac1 throughout the fusion process. These findings contribute to our understanding of the molecular mechanisms underlying myogenesis and provide valuable insights into potential therapeutic targets for muscle-related disorders.

However, it is important to acknowledge the limitations of this study. While conditional knockout mice provide a valuable model for investigating Rac1's role in muscle stem cell distribution, further studies are needed to elucidate the precise signaling pathways and downstream effectors involved. Additionally, the impact of Rac1 deficiency on muscle function and regeneration in vivo warrants further investigation.

Conclusion

In conclusion, our study highlights the critical role of Rac1 GTP-ase in myogenesis and the distribution of muscle stem cells. Through conditional mutagenesis in mice, we demonstrate that Rac1 is essential for muscle stem cell distribution, as evidenced by the reduced number of Pax7-positive satellite cells in Rac1 conditionally knockout mice. Additionally, our comparative analysis of changes in mutant myogenic cells reveals the nonredundant and partially distinct involvement of Rac1 throughout the stem cell distribution process.

In summary, our comprehensive investigation provides valuable insights into the molecular mechanisms, signaling pathways, and extracellular factors governing myogenesis, stem cell distribution, and migration. The findings contribute to our understanding of these processes in both physiological and pathological contexts, laying the groundwork for potential therapeutic interventions in muscle-related disorders.

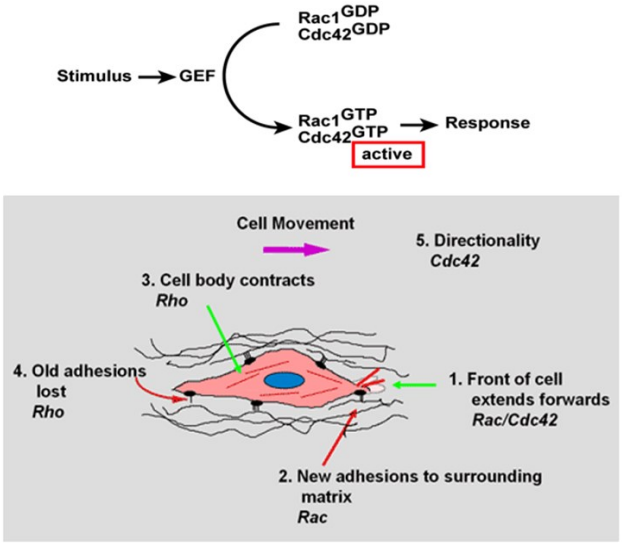

Fig.1 Rac1/-dependent cytoskeleton rearrangements and cell morphogenesis. Cell polarization Adherent junctions.

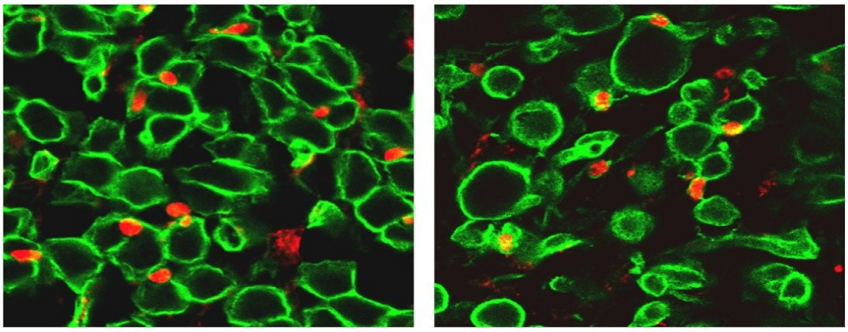

Fig 2. Fluorescence microscopy studies, Pax7+ satellite cells (red) are present in Rac1 mutant muscle. Laminin staining-green

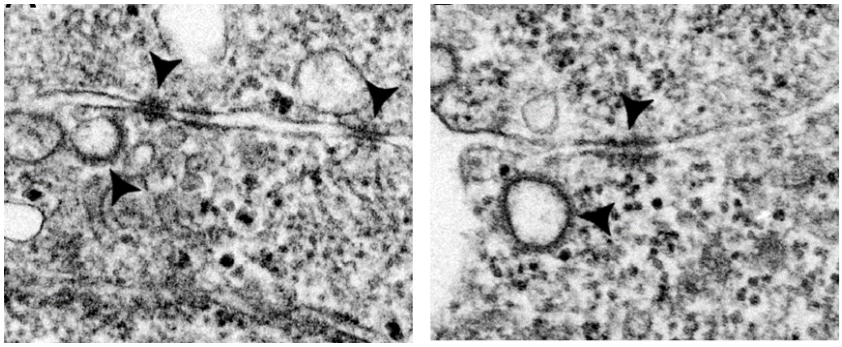

Fig.3. Transmission electron microscopy studies. A- control mouse, B-Rac1$^{fl/fl}$ mouse.

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