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Myogenesis defect due to Toca-1 knockdown can be suppressed by expression of N-WASP

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ABSTRACT

Skeletal muscle formation is a multistep process involving proliferation, differentiation, alignment and fusion of myoblasts to form myotubes which fuse with additional myoblast to form myofibers. Toca-1 (Transducer of Cdc42-dependent actin assembly), is an adaptor protein which activates N-WASP in conjunction with Cdc42 to facilitate membrane invagination, endocytosis and actin cytoskeleton remodeling. Expression of Toca-1 in mouse primary myoblasts and C2C12 myoblasts was up-regulated on day 1 of differentiation and subsequently down-regulated during differentiation. Knocking down Toca-1 expression in C2C12 cells (Toca-1^{KD} cells) resulted in a significant decrease in myotube formation and expression of shRNA-resistant Toca-1 in Toca-1^{KD} cells rescued the myogenic defect, suggesting that the knockdown was specific and Toca-1 is essential for myotube formation. Toca-1^{KD} cells exhibited elongated spindle-like morphology, expressed myogenic markers (MyoD and MyHC) and localized N-Cadherin at cell periphery similar to control cells suggesting that Toca-1 is not essential for morphological changes or expression of proteins critical for differentiation. Toca-1^{KD} cells displayed prominent actin fibers suggesting a defect in actin cytoskeleton turnover necessary for cell–cell fusion. Toca-1^{KD} cells migrated faster than control cells and had a reduced number of vinculin patches similar to N-WASP^{KO} MEF cells. Transfection of N-WASP-expressing plasmid into Toca-1^{KD} cells restored myotube formation of Toca-1^{KD} cells. Thus, our results suggest that Toca-1^{KD} cells have defects in formation of myotubes probably due to reduced activity of actin cytoskeleton regulators such as N-WASP. This is the first study to identify and characterize the role of Toca-1 in myogenesis.

1. Introduction

Skeletal muscle formation, growth, repair and regeneration are dependent on myoblast fusion, a process where mononucleated myoblasts, the muscle precursor cells, fuse into nascent multinucleated myotubes and further differentiate into myofibers [1,2]. In mammals, myoblast fusion occurs during embryo development and post-natal myogenesis begins when quiescent satellite cells are activated to become myoblasts. These myoblasts undergo differentiation and fusion, thus allowing growth and repair of muscle fibers [2]. A number of cellular processes are essential for myoblast fusion, namely cell–ECM adhesion, cell migration, cell–cell adhesion and membrane fusion [3–6]. The actin cytoskeleton, made up of polymerized F-actin and actin associated proteins, has been shown to be essential in muscle formation and regeneration [1,7–11]. The rate limiting step in actin polymerization, the formation of an actin nucleus (G-actin trimer) can be by-passed in the presence of the Arp2/3 complex. The activity of the Arp2/3 complex is regulated by the WASP family of proteins such as N-WASP [12,13].

In *Drosophila*, a number of proteins implicated in the regulation of Arp2/3-complex-dependent actin cytoskeleton remodeling has been found to be crucial for myoblast fusion [14–18]. A highly dynamic actin structure, termed the actin focus, at the site of myoblast fusion in the fusion-competent myoblast has been observed with the help of three-dimensional time-lapse imaging of *Drosophila* embryo. These actin-rich foci provide directionality for the trafficking of prefusion vesicles which are routed to ectopic membrane sites. Targeted exocytosis of prefusion vesicles represents a critical step leading to fusion with the plasma membrane [9]. Recent studies indicate the formation of finger-like protrusions from fusion competent myoblast into the apposing founder cells [19]. Additionally, F-actin-rich foci are organized by the fusion receptors and actin cytoskeleton regulators [15,16]. Actin remodeling is also essential in vertebrate myoblast fusion [7–9,20]. Knockdown or conditional knockout of N-WASP expression has been shown to reduce myoblast fusion [9,11]. Additionally, knockdown of Nap1, a member of the WAVE actin-remodeling complex, also resulted in inhibition of myogenic fusion [1]. These results highlight the importance of the actin cytoskeleton in myogenic fusion.

The BAR (Bin1-Amphysin-RVS167) family of proteins play a critical role in membrane deformation by coupling actin cytoskeleton regulators with the membrane [21]. The BAR domain proteins have been subdivided into N-BAR, F-BAR and I-BAR domain proteins [22]. Both

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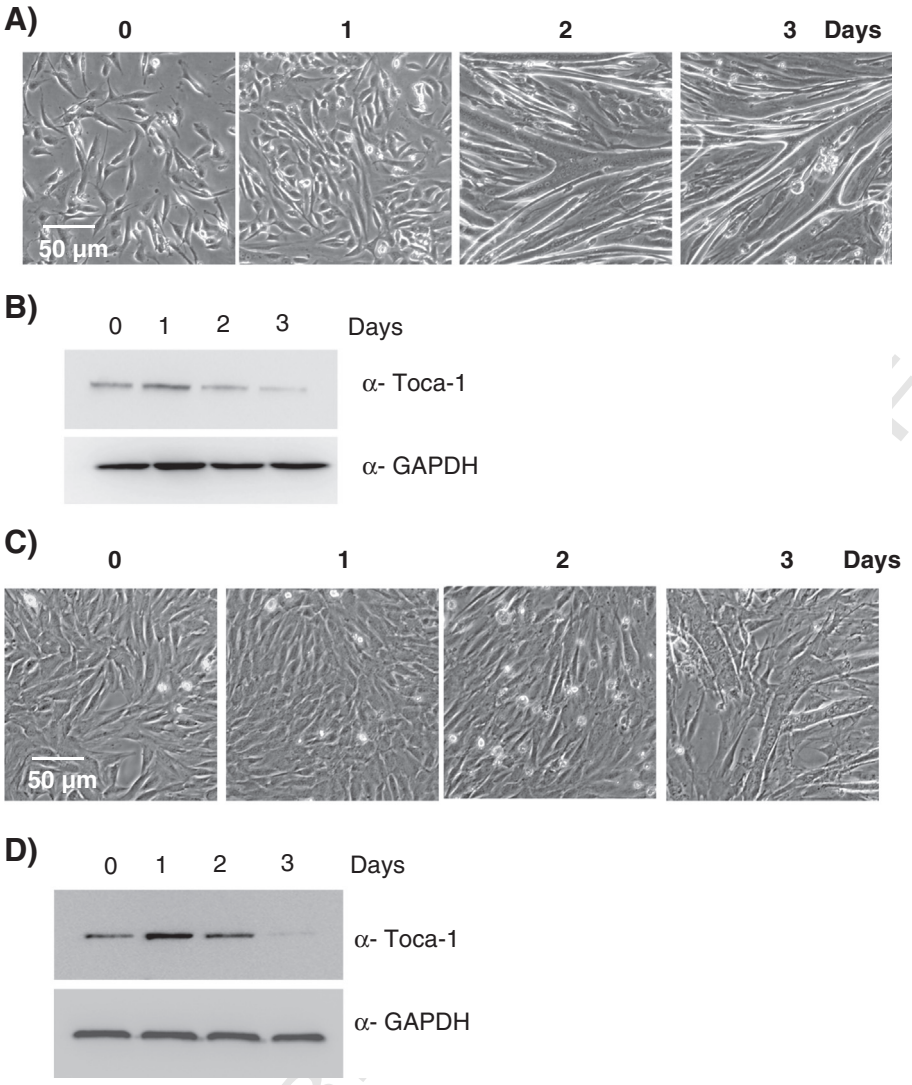

Fig. 1. Expression of Toca-1 during myogenic differentiation in primary myoblasts and C2C12 cells. A) Mouse primary myoblasts (PP2) were differentiated into myotubes by switching to differentiation medium (DMEM + 2% Horse serum) and images were captured at different days as indicated. B) Cell lysate was prepared from primary myoblasts cells undergoing differentiation and protein extracts (30 μg) were analyzed for the expression of Toca-1 and GAPDH (loading control) by western blot using anti-Toca-1 and anti-GAPDH antibodies, respectively. C) Confluent C2C12 myoblasts were differentiated into myotubes by culturing in differentiation medium (DMEM + 2% Horse serum) and images were captured at different days as indicated. D) Cell lysate was prepared from C2C12 cells undergoing differentiation and protein extracts (30 μg) were analyzed for the expression of Toca-1 and GAPDH (loading control) by western blot using anti-Toca-1 and anti-GAPDH antibodies respectively.

N-BAR and F-BAR domain proteins have been shown to induce positive curvature which supports the formation of vesicles, while I-BAR domain proteins have been shown to induce negative curvature which supports the formation of filopodia [23,24]. Toca-1 (transducer of Cdc42-dependent actin assembly) is a member of the F-BAR family of proteins and regulates the actin cytoskeleton. Toca-1 is an adaptor protein, initially identified as an essential co-factor for Cdc42-induced actin assembly via N-WASP in *Xenopus* extracts [25]. The protein consists of three functional domains; a F-BAR/EFC domain at the N terminus, an HR1 in the middle, and an SH3 domain at the C-terminus of the protein. The F-BAR/EFC domain interacts with the phosphoinositides and promotes invagination of the plasma membrane in vivo [26]. Toca-1 interacts with N-WASP and Cdc42 through the SH3 domain and the HR1, respectively. The functions of Toca-1 in myogenesis have not been defined.

Toca-1/N-WASP interaction has been shown to induce the formation of dynamic membrane tubules and vesicles [27]. In neuronal cells, Toca-1 has been shown to be involved in the regulation of neurite outgrowth [28]. In another study, knockdown of Toca-1 in A431 cells led to defects in EGF-induced filopodia and lamellipodia formation. Toca-1 was found to be required for EGF-induced migration and invasion of A431 cells which suggests that Toca-1 might play a role in the recruitment and activation of the actin nucleation machinery within lamellipodia and filopodia to enhance cell migration and invasion [29]. We have previously found that IRSp53 an I-BAR domain protein negatively regulates myogenesis. Knocking down IRSp53 expression in C2C12 cells enhanced myogenic differentiation, while over-expression of IRSp53 in C2C12 cells inhibited myogenic differentiation (Misra et al., 2012). The IRSp53 knockdown cells had increased number of vinculin patches while the IRSp53 overexpressing cells had a reduced number of vinculin patches suggesting that IRSp53 negatively regulates assembly of focal adhesion and integrin signaling in muscle (Misra et al., 2012).

Here, we have identified and characterized the role of Toca-1 in myogenic differentiation. Knocking down the expression of Toca-1 by shRNA led to a significant reduction in myotube formation, even though the differentiation was not affected as determined by the expression of myogenic differentiation markers. Toca-1^{KD} cells had a reduced number of vinculin patches and increased cell motility compared to control cells. Toca-1^{KD} cells formed cell–cell contacts similar to control cells as determined by the surface expression of N-Cadherin. The Toca-1^{KD} cells also

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